Haemostatic safety in epidural analgesia

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Title: Haemostatic safety in epidural analgesia

Epidural anaesthesia and analgesia are indicated for oesophageal surgery. A rare but serious complication is spinal haematoma, which can occur on insertion, manipulation or withdrawal of catheters. Blood tests of coagulation are often taken in the hope that they will identify patients at risk of developing a spinal haematoma. When these tests should be taken is not well defined: international guidelines are vague regarding which tests are appropriate, and how to interpret their results.

This thesis aims to advance knowledge in this area by presenting the results of five studies covering various aspects of this topic:

Paper I: Risk of epidural haematoma: pre- to postoperative dynamics of coagulative status in 358 patients undergoing oesophageal resection over ten years, and a review of the literature. APTT and PT-INR are usually slightly elevated and may not always indicate hypocoagulation. Caution is nevertheless recommended when being presented with elevated routine tests of coagulation before withdrawing an epidural catheter and a thorough clinical evaluation is important. Viscoelastic haemostatic tests may have a role in this setting but they are so far not validated.

Paper II: Correction of hypothermic and dilutional coagulopathy with concentrates of fibrinogen and factor XIII: an in vitro study with ROTEM. Iatrogenic postoperative dilutional coagulopathy is common. Fibrinogen improved in vitro haemodilution-induced coagulopathy at both 33°C and 37°C, though more efficiently after crystalloid than hydroxyethyl starch haemodilution. Factor XIII provided an additional effect, but only after crystalloid dilution.

Paper III: Thromboelastometry versus free-oscillation rheometry and enoxaparin versus tinzaparin: an in-vitro study comparing two viscoelastic haemostatic tests' dose-responses to two LMWH's at the time of withdrawing epidural catheters from ten patients after major surgery. Clot initiation time's dose-dependent prolongation by LMWH's in this study agrees with previous research, as does tinzaparin's stronger anti-coagulative effect than enoxaparin at equivalent levels of anti-FXa activity. Anti-FXa activity may not be the most appropriate assay to guide dosage of LMWH's. Significant inter-individual variation in aPTT's dose–response suggests that the relationship between dose and effect in the postoperative period is complicated. While both ROTEM® and FOR may have some role in postoperative monitoring but more research is needed.

Paper IV: Monitoring LMWH's at therapeutic levels: dose-responses of, and correlations and differences between aPTT, anti-factor Xa and thrombin generation assays. APTT displays a linear dose-respone to LMWH. There is variation between aPTT assays. Tinzaparin increases aPTT and decreases thrombin generation more than enoxaparin at any given level of anti-FXa activity, casting doubt on anti-FXa's present gold standard status. Thrombin generation with tissue factor-rich activator is a promising method for monitoring LMWH's.

Paper V: Coagulative safety of epidural catheters after major upper gastrointestinal surgery: advanced and routine coagulation analysis in 38 patients. The increase in PT-INR may be caused by decreased postoperative FVII while the elevated aPTT may be caused by low FXII. The mild postoperative hypocoagulation indicated by routine tests is not consistent with thromboelastometry. The relevance of ROTEM® and Multiplate® in the context of moderately increased routine tests remains unclear.

Further research in this area should address the inconclusivity of current research, in that the actual clinical relevance of current laboratory assays, in predicting the risk of spinal haematoma upon the withdrawal of epidural catheters, is unknown. Ideally, haemostasis in a large number of patients with spinal haematoma should be analysed in conjunction with detailed clinical histories.

Key words: epidural anaesthesia, routine coagulation testing, spinal haematoma, viscoelastic tests, coagulation factors

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Content

List of original papers

I Risk of epidural haematoma: pre- to postoperative dynamics of coagulative status in 358 patients undergoing oesophageal resection over ten years, and a review of the literature.
Thomas OD, Lybeck KE, Flisberg P, Schött U.
Submitted to Perioperative Medicine.

II Correction of hypothermic and dilutional coagulopathy with concentrates of fibrinogen and factor XIII: an in vitro study with ROTEM.
Winstedt D, Thomas OD, Nilsson F, Olanders K, Schött U. Scand J Trauma Resusc Emerg Med. 2014 Dec 16;22:73.

III Thromboelastometry versus free-oscillation rheometry and enoxaparin versus tinzaparin: an in-vitro study comparing two viscoelastic haemostatic tests' dose-responses to two low molecular weight heparins at the time of withdrawing epidural catheters from ten patients after major surgery.
Thomas OD, Larsson A, Tynngård N and Schött U.
BMC Anesthesiology 2015, 15:170

IV Monitoring low molecular weight heparins at therapeutic levels: dose-responses of, and correlations and differences between aPTT, anti-factor Xa and thrombin generation assays.
Thomas OD, Lybeck E, Strandberg K, Schött U.
2015 PLoS ONE 10(1): e0116835.

V Coagulative safety of epidural catheters after major upper gastrointestinal surgery: advanced and routine coagulation analysis in 38 patients.
Thomas OD, Rein H, Strandberg K, Schött U.
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Abbreviations and trade names

aPTT	activated partial thromboplastin time
AT	antithrombin
ETP	endogenous thrombin potential
ECMO	extracorporeal membrane oxygenation
ER	Endoplastic retiulum
ETP	Endogenous thrombin potential
FXIII	factor XIII (Cluvo®)
FI	Fibrinogen (Riastap®)
FOR	Free oscillation rheometry
HES	hydroxyethyl starch (Venofundin®, Voluven®)
LMWH	low molecular weight heparin
PCC	prothrombin complex concentrate (Ocplex®, Confidex®)
PIVKA	Proteins Induced by Vitamin K Absence
Plc	platelet count
PT-INR	prothrombin time international normalized ratio
RA	ringer's acetate
ROTEM®	rotational thromboelastography
SD	standard deviation
TAFI	thrombin activatable fibrinolysis inhibitor
TEG®	thromboelastometry
TFPI	tissue factor pathway inhibitor
TG	thrombin generation
VHT	viscoelastic haemostatic tests
vWF	von Willebrand Factor
*	$P<0.05$
**	$P<0.01$

Introduction and aims of this thesis

This thesis mainly concerns haemostasis in the postoperative period, how it affects the safety of epidural catheters and how it can be monitored with various laboratory tests.

Why is this subject interesting? Because anaesthetists and their patients have a strong interest in avoiding haemorrhage within the spinal canal, the risk of which is greatest at the time of insertion of an epidural catheter, and at the time of withdrawal when it is no longer needed.

Many factors in the perioperative period can disturb haemostasis: we know that most patients are prone to thrombosis, which is why they are treated with prophylaxic anticoagulants. Some may be hypocoagulable for a handful of different reasons such as haemodilution, hypothermia, perioperative malnutrition resulting in deficiency of vitamin K dependent coagulation factors. Blood tests are therefore frequently (and in some institutions, always) taken before manipulation of epidural catheters so as to provide some kind of insurance against a bleed caused by the catheter dislodging a blood-clot as it is withdrawn.

Guidelines describe which laboratory results are acceptable, but they are somewhat vague as to what to do when results are slightly abnormal: there is wild variation in how anaesthesiologists react when presented with an activated partial thromboplastin time (aPTT) of 45 seconds and an epidural catheter requiring withdrawal.

Following the introduction of low molecular weight heparin (LMWH) as thrombosis prophylaxis to the USA in 1993 there were many cases of spinal haematoma in conjunction with neuraxial blockade. [1]. The doses used were at the time much higher than those used in Europe, but nevertheless demonstrate that there is a need to monitor LMWH's effect in order to prevent these complications. At present there is no perfect test that can be used to monitor thrombosis prophylaxis, and there is even variability between the results given by various routine laboratory tests measuring the same parameters, particularly aPTT, due to a lack of standardization of methods and reagents.

Some disturbances of haemostasis are not reliably detected by routine tests. During recent years, viscoelastic haemostatic tests (VHT's) such as ROTEM® and TEG® have been introduced into everyday clinical practice. Despite being mentioned in many, even most, guidelines, the level of evidence for the usefulness of these is mainly based on case studies: this area is difficult to study because the incidence of complications is very low.

This project began with a small case series of patients after major surgery, in which we examined ROTEM® and platelet aggregometry in addition to routine tests of coagulation: see Appendix 1. [2] This led to a retrospective journal study after which we can present routine coagulation analysis from 358 patients who had undergone oesophagectomy, from before operation and at the time of having their epidural catheters withdrawn. The last study in this series is a series of prospectively collected laboratory studies on patients undergoing major upper gastrointestinal surgery, in which standard, thromboelastometric, platelet function and coagulation factor levels were measured before surgery and at the time of withdrawing their epidural catheters.

Finally, we present three laboratory-based studies in which we compare and contrast various methods of monitoring LMWH's and synthetic colloids. These contribute to this area of postoperative medicine by demonstrating how these tests respond to clinical and supraclinical concentrations of LMWH, and also challenge current dogmas concerning how LMWH can be monitored.

Background

Benefits and risks of epidural analgesia after major surgery

Until relatively recently, epidural analgesia was the gold standard for almost all general surgery, vascular surgery and orthopaedics of the low limbs and it certainly true that an epidural block over the correct part of the body can give effective analgesia (pain relief), even anaesthesia (loss of all sensation). Problems with epidural analgesia include there being a failure rate of probably more than 10%, in which case patients can be subjected to unnecessary pain. [3]

Over the last decade there has been considerable interest in so-called 'fast track surgery', a concept involving mobilisation of patients as quickly as possible after surgery such that they avoid complications involved with immobility. Since epidural analgesia's side-effects include weakness of the legs causing bedriddenness, and urinary retention necessitating a catheter, the benefits of an epidural for moderate general surgery are probably not worth it and adequate 'multimodal' analgesia can be given instead using a cocktail of analgesics such as paracetamol, non-steroidal anti-inflammatory drugs, slow release oral or intravenous opioids and often a peripheral nerve block or infiltration of local anaesthetic. [4]

Major surgery, especially that involving upper laparotomy and a thoracotomy is still an indication for epidural analgesia. Thoracotomy pain can be both severe and preventative of deep-breathing: good analgesia is therefore important not only for comfort but also to prevent respiratory complications such as stagnation of airway secretions, pneumonia and atelectasis. [5] Previous research indicating that cardiovascular morbidity can be decreased by epidural anaesthesia and analgesia is most likely to apply to this group of patients.

The specific side-effects and complications associated with epidural anaesthesia and analgesia are well known: sympathetic block that can potentially cause haemodynamic instability, infection at the site of catheter insertion, local anaesthetic toxicity and of course spinal haematoma: bleeding within the spinal column, which compresses nervous tissue resulting in loss of sensation and at worst paralysis.

Current Guidelines

There are various international and guidelines. See Table 1, which summarizes the major international guidelines that are currently in place. Updated Nordic guidelines from the Scandinavian Society of Anaesthesiology and Intensive Care Medicine are expected to be published soon.

Table 1

Summary of current guidelines. PT-INR: prothrombin time international normalized ratio. aPTT: activated partial thromboplastin time.

	Insertion of epidural	**Withdrawal**
PT-INR		
SSAI (Scandinavian Society for Anaesthesia and Intensive Care). [10] *	Comfort: ≤1.2. Morbidity: <1.6. Mortality: <1.8.	
ASRA (American Society of Anaesthesia) [3] *	'Normalized' and 4-5 days after warfarin discontinued.	<1.5 although 1.5-3 sometimes acceptable 'with caution'
ESA (European Society of Anaesthesia) [8]	≤1.4	Warfarin should be administered only when catheter has been removed.
aPTT		
SSAI	'Should be within the normal range'	
ASRA	"It is not necessary to routinely check the aPTT or platelet count, unless the clinician is concerned about changes in these values after prolonged administration or in patients with many comorbidities that might influence the pharmacologic expression of subcutaneous UFH."	
ESA	'aPTT should have normalized (after heparin).'	'Normal aPTT'
Platelet count (x10^6)		
SSAI	Comfort: >100 Morbidity: >80 Mortality: >50	
ASRA	"Because heparin-induced thrombocytopenia may occur during heparin administration, we recommend that patients receiving heparin for more than 4 days have a platelet count assessed before neuraxial block and catheter removal."	
ESA	Platelet count should be monitored if patient has received UFH or LMWH for 5 days or more. No threshold given.	

*Note that the Nordic guidelines cover 'disturbed haemostasis' while the ASRA and ESA Guidelines relate to patients on anticoagulative drugs. The Scandinavian guidelines recommend a 'benefit analysis' of epidural catheterization and removal of epidural catheters: the requirements for test results are more stringent in procedures that are only for comfort compared to procedures that reduce the risk for morbidity or mortality.

The Nordic guidelines are the only ones that clearly acknowledge the necessity for attempting a quantitative risk/benefit analysis depending on whether inserting or removing the catheter is to improve 'comfort', 'morbidity' or 'mortality'. Removing a catheter suspected of being infected and causing sepsis is much more strongly indicated than removing an uninfected catheter in patient with dilutional coagulopathy that can be corrected.

The Nordic guideline does mention the use of prothrombin complex concentrate (PCC) for the correction of an elevated prothrombin time international normalized ratio (PT-INR) in patients treated with warfarin in order to permit neuraxial blockade. Whether PCC is appropriate in the postoperative period is unclear given that this population of patients is already at an increased risk of thrombosis. One case study from 2005 describes a patient who was given vitamin K to correct an elevated PT-INR before withdrawal of his epidural catheter five days after cardiothoracic surgery. He unfortunately developed a thromboembolic stroke. [6, 7]

Risk of spinal haematoma in patients treated with epidural anaesthesia

Spinal haematoma is a rare complication of neuraxial blockade (ie epidural or spinal injection or catheterization for administration of analgesics). The risk of spinal haematoma varies depending on several factors which are likely not independent: at the two ends of the spectrum, the incidence of spinal haematoma in elderly patients undergoing major orthopaedic surgery is around 1 in 4000 while healthy among healthy parturients receiving spinal anaesthesia for caesarean section, the incidence is more likely 1 in 30 000: see Table 2 [8] This thesis is primarily concerned with postoperative analgesia: the risk of spinal haematoma when withdrawing epidural catheters is likely between 1:150,000 and 1:190,000. [9]

Table 2:
Factors potentially affecting the risk of spinal haematoma in conjunction with neuraxial blockade.

	Lower risk ⋯⋯➤		Higher risk
Type of manipulation	Withdrawal of catheter	One-shot injection	Catheterization
Number of puncture attemps	One		Many
Blood in catheter or cannula	No		Yes
Category of patient	Obstetric		Undergoing orthopaedic surgery
Patient age	Young		Old
Sex	Men		Women
Weight	High		Low
Renal function	Good		Reduced
Antihaemostatic medication	None	Thrombosis prophylaxis	Anticoagulation
Experience of anaesthetist	Little		Much
Spinal tumours			
Anatomical abnormalities such as Mb Bechterew and spinal stenosis.			

Overview of haemostasis

A short overview of haemostasis is necessary. Thrombosis is the formation of a plug consisting of platelets and fibrin filaments and is a requirement for life. Virchow's classic triad of haemostasis, described in the 19th century identifies three major factors that contribute to thrombosis:

Figure 1:
Virchow's triad described in 1856[10].

The triad still applies today: patients undergoing major surgery are often predisposed to thrombosis because of their underlying illness [11] and because of immobility resulting in deep vein thrombosis; endothelial injury during surgery requires adequate haemostasis to limit haemorrhage. Virchow's Triad offers no explanation of mechanism, though.

Haemostasis can be divided in to three stages: **initiation, amplification and propagation**, although this is a simplification: coagulation involves intricate and complicated feedback systems where each coagulation factor and cell may have differing effects depending on the current state of coagulation and inflammation.

Initiation involves activation of coagulation and synthesis of thrombin (FIIa) on cell surfaces, either in small amounts on tissue factor-bearing cells, which initiates the extrinsic pathway by activation of FVII, or by exposure of a negatively charged surface or collagen, which initiates the intrinsic pathway by activation of FXII. There is continuous low-level coagulative activity in healthy individuals even in the absence of thrombosis, which is limited by the presence of anticoagulant regulators such antithrombin III (AT), the protein C/S system, tissue factor pathway inhibitor (TFPI) and fibrinolysis. [12]

Once thrombin activity reaches a threshold, the regulatory systems can no longer contain the increasing activities of coagulation factors and platelets: increasing positive feedback occurs on several fronts. Thrombin activates platelets and FV and FVIII such that coagulation begins to occur on the surfaces of platelets that have been recruited to the forming thrombus. This is amplification. See Figure 2.

Propagation involves mass production of thrombin on activated platelets where FIXa and FVIIIa form the tenase complex which activates FX such that it can bind to FVa to form the prothrombinase complex, which activated prothrombin (FII) to thrombin (FIIa).

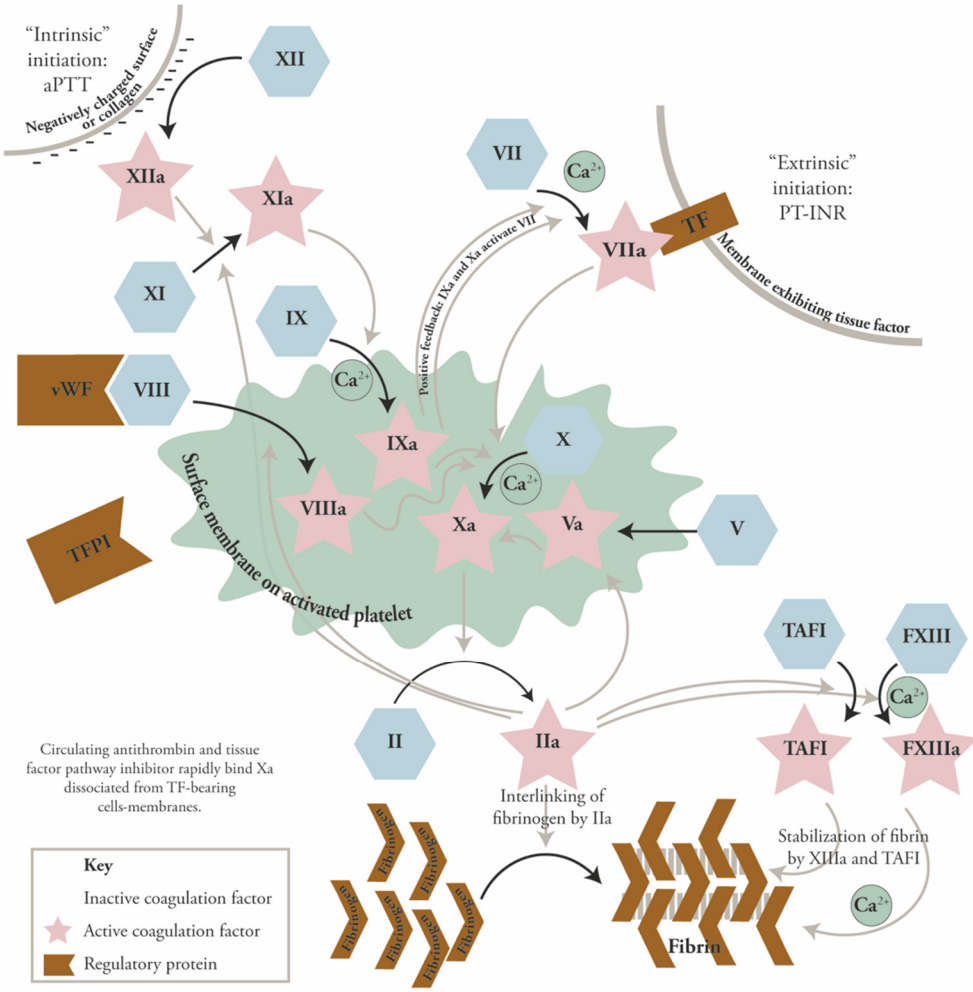


Figure 2
schematic overview of the coagulation factors involved in coagulation and where they are located during amplification and propagation of coagulation. The following factors' synthesis is dependent on vitamin K: II, VII, IX, XI, Protein C, S.

PT-INR: prothrombin time international normalized ratio

The PT-INR is a standardized test run on decalcified, platelet poor plasma, that was first described by Quick in 1935. It measures activity of the extrinsic and common coagulation paths, ie factors VII, X, V and II:

1. Coagulation is activated by addition of recombinant tissue factor and calcium. See Figure 2. This was previously referred to as 'thromboplastin', extracted from animal brain.
2. The PT is the time taken for a visible clot to form. Today's method measures this using an automatic chromatic technique.
3. Results are standardized so that the PT is converted into a ratio to an internationally defined 'normal': 1.0 means that the sample's PT is normal; <1 indicated that the PT was shorter than normal ie hypercoagulation; >1 indicates that the PT was longer than normal ie hypocoagulation.

Since there are no platelets in plasma and formation of the functional prothrombin complex (which converts fibrinogen to fibrin) requires a membrane, the PT reagent contains phospholipids that form membranes. In addition to calcium, tissue factor and phospholipids, reagents for the PT generally contain polybrene, which removes antihaemostatic activity provided by heparin or LMWH up to around 2.0 anti-Xa IU/ml.

In Scandinavia, Holland and Japan, Owren's PT is used instead of Quick's. The principle is the same other than that the reagent also contains fibrinogen. The rationale behind this is to avoid hypofibrinogenaemia incorrectly giving the impression that a hypocoagulable patient is deficient in the coagulation factors involved in the extrinsic pathway: measuring PT-INR by Owren's method, and the plasma level of fibrinogen, gives more information than the PT-INR by Quick's method and the plasma level of fibrinogen.

aPTT: activated partial thromboplastin time

This test, also run on platelet-poor plasma, was described by Proctor and Rapaport in 1961 and is sensitive to deficiencies in the factors involved in the intrinsic and common pathways: I, II, V, VIII, IX, X, XI and XII. It is also sensitive to heparin by its inhibition of FII and FX, antiphospholipid antibodies such as the lupus anticoagulant and coagulation factor inhibitors.

Coagulation is activated by maximal activation of FXII, which is the first step of the intrinsic pathway. Any of several reagents can be used: silica, a glass surface, kaolin.

Recalcification starts coagulation and, similar to the PT, the aPTT is the time taken for a visible or chromatically-detected fibrin clot to form. Also similar to the PT assay, phospholipid membranes are needed in order for functional prothrombin to form, so the reagents for the aPTT also contain phospholipids.

A problem with the aPTT is that it unlike the PT-INR in that it is not a standardized test: aPTT measured on a particular blood sample varies depending on which reagent is used and what kind of apparatus. An additional aspect of both the PT and aPTT assays is that they both involve maximal activation of coagulation, which is not physiological. Reagents may also vary in how strongly they activate coagulation.

Plc: platelet count and platelet function tests

Platelet count is simply a measure of the number of platelets present in each millilitre of blood. Often expressed in 'millions per millilitre', measurement is done by manual or automatic microscopy.

Platelet function can be measured by several methods: the most common at present is platelet aggregometry (Multiplate®, VerifyNow®, 'ROTEM Platelet®') although other assays such as PFO-100® and PlateletWorks® do exist.

Platelet aggregometry is based on the principle that the impedance between electrodes placed in coagulating blood decreases as the platelet aggregate on the electrodes. A curve showing impedance against time is generally created, and the area under this curve is taken as a measure of platelet activity. Ideally, this is independent of the concentration of platelets in blood: in reality, however, the area under the curve is actually decreased in thrombocytopenia.

Platelet function tests' primary use is in patients who are, or have been treated with platelet inhibitors: clopidogrel (Plavix®), for example, is an effective platelet inhibitor given after stenting of the coronary arteries, but a clinical problem is that some patients are 'clopidogrel resistant' so that their risk of coronary artery events is not decreased despite taking a standard dose of this medicine. Platelet aggregometry can be run using an initiating reagent that stimulates the ADP-receptor which is clopidogrel's target-site to identify patients who are non-responders. Non-responders are not a problem associated with the most recent platelet-inhibitors such as ticagrelor (Brilique®) and prasugrel (Efient®).

A second use of platelet aggregometry in patients treated with platelet-inhibitors is to check that residual platelet-inhibition has ceased before administering regional anaesthesia or surgery. Guidelines exist for the interval between the last dose of platelet inhibitor and neuraxial blockade, for example, but platelet function tests can be used to confirm that the effect of these drugs has worn off if there is suspicion of residual effect or if regional anaesthesia is strongly indicated before the recommended interval between drug and puncture has passed or in the case of suspicion of prolonged anti-platelet effect.

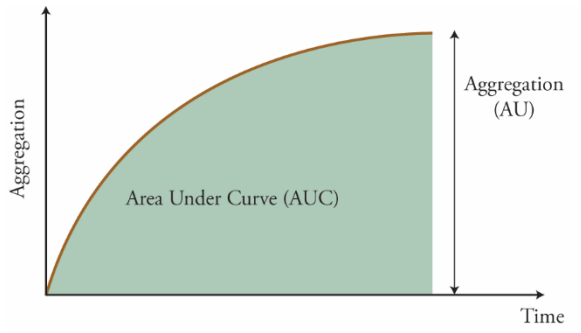


Figure 3:
Appearance of a Multiplate® platelet aggregation curve: aggregation is measured in arbitrary units, 'velocity' is a measure of the initial slope of the curve. AUC is the area under the curve.

Fibrinogen and its measurement by the Clauss method

Fibrinogen, also called Factor I, is a glycoprotein synthesized in the liver consisting of three chains. It is polymerized to fibrin filaments by thrombin (FIIa) in the final stage of coagulation (see Figure 2). It is an acute-phase protein, increasing in systemic inflammation; it binds to platelets' GPIIb/IIIa receptors, contributing to platelet aggregation and activation. [13]

The most usual method for measuring fibrinogen concentration is the optical Clauss method, in which fibrin formation in platelet-poor plasma is directly initiated by the addition of a high concentration of thrombin (FIIa). The time for clot formation is compared to a reference values for plasma containing known concentrations of fibrinogen. The reagent also contains phospholipid and calcium in order to facilitate thrombin's polymerization of fibrinogen to fibrin. [14]

The plasma is generally diluted (1:10) before the initiation of coagulation in order or to reduce or eliminate the effects of heparin or LMWH which might have inhibited the thrombin, but high levels of heparin may still affect this assay's results in situations where high concentrations of heparin are used such as during cardiac surgery or extra-corporeal membrane oxygenation (ECMO). One more potential source of error is that synthetic colloids, particularly hydroxyethyl starch (HES), may interfere with some thrombin concentrates used in the Clauss assay when optical methods are used to measure the time to clot formation such that the fibrinogen level is overestimated. [15]

Heparin, low molecular weight heparins and the anti-FXa assay

Heparin is a drug extracted from animal lung or intestine, which activates antithrombin III (AT) so that it binds to FXa; and FIIa by forming a complex with AT, Xa and IIa. [16]

Low molecular weight heparins (LMWH) are saccharides manufactured from the lysis of heparin and inhibit FXa by binding to the same site on AT as heparin. LMWH's vary in their ability to inhibit FIIa: those with shorter molecules lack binding sites for FIIa: they are more specific for inhibition of FXa. See Table 3. [17]

The current gold standard for measuring LMWH activity or 'concentration' is the anti-FXa assay, described in Study IV, which does not measure anti-FIIa activity. They are also generally dosed in 'units of anti-FXa' activity, which is confusing because although all LMWH's inhibit FXa, their varying inhibitions of FIIa mean that '10 000 international units' of tinzaparin have a stronger anticoagulative effect than enoxaparin with the same anti-FXa activity because the tinzaparin exerts a stronger inhibition of FIIa than enoxaparin:

Table 3:
heparin and LMWH's different specificies for thrombin (FIIa) mean that monitoring LMWH in units of anti-FXa activity is confusing.

Agent	Trade name in Sweden	Mean molecular mass	Anti FXa/FIIa (IU/ml)	Anti FXa/FIIa ratio
Unfractionated heparin (UFH)	Heparin	15 kDa	193/193	1
Tinzaparin	Innohep®	6.8 kDa	90/45	2.0
Dalteparin	Fragmin®	6.0 kDa	130/52	2.5
Enoxaparin	Klexane®	4.2 kDa	100/25	3.9
Fondaparinux	Arixtra®	1.7 kDa	930/0	∞

Viscoelastic tests

Also known as viscoelastic haemostatic assays (VHA's), these are mechanical tests run on whole blood. The most established brands are ROTEM® and TEG®, which both measure forming clots' resistance to rotational (shear) forces; other available viscoelastographs are free-oscillation rheometry (ReoRox®) and the SonoClot®. VHA's have been shown to be useful in the guidance of transfusion in cardiac surgery and major haemorrhage [18, 19], and they can

ROTEM®, rotational thromboelastometry, makes use of a pin suspended in a plastic cuvette. The pin rotates to and fro every 6 seconds and the resistance to rotation is plotted against time to give a curve displaying impedance to rotation 'amplitude' against time:

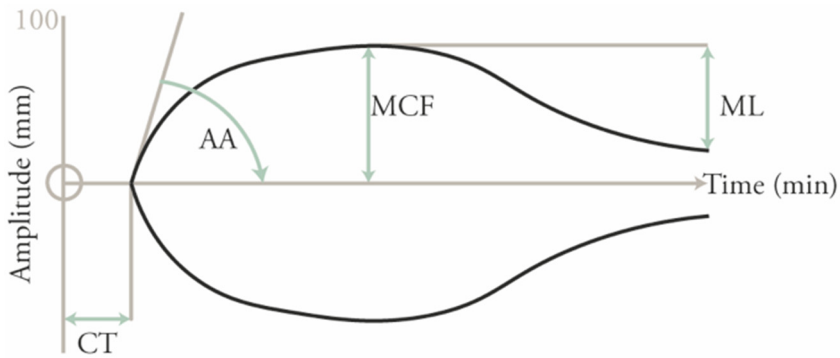


Figure 4:
Thromboelastometry curve with parameters as they are named by ROTEM®. CT: clotting time reflecting initiation. AA: alpha angle reflecting the rate of clot formation. MCF: maximum clot firmness. ML: Maximum lysis, a measure of fibrinolysis. CFT, not shown above, is the clot formation time: time to reach an amplitude of 20mm.

TEG® also uses measures impedance to forced rotational forces, but rather than using a pin immersed in an immobile cuvette as ROTEM® does, the TEG® cuvette rotates to and fro around an immobile pin.

Sonoclot® is a less common viscoelastic test, which is not used in the articles in this thesis. It makes use of an ultrasonic transducer vibrating at 200Hz, submersed in a sample of blood or plasma in a plastic cuvette. As the clot forms, fibrin strands adhere to the probe and impede its vibration. This is detected by the electronics driving the probe, and an output curve is generated showing impedance to vibration against time. [20]

Free oscillation rheometry (FOR) in common with Sonoclot is only extremely rarely seen in clinical use. The FOR apparatus (ReoRox®) produces an output chart consisting of two curves representing viscosity and elasticity. This technique is somewhat different from ROTEM® and TEG® in that the blood sample is placed in a cuvette that can rotate freely, but which is attached to a torsion wire that causes the cuvette to oscillate backwards and forwards. A gold plated bob is suspended in the sample such that the oscillations' characteristics are affected by adhesion of fibrin filaments to bob and the cuvette wall.

The cuvette is set into rotation every 2.5 seconds and an optical sensor measures both the frequency and the amplitude of the oscillations following each initiation. The characteristics of the oscillations' amplitude decreases during initiation due to an increase in viscosity. During propagation, the frequency of oscillation increases due to increasing elasticity: [21, 22]

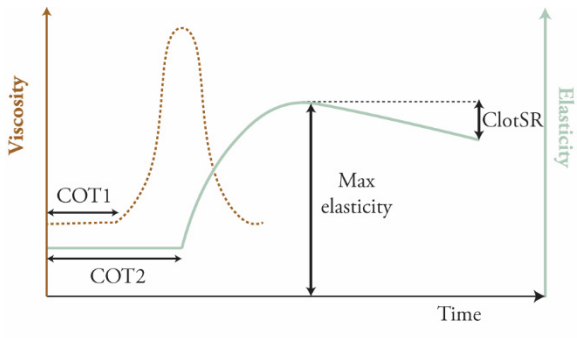


Figure 5:
A normal FOR curve. COT1 and COT2 indicate clot initiation and propagation; ClotSR is a measure of fibrinolysis.

FOR and ROTEM® measures of clot firmness and lysis correlate well, at least in the setting of acute trauma. [23] Measures of clot initiation as measures by the two tests may not correlate so well, which may be the result of ROTEM® applying constant shear agitation to the forming clot while FOR applies an intermittent transient force at the start of each oscillation. Other reasons for differences are that the reagents used to initiate coagulation in are not standardized and that the surfaces that blood is exposed to in the two assays differ: ROTEM® is plastic while the ReoRox® FOR surfaces are gold plated. Neither of the tests provides an environment similar to the spinal canal surrounding a haemorrhage caused by a needle or dislodgement of a clot by withdrawing an epidural catheter.

Thrombin generation

The production of thrombin can be seen as a functional measure of coagulative activity or potential, since it is thrombin which polymerizes fibrinogen to fibrin filaments. It does not, however, measure clot stability or fibrinolysis. The assay used in Study IV is a fluorescent method: functional thrombin produced after initiation by tissue factor, phospholipids and calcium cleaves a substrate so that it releases a fluorophore (a substance that re-emits light upon excitation by light) that can be optically detected so as to quantify the 'endogenous thrombin potential' (ETP), which is a measure of the quantity of thrombin produced. The rate of thrombin formation is obtained by differentiating the curve such that initiation, propagation and termination of coagulation are shown.

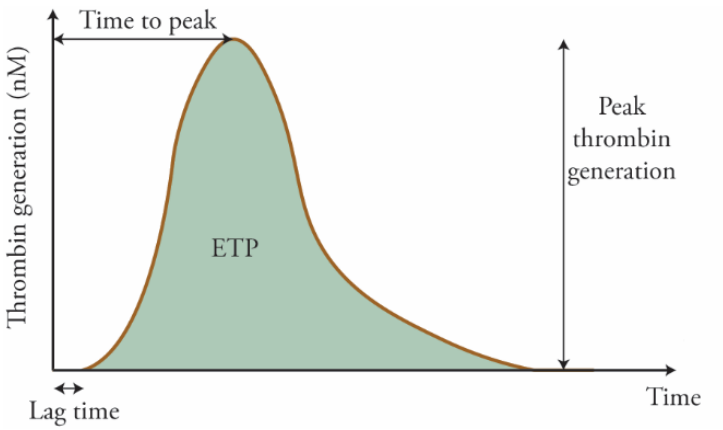


Figure 6:
Thrombin generation curve showing fluorescence against time ie the rate of thrombin generation against time. The integral of the curve (the area under the curve), also called 'Endogenous Thrombin Potential' is a measure of the total amount of thrombin produced.

Vitamin K, the vitamin K cycle and proteins induced by vitamin K absence (PIVKA)

Vitamin K is required for the posttranslational synthesis of several coagulation factors and regulatory proteins (II, VII, IX, XI, Protein C and S): it is a substrate in the carboxylation of glutamate to γ-carboxyl glutamate. The carboxyl groups of γ-carboxyl glutamate bind calcium ions glutamate does not.

As early as 1974, Stenflo *et al.* found that functional prothrombin (FII) must be able to bind calcium ion if it is to be activated to functional thrombin (FIIa). Calcium binding sites are provided by carboxyl groups on vitamin K-dependent coagulation factors. Without the interaction between calcium ions and these binding sites, the protein chains adopt the wrong conformation and are not able to interact with phospholipid membranes such as the platelet surface. Since coagulation occurs when the various coagulation factors are brought together (mainly) on platelet surfaces, coagulation factors unable to interact with these membranes are unlikely to form functional complexes with the other factors and coagulation will not occur.

Vitamin K's critical role in the synthesis of coagulation factors is that it is required for the carboxylation of glutamate (Glu) to γ-carboxyl glutamate (Gla) in coagulation factors' precursors: if it is deficient, partially carboxylated coagulation factors with reduced or absent activity are synthesized. These are called Proteins Induced by Vitamin K Absence (PIVKA) and can be measured using an ELISA method (enzyme-linked immunosorbent assay) [24, 25].

Overview of the Vitamin K Cycle

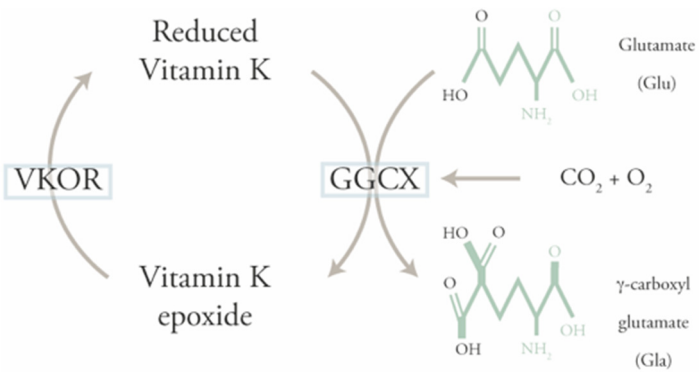


Figure 7:
Simplified diagram showing the vitamin K cycle in the endoplasmic reticulum facilitating carboxylation of Glu to Gla on precursors to coagulation factors. GGCX: gamma-carboxyglutamyl carboxylase. Warfarin exerts its effect by inhibiting VKOR (vitamin K epoxide reductase).

Vitamin K1 is the form found in green leafy plants since it is involved in photosynthesis: patients taking vitamin K antagonists such as warfarin find that their degree of anticoagulation decreases significantly if they consume large amounts of kale. The form found in animals is vitamin K2, which is mainly synthesized by bacteria in the gut. K2 exists as a variety of related subtypes with slightly varying lengths of carbon side chains. Synthetic forms of vitamin K include K3, 4 and 5.

The enzyme which carboxylates Glu to Gla is γ-carboxyglutamyl carboxylase (GGCX), which is located in the endoplastic reticulum (ER). Carboxylation of Glu by GGCX requires three substrates: reduced vitamin K (vitamin K hydroquinone), carbon dioxide and oxygen: it involves the transfer of a hydrogen ion from Glu to vitamin K, resulting in vitamin K epoxide.

Reduced vitamin K is therefore consumed in the production of vitamin K dependent coagulation factors and must be recycled by the enzyme vitamin K epoxide reductase (VKOR), also in the ER. [26] This is of clinical interest because warfarin exerts its effect by inhibiting VKOR: it prevents the recycling of vitamin K. Polymorphism in the gene encoding VKOR has been demonstrated, offering an explanation for variation between individuals in response to treatment with warfarin. [27]

This is of course a simplification of a complex and relatively recently described biochemical process: active reduction of vitamin K epoxide by VKOR occurs in two stages and requires interaction by several other proteins: protein disulphide isomerase, TMX and endoplasmic reticulum oxioreductin 1. An endogenous regulator, calumenin, interrupts these interactions and decreases production of reduced vitamin K. As a point of interest, calumenin is inactivated in order to increase the yield, in the commercial recombinant production of Gla-proteins such as FIX and FVIIa (Novoseven®). [28, 29]

Assays to measure deficiency of vitamin K include proteins induced by vitamin K absence (PIVKA)

All assays that measure activity or levels of vitamin K dependent coagulation factors ought to be affected by severe deficiency of this vitamin. Given that many coagulation factor levels can be reduced by more than 50% without any clinical decrease in coagulation, PT, aPTT and viscoelastic tests are insensitive to mild deficiency of vitamin K. Coagulation factors can be measured directly, but levels of these can be reduced because of other factors than deficiency of vitamin K. PIVKA is therefore theoretically an attractive test since they should only be elevated with precursors to coagulation factors have been synthesized yet the last stage of carboxylation has not occurred.

Interest in measuring PIVKA has been greatest in neonates, who in many parts of the world are given vitamin K as prophylaxis against haemorrhagic disease of the newborn. [30] The assay used in our research is PIVKA-II: inadequately carboxylated FII (thrombin). This assay has never become widespread and the literature concerning its clinical usefulness in the new-born is conflicting. [31, 32]

Materials and methods

The studies included in this thesis involve four types of research: literature review, a retrospective study of patients' notes, laboratory analysis of blood samples to investigate normal postoperative coagulation, and analysis of manipulate blood samples in order to test different laboratory methods' responses to change in coagulation.

Summary of methods used in Study I

Risk of epidural haematoma: pre- to postoperative dynamics of coagulative status in 358 patients undergoing oesophageal resection over ten years, and a review of the literature.

This study consists primarily of a retrospective review of all the patients who underwent oesophageal resection in Lund between 2002 and 2012. Patient notes were used to retrieve routine laboratory results and data concerning dosage of LMWH, when and why epidural catheters were withdrawn:

Table 4
results recorded in Study I

Test
Prothrombin time international normalized ratio (PT-INR)
Activated partial thromboplastin time (aPTT)
Platelet count (Plc)

Literature search

No suitable MESH terms were not found, so a literature search was conducted using the PubMed terms 'epidural' AND 'coagulation' AND English language. Relevant articles published in 2000 and after were included.

Summary of methods used in Study II

Correction of hypothermic and dilutional coagulopathy with concentrates of fibrinogen and factor XIII: an in vitro study with ROTEM®.

Coagulation was monitored with thromboelastometry (ROTEM®) in blood from 10 volunteers in 14 different conditions.

The following parameters measuring coagulation initiated by the extrinsic pathway, both with and without a platelet inhibitor, were recorded:

Table 5:
ROTEM® parameters recorded in Study II

EXTEM-CT (reagent activating extrinsic pathway, clotting time)
EXTEM-CFT (clot formation time)
EXTEM-MCF (maximum clot firmness);
FIBTEM-MCF (same activating reagent as EXTEM, with addition of a platelet inhibitor, maximum clot firmness)

Figure 8 shows the fourteen different conditions that were tested. Blood was collected in citrated vials and incubated at 33°C or 37°C.

To investigate the effect of haemodilution during normothermia and hypothermia, samples were diluted with ringer's acetate or 6% hydroxyethyl starch (HES) in 0.9% saline, such that 2/3 of the resultant mixtures consisted of citrated blood and 1/3 consisted of RA or HES.

Next, to investigate the effect of addition of fibrinogen and factor XIII on the aforementioned normo- or hypothermic, diluted or undiluted samples, concentrates of either fibrinogen alone or fibrinogen and FXIII were added in separate vials. The amounts of fibrinogen and FXIII corresponded to the clinical doses given to a patient weighing 70kg in massive haemorrhage (4g) and haemorrhage in patients with congenital FXIII deficiency (1550 IU).

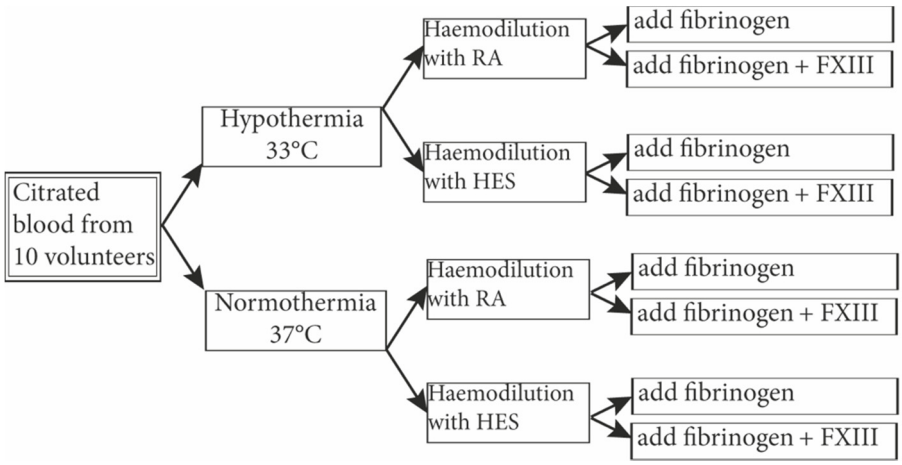


Figure 8
Diagram showing the 14 different states of hypothermia and haemodilution examined with thromboelastometry in Study II.

Summary of methods used in Study III

Thromboelastometry versus free-oscillation rheometry and enoxaparin versus tinzaparin: an in-vitro study comparing two viscoelastic haemostatic tests' dose-responses to two low molecular weight heparins at the time of withdrawing epidural catheters from ten patients after major surgery.

Coagulation was monitored at 37°C with thromboelastometry (ROTEM®) and free oscillation rheometry (FOR: ReoRox®) in blood collected from 10 patients who had undergone oesophageal resection, at the time of withdrawing their epidural catheters.

The samples were spiked with enoxaparin or tinzaparin to plasma concentrations of 0, 0.5, 1.0 and 1.5 IU/ml, a range which comprises both standard levels for thrombosis prophylaxis (0.2-0.4 IU/ml) and treatment levels for thromboembolism (0.5-1.0 IU/ml).

Coagulation was initiated with reagents that activate the intrinsic path of coagulation. The parameters shown in Table 6 were recorded.

Table 6:
parameters recorded in Study III from the blood samples spiked with various doses of enoxaparin or tinzaparin.

ROTEM activated with INTEM® (partial thromboplastin and ellagic acid):	**ReoRox® activated with thromboplastin:**
CT (clotting time)	COT1
CFT (clot formation time)	COT2
AA (alpha angle)	Slope
MCF (maximum clot firmness)	G'max
ML (maximal lysis)	ClotSR

Summary of methods used in Study IV

Monitoring low molecular weight heparins at therapeutic levels: dose-responses of, and correlations and differences between aPTT, anti-factor Xa and thrombin generation assays.

Blood was collected in citrated vials, from 10 healthy volunteers and spiked to concentrations of enoxaparin and tinzaparin of 0, 0.5, 1.0 and 1.5 IE/ml, a range which covers standard levels for thrombosis prophylaxis (0.2-0.4 IU/ml) and treatment levels for thromboembolism (0.5-1.0 IU/ml).

Coagulation was monitored with four different methods of measuring aPTT, one method of anti-FXa activity, and thrombin generation using initiating agents containing two different concentrations of tissue factor, in blood from 10 healthy volunteers:

Table 7:
Methods used and parameters recorded in Study IV.

Parameter recorded	**Type of analysis and apparatus**	**Reagent**
aPTT	Whole blood: ReoRox® free-oscillation rheometry (FOR).	Medirox MRX931®
	Whole blood: Hemochron Jr®	Hemochron Jr®
	Optical plasma analysis: Siemens BCS-XP®.	ActinFSL®
		PTT-Automat®
Anti-FXa	Optical plasma analysis: Siemens BCS-XP®.	Coamatic Heparin®
Thrombin generation = endogenour thrombin potential (ETP)	Fluorescent plasma analysis: Ceveron Alpha®	TGA-RB® (contains 2pM human tissue factor)
		TGA-RC® (contains 5pM human tissue factor)

Summary of methods used in Study V

Coagulative safety of epidural catheters after major upper gastrointestinal surgery: advanced and routine coagulation analysis in 38 patients.

Coagulation was monitored with routine tests of coagulation, thromboelastometry (ROTEM®) and coagulation factor measurement in blood from 38 patients undergoing elective major upper gastrointestinal surgery. Tests were taken on two occasions: immediately before surgery and at the time of withdrawing the patients' epidural catheters:

Table 8:
Assays run on each sample taken in Study V.

Parameter
ROTEM® EXTEM-MCF (Maximal clot firmness)
ROTEM® EXTEM-CT (Clotting time)
ROTEM® INTEM-MCF
ROTEM® INTEM-CT
ROTEM® FIBTEM-MCF
ROTEM® HEPTEM-MCF
ROTEM® HEPTEM-CT
Multiplate® AUC (Area under curve): ADPtest
Multiplate® AUC: COLtest
Multiplate® AUC: TRAPtest
Multiplate® AUC: ASPItest
P-FII
P-FVII
P-FX
P-FIX
P-FXI
P-FXII
P-FXIII
PT-INR (prothrombin time international normalized ratio)
aPTT (activated partial thromboplastin time)
Platelet count (Plc)
P-Fibrinogen
D-dimer
Gamma-Glutamyltransferase (GT)
C-reactive protein (CRP)
Bilirubin
Alkaline phosphatase (ALP)
Creatinine (Crea)
Haemoglobin (Hb)
PIVKA-II (Protein induced by vitamin K absence)

Main results

Study I

Risk of epidural haematoma: pre- to postoperative dynamics of coagulative status in 358 patients undergoing oesophageal resection over ten years, and a review of the literature.

Review of the literature

Figure 9 shows the number and topics of the articles that were found. Complete results are shown in the manuscript: an overview of the results is presented here.

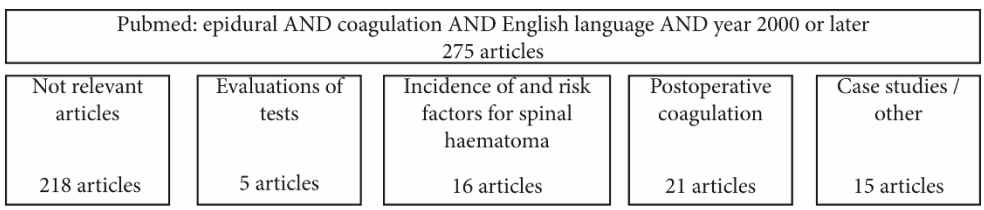


Figure 9:
Summary of literature search in Study I.

Articles concerning the evaluation of tests in the specific context of epidural analgesia

There is a discrepancy between routine and viscoelastic tests in the postoperative period: aPTT and PT-INR indicate hypocoagulation whereas ROTEM® indicates normocogulation or hypercoagulation, even in the first postoperative days after major surgery when routine tests typically show coagulopathy, and in patients treated with warfarin. [2, 33]

Two studies suggest that viscoelastic tests can likely be used to monitor LMWH. [34, 35]

Articles concerning the incidence of, and risk factors for spinal haemorrhage

Sixteen articles were found, which generally use one or more of the following strategies:

1. **Observational studies of routine care**. Few or no spinal haematomas occur. Confidence intervals for the actual risk of spinal haematoma are calculated, sometimes for subgroups in which the risk of haematoma ought to be increased, such as those with incidentally elevated PT-INR. No study observes enough adverse events to be able to analyse causes of spinal haematomas [36-41].Two of the studies' authors conclude that test results are abnormal in so few patients without clinical risk factors for coagulopathy, that it is not worth screening with them. [37, 39] They do not, however, address what to do when a patient *does* have risk factors and then turns out to have abnormal routine tests of coagulation. The only laboratory test which is accepted by all to be indicated is a platelet count after treatment with heparin or LMWH for more than three days, to exclude heparin induced thrombocytopenia (HIT).
2. **Experimental or observational pharmacological studies**. Kassis measured anti-FXa and anti-FIIa activity in blood tests at intervals before, 2 and 4 hours after administering subcutaneous heparin, to reach the conclusion that it is safe to place or withdraw an epidural catheter 2 hours after administering subcutaneous heparin. [42] Leonard investigated the antiplatelet effect of levobupivacaine in concentrations equivalent to plasma after an epidural bolus, and in the epidural space when a blood patch is given. Systemic levobupivacaine shouldn't affect platelet function but levobupivacaine in a blood patch may do. [43]
3. **Identification of risk factors for spinal haematoma by finding small numbers of patients with a spinal haematoma in large registries of patients**. These studies identify the clinical risk factors shown below. [39-41]
4. **Observational studies of controversial protocols that cannot be described as *lege artis***. For example Pastor and Canto place epidural catheters one hour before full heparinization for extra corporeal circulation for heart operations; Liu withdrawew epidural catheters despite the PT-INR being as high as 7.1 [44-46]. The same statistics are applied as in strategy 1: these studies are relatively small and do not report any spinal haematomas. 95% confidence limits for the actual risk of haemorrhage are calculated and are in the region of 0-1:300.

Risk factors for spinal haematoma associated with epidural analgesia (see above):

- Liver failure or hepatic resection
- Major intraoperative haemorrhage or transfusion of blood products
- Drugs that may affect haemostasis, such as aspirin, NSAID's, tricyclic antidepressants or antiplatelet drugs
- Having abnormal anatomy, in particular spinal stenosis.
- Female sex
- Impaired renal function
- Type of surgery
- Thrombocytopenia or platelet dysfunction
- Patient age

Articles concerning the clinical pathophysiology of postoperative coagulation in the context of epidural analgesia

Nineteen articles were found, mainly concerning coagulation in patients who had undergone hepatectomy. Just one study included patients who had undergone oesophagectomy. Almost all the studies recorded PT or PT-INR, aPTT and Plc daily for the first week after surgery. Almost without exception they found a transient dip in Plc during the first 2-3 postoperative days, accompanied by a slightly shorter prolongation of the PT and sometimes aPTT.

Tsui investigated the proportion of patients with coagulopathy necessitating delaying withdrawal of an epidural catheter [47]. The risk was highest after hepatic resection, lower in major abdominal surgery and lowest in orthopaedics.

Bergman replies to Weinberg, that 'prothrombin is not the whole story' in postoperative coagulation after hepatic resection [48, 49] and points out that these patients are prone to thrombosis despite elevated PT-INR and aPTT. This may be due to deficiencies in antithrombin or protein C, which are not detected by the PT assay. Mohammed describes a prospective study of patients undergoing hepatectomy for liver donation, in which ROTEM® is compared with routine tests of coagulation. The transient hypocoagulability indicated by routine tests was not demonstrated by ROTEM® but ROTEM® did not indicate postoperative hypercoagulability either. [50]

Articles presenting case reports or other relevant research

9 relevant case studies, 4 reviews and a report from a postal survey were found: see Supplementary File 3. Two reports describe complications arising as a result of treating a laboratory test indicative of hypocoagulability, in an attempt to reduce the risk of spinal haematoma: Chaney describes administering vitamin K to a patient to correct an

elevated PT-INR five days after cardiac surgery, after which the patient had a thromboembolic stroke. [7] Lim describes a series of patients who received plasma transfusions to correct elevated PT-INR's. One developed anaphylaxis. [51]

Chung, Fakouri and Goswami describe patients who would not be expected to be at a high risk of spinal haematoma, but who developed one anyway. [52-54] Ladha presents a case of a patient with elevated liver function tests but not coagulopathy before gastrectomy with epidural analgesia. She developed a postoperative deficiency of vitamin K dependent coagulation factors and required urgent decompression of a spinal haematoma. [55]

Finally, there are two studies describing cases where spinal haematoma was not expected, but was preceded by an abnormal course of events: Özdemir describes a patient who developed a late chronic intracranial subdural haematoma after inadvertent dura puncture at epidural catheterization. [56]. Walker describes a spinal haematoma in a patient with normal routine coagulation status but various other risk factors for spinal haematoma: major haemorrhage with massive transfusion, multiple attempts at epidural catheterization including a bloody tap and ROTEM® parameters indicative of coagulopathy [57].

Results from the retrospective journal study

307 patients received a thoracic epidural infusion with bupivacaine and morphine while 51 received an intravenous morphine infusion. Tests taken preoperatively and before planned withdrawal of the epidural catheter demonstrated increases in all three measures: aPTT (activated partial thromboplastin time), PT-INR (prothrombin international normalized ratio) and platelet count (Plc). Postoperative thrombocytopenia was almost non-existent while aPTT or PT-INR were elevated in around half of patients: aPTT was elevated in 97 of the 202 patients whose test results were complete. See Figures 10 and 11.

See the original manuscript for details of epidemiological data and other laboratory test results.

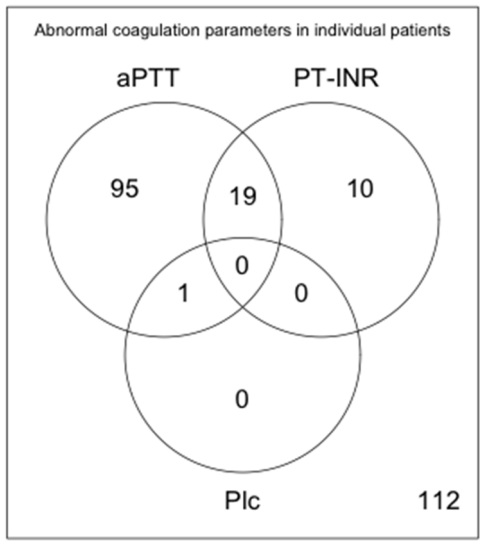


Figure 10:

Venn diagram showing which, and how many, routine coagulation tests in Study I indicated coagulopathy in the patients whose coagulation status was complete.

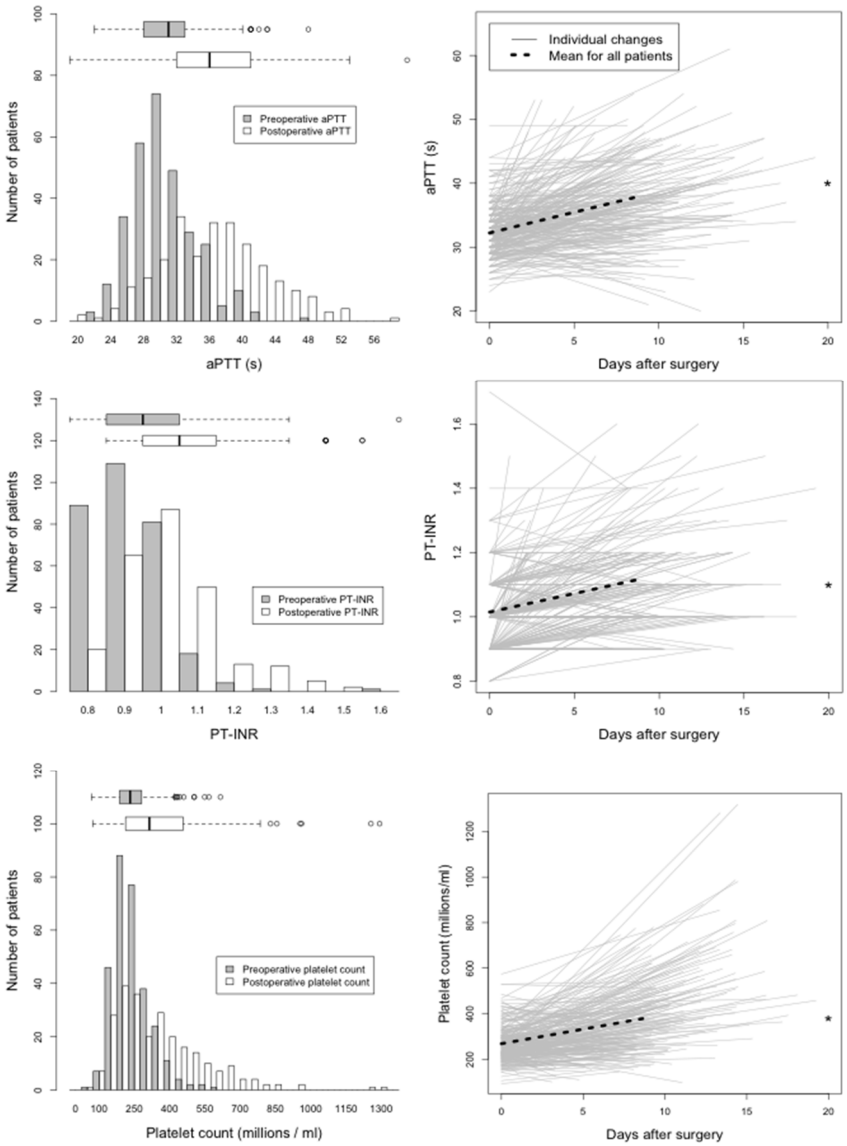


Figure 11:
Results from Study I: dynamics of results of routine tests of coagulation. Thin grey lines: individual patient's test results. Thick dotted lines: mean changes. * $p<0.05$.

Study II:

Correction of hypothermic and dilutional coagulopathy with concentrates of fibrinogen and factor XIII: an in vitro study with ROTEM.

Activation of coagulation was impeded by hypothermia but values were mainly within the manufacturers normal ranges; clot stability was significantly lower in hypothermia only in the haemodiluted samples:

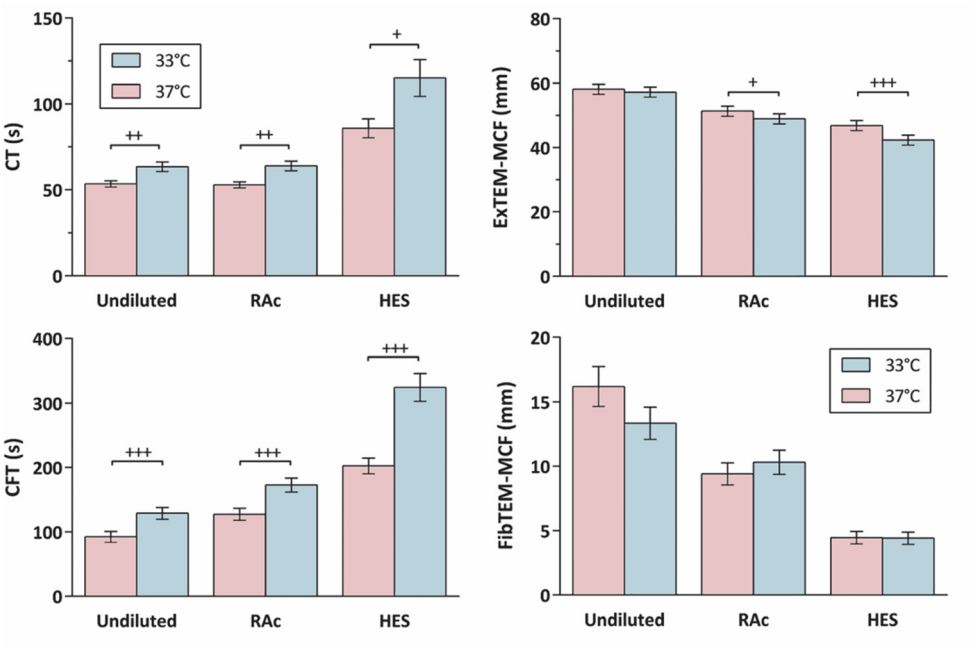


Figure 12:

Results from study II: hypothermia significantly slows clot activation and clot formation rate but not clot stability. Haemodilution with 30% ringer's acetate (RA) or HES (hydroxyethyl starch) decreases clot formation rate and stability.

Fibrinogen with or without factor XIII improved almost all ROTEM® parameters: irrespective of temperature. Addition of fibrinogen concentrate increased FIBTEM-MCF more in the samples diluted with RA than HES, particularly in presence of factor XIII:

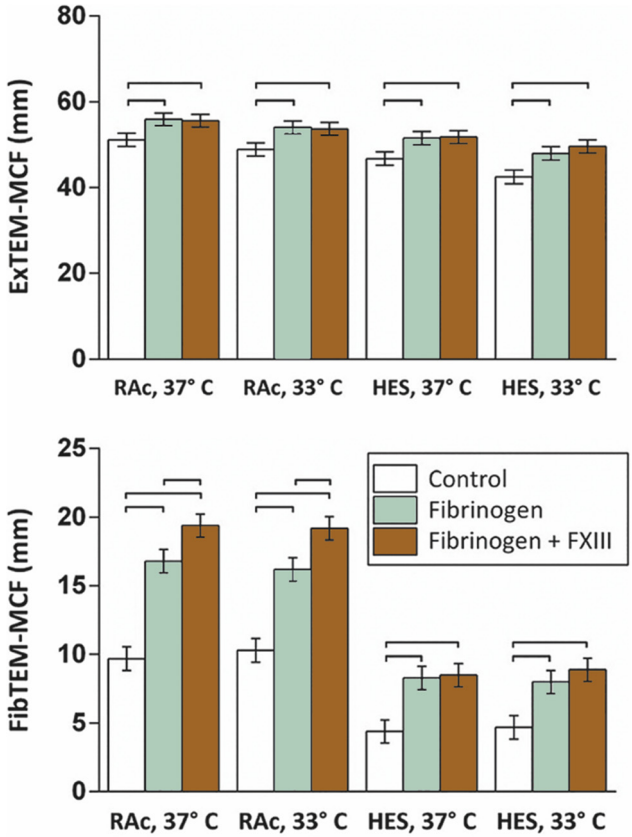


Figure 13:

Results from Study II: the low clot stability caused by 30% haemodilution with ringer's acetate (RAc) and HES (hydroxyethyl starch) is corrrect in vitro by addition of fibrinogen or fibrinogen and factor XIII. The magnitude of correction provided by fibrinogen + FXIII was significantly greater than fibrinogen alone when the haemodilution had been caused by RA but not HES.

Study III

Thromboelastometry versus free-oscillation rheometry and enoxaparin versus tinzaparin: an in-vitro study comparing two viscoelastic haemostatic tests' dose-responses to two low molecular weight heparins at the time of withdrawing epidural catheters from ten patients after major surgery.

Clot initiation time was increased significantly by addition of each of the LMWH's tested, although there was significant inter-individual variation. Tinzaparin prolonged clot initiation times more than enoxaparin when the two drugs were given in equal doses of anti-FXa units.

None of the measures of clot stability or clot formation rate showed a dose-response to either LMWH. Clot lysis showed a tentative negative dose-response to the drugs.

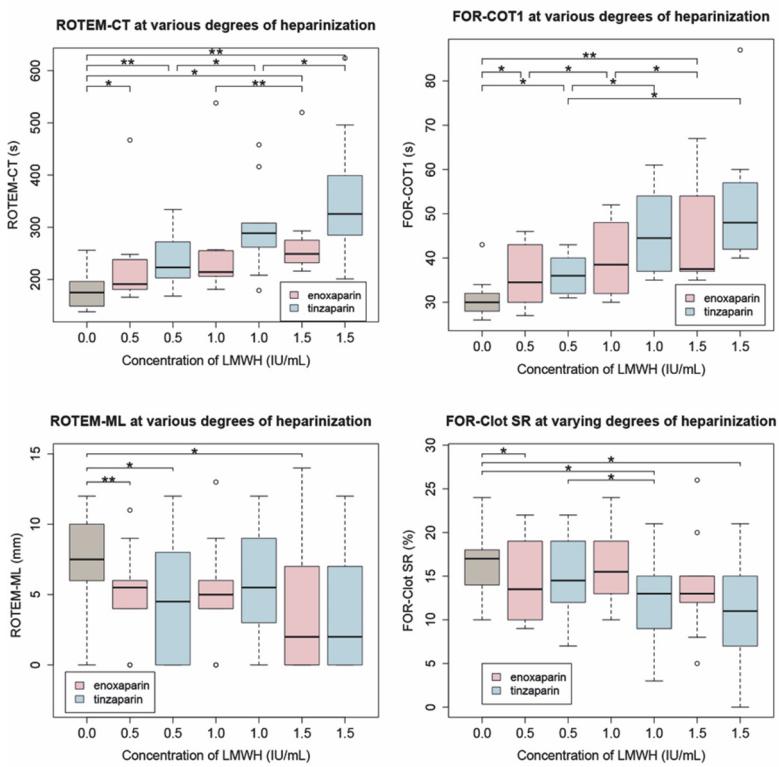


Figure 14:
Dose-response of measures of clot initiation to LMWH and tentative negative dose-response of measures of clot lysis to concentration of LMWH.

Study IV

Monitoring low molecular weight heparins at therapeutic levels: dose-responses of, and correlations and differences between aPTT, anti-factor Xa and thrombin generation assays.

There was no significant difference between the anti-FXa assay's dose-responses to tinzaparin or enoxaparin:

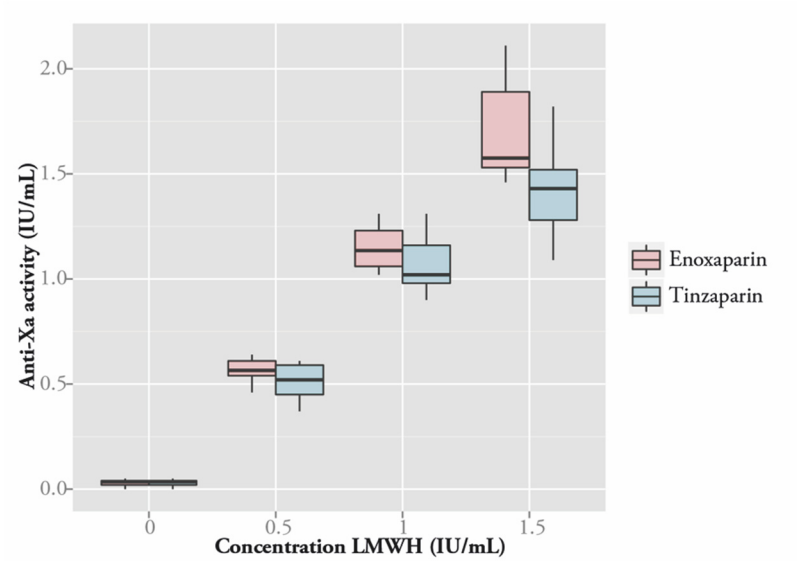


Figure 15:
Anti-Xa test's dose response to enoxaparin and tinzaparin dosed in international units of anti-Xa activity.

All the aPTT assays exhibited a linear dose-response to LMWH but the individual methods give somewhat different results, particularly at higher concentrations of LMWH: the whole-blood methods (Hemochron Jr and free-oscillation rheometry) gave on average significantly higher results than the optical methods. See Figure 16.

Methods' mean aPTT at 1.0 IU/mL LMWH varied between 54s (SD 11) and 69s (SD 14) for enoxaparin and between 101s (SD 21) and 140s (SD 28) for tinzaparin. Tinzaparin resulted in greater inhibition of coagulation than enoxaparin, as measured by aPTT or TG, when given in equal units of units of anti-FXa activity.

Thrombin generation (TG; ETP) with the reagent containing more tissue factor showed a negative exponential dose-response to LMWH and is sensitive over the therapeutic range of LMWH concentrations. See Figure 17.

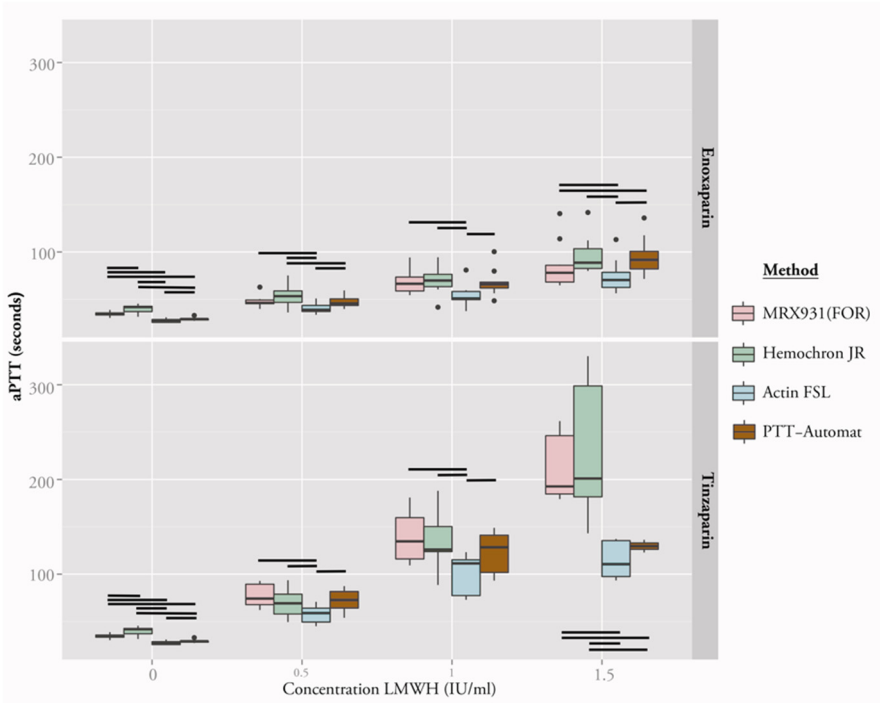


Figure 16:
Dose-response of aPTT to increasing doses of enoxaparin and tinzaparin, using four different methods:

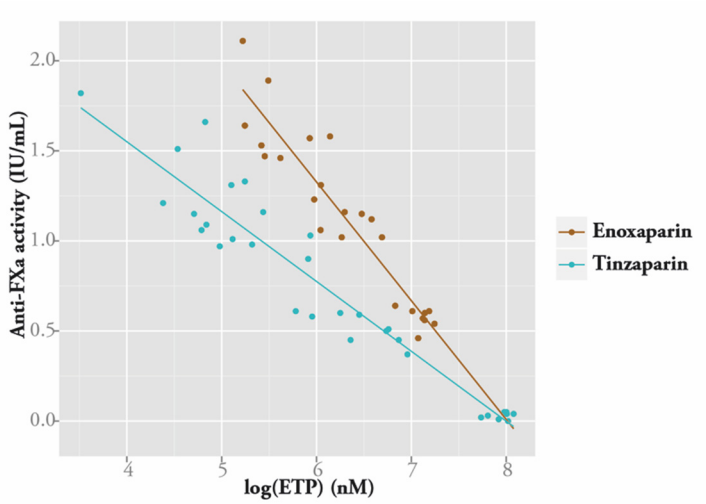


Figure 17:
Thrombin generation is decreased by increasing doses of anti-FXa in a negative logarithmic fashion that also reflects tinzaparin's stronger anti-FIIa effect compared to enoxaparin:

Study V

Coagulative safety of epidural catheters after major upper gastrointestinal surgery: advanced and routine coagulation analysis in 38 patients.

Patients' mean age was 70 +/- 7 years, the average length of operation was 10.4 hours and the mean time between operation and withdrawing epidural catheter was 6.2 days (range 2 to 15 days). The most common indication for operation was gastric cancer.

Routine tests of coagulation at the time of withdrawal of epidural catheters showed thrombocytosis and hyperfibrinogenaemia, and slightly elevated results for PT-INR and aPTT (see Figure 18 and Table 9). The mean PT-INR at the time of withdrawal of epidural catheters was 1.2 ± 0.2 and the mean aPTT was 30 ± 4s: slightly elevated compared to the preoperative tests.

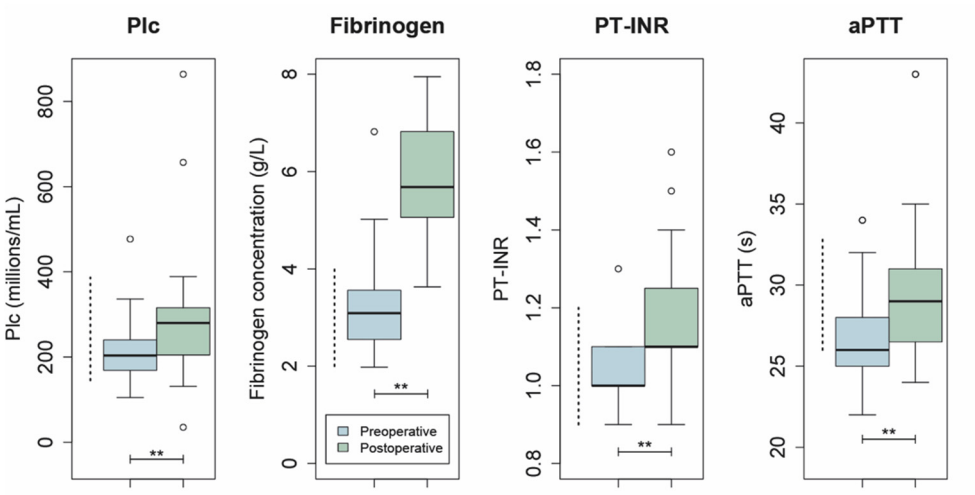


Figure 18:
Routine tests results taken prior to operation and at the time of withdrawal of patients' epidural catheters. **: P<0.05:

CRP increased pre- to postoperatively, but liver function tests only increased slightly and serum creatinine actually decreased. Average serum albumin was within the reference interval:

Table 9:

Routine laboratory test results: postoperative results having been taken at the time of withdrawal of epidural catheters. Stars indicate significant differences between pre- and postoperative results (*:P<0.05, **:P<0.01).

	Preoperative results	**Postoperative results**	**Reference range**
PT-INR	1.0 ± 0.1	1.2 ± 0.2 **	0.9-1.1
aPTT (s)	27 ± 3	30 ± 4 **	26-33
Plc (x 109/L)	213 ± 153	283 ± 153 *	145–387
Fibrinogen (g/L)	3.2 ± 0.9	5.8 ± 1.1	2.0-4.0
CRP (mg/L)	6.8 ± 9.4	92 ± 60	<3.0
Serum albumin (g/L)	36.6 ± 3.7	-	36-45
Serum creatinine (µmol/L)	73.4 ± 19	65.2 ± 18.3 *	45-105
Serum bilirubin (µmol/L)	14 ± 22	10 ± 16 *	5-25
ALP (µkat/L)	1.64 ± 1.1	2.30 ± 1.3 *	0.6-1.8
Bilirubin (µmol/L)	14 ± 22	11 ± 16 *	5-25
GT (µkat/L)	1.3 ± 2.0	2.1 ± 1.9 *	0.2-1.9
Blood haemoglobin (g/L)	119 ± 16 g/L	113 ± 12 g/L	Men: 134-170; women: 117-153

Whole blood tests of analysis: ROTEM® and Multiplate® results are shown graphically in Figure 19. ROTEM® Clotting Times, measures of clot initiation, were not significantly different postoperatively compared to preoperatively, but Maximum Clot Firmness increased significantly. Interestingly, there was no demonstrably significant correlation between the measured level of fibrinogen and ROTEM®-MCF when clot initiation was provided by the FIBTEM reagent which contains a platelet inhibitor.

Multiplate® measures of platelet function increased significantly when activation was provided by the COLtest and ASPItest reagents, which contain collagen and arachidonic acid respectively.

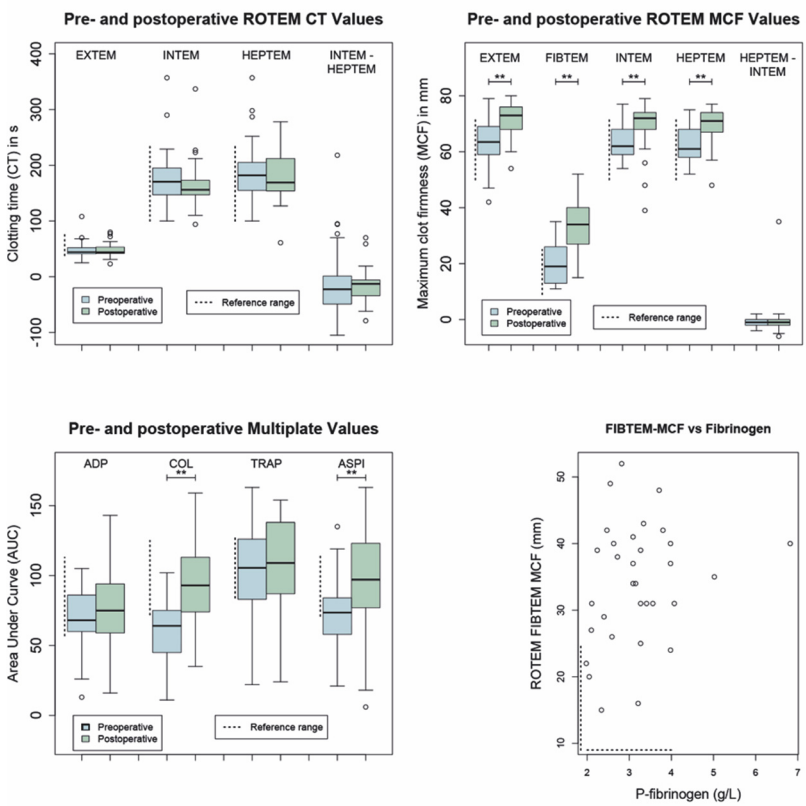


Figure 19:

ROTEM® and Multiplate® results showing pre- to postoperative changes. Postoperative tests were taken at the time of withdrawing epidural catheters. Note that (HEPTEM-INTEM) results are centred around 0, and that was no significant correlation between ROTEM-FIBTEM-MCF and fibrinogen levels. Stars indicate significant differences between pre- and postoperative results (*:P<0.05, **:P<0.01).

Figure 20: shows the changes in levels of individual coagulation factors over the perioperative course: the postoperative values represent tests taken at the time of withdrawal of epidural catheters. We expected a significant decrease in the levels of vitamin K dependent coagulation factors (FII, FVII, FIX, FXI) but this was not the case. In fact the level of FIX increased significantly. The level of factor XII decreased significantly.

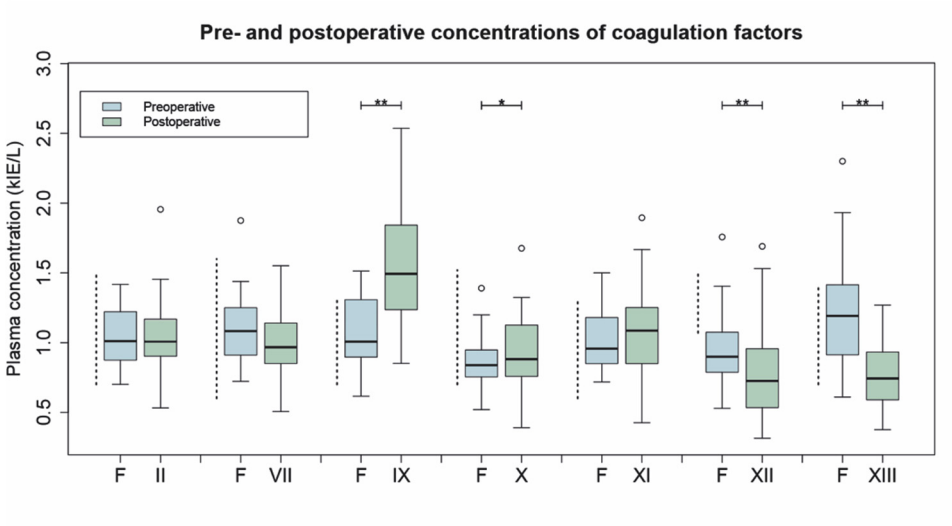


Figure 20:
Individual coagulation factor levels before surgery and at time of withdrawal of epidural catheters. The dotted lines indicate the reference interval. *=P<0.05; **=P<0.01.

See original manuscript for plots showing relationships between coagulation factor levels and other measures of haemostasis: there are significant but weak correlations between FVII and PT-INR and EXTEM-CT; and between levels of FII, FX, FXI, FXII and aPTT.

Discussion

Coagulation after major surgery is biphasic: first relative hypocoagulation then hyperfibrinogenaemia and thrombocytosis

Major surgery causes a coagulopathy by several mechanisms including haemorrhage, hypothermia and haemodilution. This period is most likely the most dangerous period to place or manipulate an epidural catheter: a careful clinical assessment and comprehensive testing of coagulation should be done if necessary. There is insufficient evidence to say that one test is more reliable than another in this setting.

Following this period, systemic inflammation takes over and patients become prone to thrombosis despite both aPTT and PT-INR indicating mild coagulopathy. It is likely that thrombocytosis and hyperfibrinogenaemia compensate for relative deficiencies of coagulation factors, although the levels observed in Study V were probably not depleted enough to cause hypocoagulation.

An unresearched hypothesis would be that these tests demonstrate a potential coagulopathy that is evident ex vivo in platelet-free plasma, that is compensatd for by thrombocytosis and hyperfibrinogenaemia. The case study found in Study I, where a subjectively healthy person unexpectedly developed a deficiency of vitamin K dependent clotting factors, meaning that a high level of suspicion is necessary, particularly in patient groups that may be malnutritioned. [55]

Why do PT-INR and aPTT indicate hypocoagulation but platelets and viscoelastic tests show normo- or hypercoagulation?

PT-INR and aPTT are not greatly affected by fibrinogen levels or platelet count, both of which increase in postoperative inflammation. Neither do they measure the fibrin-stabilizing effect of FXIII. Tentative explanations from Study V's results are that these assays results are prolonged by decreases in factors VII and XII respectively. Levels of FXII have previously been shown to be decreased in inflammation [58] which is theoretically consistent with the postoperative state. Postoperative thrombocytosis is a known component of postoperative inflammation [59].

Given that the risk of thrombosis is increased in the postoperative period, necessitating thrombosis prophylaxis, and that these tests have not been shown to be predictive of haemorrhage in surgery, the slightly increased PT-INR and aPTT results which appear to be part of the normal postoperative response would appear to be unreliable predictive tests for spinal haematoma upon withdrawal of epidural catheters. There is, however, no empirical evidence of high quality supporting this conclusion.

Risk-benefit ratio: estimating the risk of rare events from series of non-events make this a difficult area to research

Major surgery is a high-risk activity, which patients choose to undergo because of the potential benefits of being cured of serious conditions, in the context of this thesis mainly upper gastrointestinal cancer. Each component of a patient's therapy, including analgesia by epidural catheterisation, ought to be subjected to a risk benefit analysis and only administered when the magnitude and chances of benefit exceed the risks, and their severity. During the past decade epidural analgesia has lost its gold standard status for analgesia after some types of surgery since an unwanted effect of epidural infusions is that they may impede mobilisation, which increases the risk of some post-operative complications:

Table 10:
Comparison of the risks and benefits of epidural and 'multimodal' analgesia

	Epidural analgesia	Intravenous and enteral analgesia with combination of drugs and sometimes a peripheral block ('multimodal analgesia')
Benefits	Ideally total analgesia at site of operation Avoids some opiate side-effects Decreased risk of pulmonary pneumonia in high-risk patients [60]. Increased peristalsis (in addition to absence of opiate side-effects). Likely decreases risk of myocardial infarction after major vascular surgery	Technically easier to administer Potentially less complicated weaning resulting is faster mobilisation in some patients Less labour intensive
Risks/ Side effects	Risk of incomplete of failed block Risk of haemodynamic compromise Risk of respiratory depression if opiates are added, particularly in elderly patients. Risk of spinal haematoma Risk of immobilisation increasing the risk of post-operative thrombosis and respiratory tract infection. Labour intensive, expensive	Opiate side-effects: somnolence, respiratory depression, gastric retention and decreased peristalsis. Increased risk of atelectasis and pneumonia if pain restricts deep breathing.

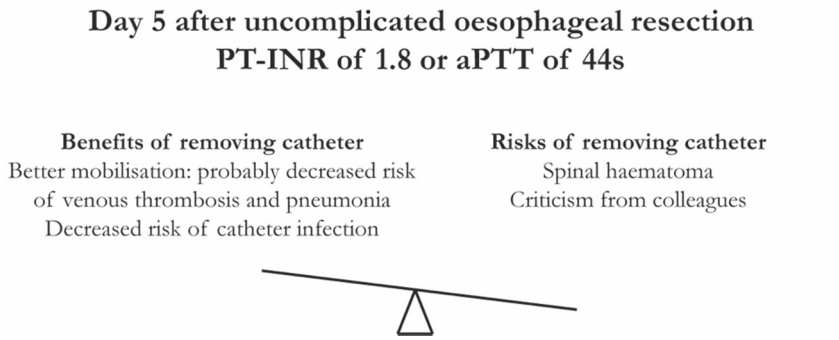
Adapted from Rawal, 2012 [61]

The rationale for epidural analgesia being removed from some standard treatment pathways is that risk/benefit analyses favour multimodal analgesia over epidural analgesia. The PROSPECT Study, for example, found this to be the case in patients undergoing laparoscopic colorectal surgery. [4]

Even in the types of surgery where epidural analgesia is clearly indicated, patients who have experienced a serious haemorrhagic complication are not so much interested in population statistics as that they could have received a less invasive form of analgesia and avoided their complication, even if they had run a slightly higher risk of other complications. The risk of spinal haematoma in patients treated with neuraxial blocks is discussed earlier and we can expect more data of high quality on this subject from prospective national and international studies and quality registers such as Sweden's SPOR (Swedish Perioperative Registry www.periop.se.)

This thesis is concerned primarily with the risk of spinal haematoma when an epidural catheter has been in place for a number of days after major surgery and routine tests show slightly abnormal values:

Clinical scenario demonstrating the risk-benefit dilemma of finding slightly elevated routine coagulation parameters in a patient who no longer needs epidural analgesia:



This clinical scenario is the crux of this thesis: when the acute pain team's clinical assessment is that it's time to remove the epidural catheter, delaying so may actually harm the patient. If delayed withdrawal is combined with holding thrombosis prophylaxis or even administration of prothrombotic products such as plasma or prothrombin complex concentrate, the patient's risk of thrombotic complications such as deep vein thrombosis or pulmonary embolus would also be expected to increase.

The source of the clinician's dilemma in the above scenario is that the actual risks of the benefits and risks are not quantified: in fact, the slightly slow activation of clotting that is indicated by the elevated PT-INR and aPTT may well be compensated for by the postoperative hypercoagulable state such that there is actually no risk associated with withdrawing the catheter. [62]

Correction of PT-INR with vitamin K and prothrombin concentrate and plasma

The Nordic guidelines recommend correcting an increased PT-INR identified the day before planned epidural catheterization by administering vitamin K which may exert its effect overnight, or in emergencies PCC (prothrombin complex: Ocplex®, Confidex®). [63] PCC contains the vitamin K dependent procoagulants FII, FVII, FIX and FX, the anticoagulant Protein C and its cofactor Protein S and a small amount of heparin. Data enabling a risk-benefit analysis of administration of PCC to correct a slightly elevated PT-INR on day 5 after major surgery is unclear: neither the risk-reduction for spinal haematoma on withdrawal of an epidural catheter, nor the increase in risk of deep vein thrombosis is known in this situation. [6]

Estimating risk from a series of non-events

People generally subjectively underestimate risk from their own experience of a limited number of events: having watched 30 people jump from a 3 metre cliff into a lake one at a time, all without injury, most of us would be happy to do the same. There may, however, be an underwater rock that unbeknown to us often causes unsuspecting tourists to limp away with a broken ankle. How high a risk of injury on each jump would be considered acceptable given that the lake is jumped in to by 50 people per day during the whole summer (ie 5000 jumps per year?) 50%? 10%? 1%? 1‰? 0.1‰? These risks equate to 2500, 500, 50 and 5 and 0.5 broken ankles during an average season.

Assuming that injury occurs randomly, independently and with fixed risk, Hanley's simple formula can be applied: the upper limit of the 95% confidence interval for the actual risk of the negative event occurring is $3/n$ where n is the number of 'non-events'. In this example, there is a 5% chance that the risk of injury on each jump from the cliff is more than 3/30 = 10%, so we can be fairly confident that the risk is between 0 and 10%. [64]

If we wanted to be sure that the risk of breaking an ankle was less than 1‰ or 0.1‰ respectively, we would have to observe 300 or 3000 jumps without injury. Given that the water level, and therefore the risk of injury, can vary considerably during a season and between years, simply observing jumps is not a suitable way of estimating the risk: investigating the depth and underwater appearance of the lake would be required, for example by sounding or diving.

This analogy applies to the risk of spinal haematoma in the presence of elevated routine tests of coagulation: Liu et al. [45] tested the hypothesis that PT-INR during the first days of thrombosis prophylaxis with warfarin in patients with an epidural catheter in place would not be predictive of spinal haematoma. 4365 patients were included, of which 700 had a PT-INR of 2.0 or more and 89 had a PT-INR of 3.0 or more. The upper limit of the 95% confidence interval for the true risk of spinal haematoma can therefore be calculated with Hanley's formula to be 0.4% and 3.4% respectively.

The actual risk may well be lower than these values, but they are in any case well above the acceptable risk of spinal haematoma and the ethics of subjecting patients to an unknown risk of a serious complication, that could be as high as 3%, against the advice of current guidelines would be problematic. An additional problem of applying risk analyses reached in observational studies to today's and tomorrow's patients, analogous to the water level in the above-described hypothetical lake, is that the risk of spinal haematoma may well not be constant from year to year since there are many factors potentially affecting the risk of this complication:

Table 11:
Potential factors affecting risk of spinal haematoma, that may vary between past clinical series' findings and today's and tomorrow's clinical praxis

Differences in fluid treatment regimes: more use of synthetic colloids in older studies, less tendency to accept dilutional coagulopathy in Scandinavia.
Differences in use of blood products and concentrates of coagulation factors.
Varying use of synthetic colloids such as HES.
Different traditions of thrombosis prophylaxis (some American institutions use vitamin K-antagonists while these are recommended against in European studies). Recommended doses of LMWH can vary.
Probably less attention to adequate postoperative hydration and renal function in older observational studies.
Varying equipment: needles and epidural catheters different between centres and over time.
How is diagnosis 'spinal haematoma' reached? Varying tendency to investigate with MRI (magnetic resonance imaging) in older studies?

Specific and whole-blood tests of LMWH effect.

Studies III and IV compare various methods of measuring LMWH activity. That anti-FXa activity showed a linear correlation to the number of units of anti-FXa activity was reassuring. A somewhat surprising find was that aPTT correlated reasonably well to the concentration of LMWH. Tinzaparin's greater effect on aPTT than enoxaparin when dosed in units of anti-Xa activity is in theoretical agreement with tinzaparin having more anti-FIIa activity than enoxaparin, and this would go some way to support the hypothesis that aPTT a clinically relevant test of coagulation in the postoperative period.

The results confirm that different aPTT assays vary somewhat in their sensitivity to LMWH, which is a result of the methods and reagents not being standardized. Is there then a role for aPTT in risk-reduction or risk-elimination around the time of withdrawing an epidural catheter? Given that aPTT is universally available and inexpensive, it may be used as a screening test for postoperative coagulopathy. An elevated of aPTT more than an arbitrary 30% above the upper limit of the reference local interval ought to raise suspicion of coagulopathy. The following should be borne in mind:

1. A slightly elevated aPTT is normal in the immediate postoperative period despite these patients being prone to thrombosis.
2. An aPTT may also be indicative of excessive anti-FXa or anti-FIIa activity by accumulation or overdosage of LMWH.
3. A clinical assessment of the patient's coagulative state is necessary.

The only ROTEM® and FOR parameters that were significantly affected by LMWH were measures of clot initiation. A clear advantage of so-called 'patient-near' assays is that they give a preliminary result within minutes whereas routine hospital tests

generally take at least 45 minutes. This is of major significance in acute situations such as continuing haemorrhage or cardiothoracic surgery. One can imagine a situation in which a patient with renal failure and a strong indication for epidural catheterization, and who is treated with LMWH, arrives to the operating theatre without having taken preoperative tests. Waiting 10 minutes for a ROTEM® INTEM CT within the normal range would probably be acceptable but sending a routine aPTT or anti-FXa, stalling the operating theatre for over half an hour would be unacceptable.

Relevance of Factor XIII for epidural analgesia in the postoperative period, and the impracticality and cost of screening for tentative risk factors for haemorrhage

In study V, FXIII levels were significantly lower at the time of withdrawing epidural catheters than before surgery. Whether this is clinically significant is unclear: the ROTEM® measure of clot stability, MCF, showed a weak correlation to FXIII levels but none of the patients actually had a low MCF. Nevertheless, previous case studies of patients with clinically apparent deficiencies of FXIII had lower levels than some of the patients in this study. [65]

Factor XIII concentrate can be given intravenously (Cluvo®) but the work presented in this thesis cannot provide answers to the very relevant questions of whether this is ever clinically indicated, and how to identify patients who would benefit from a FXIII infusion. The assay for FXIII currently costs around 650SEK (58GBP) such that even if this test were to demonstrate sensitivity and specificity for spinal haematoma nearing 100%, the cost of testing for FXIII level on enough patients to likely include one spinal hematoma (ie 40 000 tests) would be in excess of £2 million. At present the only approved indication for transfusion of FXIII is congenital deficiency: there are six known patients with this condition in Sweden.

Future aspects

The above discussion of FXIII exemplifies how difficult it is to answer simple questions in this field: the small-scale yet fairly expensive research described in this thesis has generated the theoretically attractive hypothesis that FXIII deficiency may be a risk factor for spinal haematoma upon withdrawal of epidural catheters. A prospective study large enough to include even a small number of actual haematomas would economically indefensible. A more realistic approach to advancing knowledge in the empirical

laboratory findings associated with spinal haematoma would be to prospectively include patients who have been unfortunate enough to have had a spinal haematoma, and to compare their laboratory results with matched controls.

Given a rate of spinal haematoma of 1/40 000 and an annual incidence of major upper gastrointestinal surgery within the population of 1/3 000, the population needing to be studied for a period of one year in order to observe 50 spinal haematomas in patients after major gastrointestinal surgery would be 50 * 3 000 * 40 000 = six thousand million people. The population of the EU is around 500 million people, such that a prospective study in which patients with spinal haematomas were included, would take around 10 years. Maintaining interest in a continental-wide prospective study for this period may be problematic.

A clinical scoring system or algorithm for risk-stratification provided as part of international guidelines may be appropriate to decide when testing is appropriate, and which tests should be run rather than just when an epidural catheter may be withdrawn. The above difficulties in carrying out research in this area mean that any such scoring system must consist of 'expert opinion' rather than the results of empirical research.

Such an algorithm would take into account:

1. 'Immediately postoperative' risk factors for hypocoagulation during the first few days after surgery, for example dilutional coagulopathy and hypocalcaemia after transfusion. Routine and possibly viscoelastic testing after complicated surgery is likely indicated in this period.
2. The probable unnecessity of testing PT-INR and aPTT in the following period provided that the patient is not malnutritioned or septic, in renal failure, treated with high doses of LMWH or otherwise not recovering as expected.
3. The risk of heparin induced thrombocytopenia (HIT) after more than 3 days' treatment with LMWH or UFH, necessitating screening with platelet count.
4. That patients who have been given platelet inhibitors or oral anticoagulants deserve special attention and testing.
5. That the risk of spinal haematoma is greater in certain categories of patient than others, and after some types of surgery more than others.

The major advantage of such a scoring system would be that it could be prospectively evaluated and validated, rather like the CHADS system for predicting risk of stroke due to atrial fibrillation. [66]

That the universally accepted gold standard for perioperative thrombosis prophylaxis is still given in the form of a subcutaneous injection is remarkable: the practice is expensive in terms of nurse-labour, and uncomfortable for patients. It is therefore likely that non-vitamin K dependent oral anticoagulants (NOAC) will replace LMWH, which will place new demands on perioperative clinicians if these drugs are to be given safely. A detailed discussion of these drugs is outside the scope of this thesis, but attention must be paid to the fact that these drugs have varying target sites (some inhibit FXa, others FIIa), pharmacokinetics and responses to laboratory tests. [67]

Key points

1. Spinal haematoma is a serious and feared complication of epidural analgesia which is mostly likely to occur when catheters are placed or removed.
2. Epidural analgesia likely decreases mortality and morbidity in patients undergoing major upper gastrointestinal surgery but a quantitative risk-benefit analysis is not possible.
3. Risk factors for spinal haematoma include abnormalities in coagulation, patient characteristics and whether the insertion of the catheter was traumatic.
4. Of the three current routine tests of coagulation, only the platelet count is of definite benefit since it can exclude heparin induced thrombocytopenia (HIT).
5. Coagulation in the postoperative period is biphasic: an initial hypocoagulation lasts for a few days and may be caused by factors related to major surgery: haemodilution, hypothermia, loss of coagulation factors and platelets in haemorrhage. Thereafter follows an inflammatory state characterized by thrombocytosis and hyperfibrinogenaemia in which PT-INR and aPTT are generally still slightly elevated.
6. The reason for aPTT and PT-INR being slightly elevated in the postoperative period is likely due to decreases in FXII and FVII respectively.
7. Because of this, PT-INR and aPTT probably do not have a role in screening for coagulopathy in patients after the third or fourth postoperative day, provided that they do not have other risk factors for abnormal haemostasis.
8. A clinical assessment of patients' risk of spinal haematoma should be part of routine care. Risk factors including age, female, renal failure, traumatic insertion of epidural catheter, major surgery especially if haemorrhage and transfusion of blood products occurred, should lead to intensified laboratory testing even interpretation of laboratory results is difficult.
9. An elevated aPTT may be indicative of accumulation of LMWH but a normal value cannot exclude this.
10. There is variation in the results given by different methods and reagents for measuring aPTT.

11. Thrombin generation may be a more suitable assay for measuring LMWH activity than anti-FXa activity and aPTT since unlike the anti-FXa assay, it detects anti-IIa activity without showing so much variation as the aPTT.

12. Viscoelastic haemostatic tests and tests of platelet function may be used although they cannot be said to be validated for estimation of risk of spinal haematoma.

13. A comparison of free-oscillation rheometry (FOR, ReoRox®) and rotational thromboelastometry (ROTEM®) showed that both can detect prolonged clot initiation by LMWH. We could not show that one method was more sensitive than the other.

14. Acquired deficiencies of factor XIII may be clinically important and have not been researched in this setting.

15. Although we could not show that levels of vitamin K dependent coagulation factors decreased after major surgery in patients with upper gastrointestinal pathology, the PIVKA assay (proteins induced by vitamin K absence) increased significantly. Whether this is of clinical relevance is unclear. Vitamin K may be given to patients with postoperative coagulopathy even if it is not caused by vitamin K antagonists: it is unlikely to have adverse effects but its efficacy is uncertain.

16. Future research ought to include advanced analysis of coagulation in patients who have had a spinal haematoma. Tests should preferably be taken before products affecting coagulation are given, which would be significant logistic challenge.

Populärvetenskaplig sammanfattning

Epidural anestesi och analgesi ('ryggbedövning') är indicerade vid esofagusresektion ('kirurgi på matstrupen'). En ovanlig men mycket allvarlig komplikation är spinalt hematom ('blödning i ryggraden'), vilket kan uppstå vid inläggning eller bortdragning av epiduralkatetrar. Koagulationsblodprover tas vanligen i syftet att identifiera de patienter, som löper risk att utveckla spinala hematom, ibland inför borttagande av epiduralkatetrar. Internationella riktlinjer är dock något oklara vilka prover ska tas och hur dess svar ska tolkas.

Denna avhandling, som består av fem studier, undersöker olika aspekter kring detta ämne:

Studie I: Risk för spinalt hematom: pre- till postoperativa förändringar i rutinkoagulationsprovsvar hos 358 patienter som genomgick esofagusresektion under en period på 10 år.

Trombocytantalet (antalet blodplättar i blodet) steg från provtagningen inför operationerna, till dess att det var dags att dra bort patienternas epiduralkatetrar. Detta talar för att blodet levrar sig starkt. De övriga två rutinproverna aPTT (activated partial prothrombin time) och PT-INR (prothrombin time international normalized ratio) steg något under det peroperativa förloppet, vilket skulle kunna tala för en rubbning i blodlevringen.

Det är inte säkert att trenden i aPTT och PT-INR alltid visar en kliniskt relevant rubbning i koagulationen då patienter vanligtvis är benägna att utveckla blodproppar efter stora operationer. Vi rekommenderar trots detta försiktighet i fall man som kliniker ska handlägga en patient var epiduralkateter behöver dras bort samtidigt som rutinprover indicerar koagulopati. Patientnära tester såsom ROTEM® och Multiplate® har eventuellt en roll i detta sammanhang men de är än så länge inte på något sätt validerade.

Studie II: korrektion av blödningsrubbningar orsakade av hypotermi (kyla) och utspädning, med fibrinogen och faktor XIII: en in-vitro studie med ROTEM®.

Blödningsrubbning orsakad av att sjukvårdspersonal spär ut patienters koagulationsfaktorer och blodplättar med vätskor är vanlig. Fibrinogen korrigerar en sådan blödningsrubbning orsakad av utspädning av blod med antingen kristalloid vätska ('saltlösning') eller hydroxyetyl-stärkelse ('potatisstärkelseblandning'). Detta gäller både vid 33°C och 37°C, särskilt när utspädningen orsakats av kristalloidvätska. Tillsättning av faktor XIII korrigerar blödningsrubbningen ytterligare dock endast när utspädningen orsakades av kristalloid vätska.

Studie III: tromboelastometri jämfört med frioscillationsreometri, samt enoxaparin jämfört med tinzaparin: en in-vitro studie som jämför två viskoelastiska metoders dos-respons på två olika låg molekylärvikts hepariner.

Blodprover togs från 10 patienter som hade genomgått stor kirurgi, när det var dags att dra bort detas epiduralkatetrar. Två olika LMWH (låg molekylärviktshepariner), enoxaparin och tinzaparin tillsättes i olika koncentrationer och blodet analyserades med ROTEM® och FOR: två olika helblodsmetoder för att mäta koagulation.

Både ROTEM® och FOR var känsliga för ökande doser LMWH's effekt på initiering av koagulation. Det fanns dessutom skillnader i individers svar på dessa läkemedel. Tinzaparin hade en starkare effekt än enoxaparin. Detta beror på att både läkemedel hämmar koagulationfaktorer FXa och FIIa. De doseras dock i aktivitet mot FXa. Inom att enoxaparin är mer FXa specifik än tinzaparin, ger den en svagare total hämning av koagulation när doseringen sker i aktivitet mot enbart FXa. Anti-FXa aktivitet är därför förmodligen inte det bästa prov att ta i fall man vill få en 'helhetsmått' på hur mycket blodlevringen hämmas av dessa läkemedel.

Slutsatsen är att dessa läkemedel möjligen kan ha en plats i den postoperativa monitorering av LMWH, men att mer forskning behövs.

Studie IV: Monitorering av LMWH vid terapeutiska nivåer med olika metoder: dos-responsen, korrelationer och skillnader mellan aPTT, anti-FXa aktivitet och trombingenerationsanalyser.

Fyra olika metoder för mätning av aPTT prövades i blod till vilket det hade tillsatts olika halter av tinzaparin eller enoxaparin. Det fanns signifikanta skillnader mellan svaren som gavs av de olika aPTT metoder, även om de skulle mäta samma sak (koagulation initierad av maximal stimulering av av faktor XII). Tinzaparin förlängde både aPTT och trombingeneration mer än enoxaparin men de hade samma effekt på anti-FXa aktivitet, vilket beror på att de doserar i enheter anti-FXa aktivitet.

Man kan därför ifrågasätta anti-FXa aktivitet då denna analysmetod inte är känslig för anti-IIa aktivitet. Trombingeneration är en metod som potentiellt kan användas för att ge en 'helhetsbild' på LMWH då den är känslig för anti-IIa aktivitet, utan aPTT's nackdel att den kan vara opålitlig.

Studie V: koagulativ säkerhet kring epiduralbedövning efter stor gastrointestinal kirurgi: avancerad- och rutinkoagulationanalys hos 38 patienter.

Avancerad koagulationsanalys utfördes på blod taget från 38 patienter som hade genomgått stor kirurgi, både strax innan operation samt när det var dags att dra bort deras epiduralkatetrar. Liksom i Studie I förlängdes aPTT och PT-INR något medan trombocytantalet och fibrinogennivån steg signifikant i så gott som alla patienter. Analys av koagulationsfaktorer visade inte att låga nivåer på de faktorer vars syntes kräver K vitamin. Orsaken till de lätt förlängda aPTT och PT-INR kan vara minskade nivåer FXII respektive VII. ROTEM® och Multiplate®, en metod som mäter funktionen på blodplättarna, talar för en ökad benägenhet till blodlevring. Om dessa metoder kan användas i skattningen av risken för spinalt hematom är inte helt klarlagt: Multiplate® resultaten följde trombocytantalet väldigt väl, vilket talar emot att denna metod kan tillföra så mycket så länge patienter inte givits eller stått på läkemedel som hämmar trombocyter. ROTEM® kan dock ha en plats i detta sammanhang – den visar en blodlevringsbenägenhet som stämmer överens med att dessa patienter är allmänt sätt trombosbenägna, samtidigt som denna metod visat en viss känslighet för LMWH i Studier 3 och 4. Det finns även en fallbeskrivning på en patient i den Brittiska militären, vars ROTEM® dock inte rutinprover talade för nedsatt blodlevringsbenägenhet när hans epiduralkateter drogs bort. Han utvecklade sedan ett spinalt hematom.

Sammanfattningsvis är evidensnivån för vilka prover ska tas när det önskas att dra bort en epiduralkateter, ganska låg. Fortsatt forskning borde gå ut på att kartlägga hur koagulation och övriga kliniska förhållanden faktiskt varit, hos patienter som fått spinala hematom. Inom att sådana hematom är ytterst sällsynta är detta inte en lätt uppgift!

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RESEARCH ARTICLE Open Access

Rotational thromboelastometry and multiple electrode platelet aggregometry in four patients with abnormal routine coagulation studies before removal of epidural catheters after major surgery: a case series and research study

Owain D Thomas[1,2*], Anna Gustafsson[3] and Ulf Schött[1,2]

Abstract

Introduction: Routine coagulation tests have a low predictability for perioperative bleeding complications, and spinal hematoma after removal of epidural catheters is very infrequent. Thromboelastometry and point-of-care platelet aggregometry may improve hemostatic monitoring but have not been studied in the context of safety around epidural removal.

Methods: Twenty patients who received an epidural catheter for major thoracoabdominal and abdominal surgery were included prospectively. In addition to routine coagulation tests, rotational thromboelastometry and multiple electrode platelet aggregometry were carried out.

Results: A coagulation deficit was suggested by routine coagulation tests on the intended day of epidural catheter removal in four out of 20 patients. Prothrombin time-international normalized ratio was elevated to 1.5 in one patient (normal range: 0.9 to 1.2) while rotational thromboelastometry and multiple electrode platelet aggregometry parameters were within normal limits. Activated partial thromboplastin time was elevated to 47 to 50 seconds in the remaining three patients (normal range 28 to 45 seconds). Rotational thromboelastometry showed that one of the patients' results was due to heparin effect: the clotting time with the HEPTEM® activator was 154 seconds as compared to 261 seconds with INTEM. The three remaining patients with prolonged routine coagulation test results had all received over 1L of hydroxyethyl starch (Venofundin®) and thrombosis prophylaxis with low-molecular-weight heparin (enoxaparin). Rotational thromboelastometry and multiple electrode platelet aggregometrygave normal or hypercoagulative signals in most patients.

Conclusions: This case series is new in that it examines rotational thromboelastometry and multiple electrode platelet aggregometry postoperatively in the context of epidural analgesia and shows that they may be clinically useful. These methods should be validated before they can be used for standard patient care.

Keywords: aPTT, Epidural anesthesia, Epidural hematoma, Hydroxyethyl starch, Multiplate®, Platelet aggregometry, PT-INR, ROTEM®, Thromboelastography

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Introduction

Analgesia and anesthesia by administration of a local anesthetic and an opiate through an epidural catheter provide effective pain control during and after major surgery, and are routinely used at our hospital. Hematoma within the spinal canal is a serious complication of epidural analgesia causing neurological damage and requiring urgent surgical decompression. They are most likely at the time of epidural catheterization, when the risk is estimated to be between 1:4000 and 1:30,000, and at the time of removal or manipulation of epidural catheters when the risk is estimated to be between 1:150,000 and 1:190,000 [1,2]. Patients who have undergone major surgery often have a coagulation deficit which may be caused by loss of coagulation factors and platelets due to surgical hemorrhage, preoperative malnutrition, systemic inflammation response syndrome, or due to accumulation or overdosing of thrombosis prophylaxis.

It is uncontroversial that preoperative coagulation deficits predispose to spinal hematoma at the time of epidural catheterization, but the sensitivity and specificity of routine coagulation tests, usually the prothrombin time-international normalized ratio (PT-INR), activated partial thromboplastin time (aPTT) and platelet count (Plc), in this context are unknown. These tests' usefulness is questionable in patients who lack risk factors for perioperative bleeding, such as a history of bleeding or taking anticoagulant drugs [3-5]. It is more uncertain whether these tests can indicate the risk of hemorrhagic complications related to postoperative manipulation and removal of epidural catheters. A number of case reports suggest that point-of-care tests measuring whole blood viscoelasticity (e.g. thromboelastography (TEG®) and rotational thromboelastometry (ROTEM®)) and platelet aggregometry (e.g. multiple electrode platelet aggregometry (Multiplate®) and VerifyNow®) may be of use in regional anesthesia but evidence here is scarce.

Ahead of a larger study which is currently in progress, we carried out a pilot study, approved by The Swedish Central Ethical Review Board (Lund, DNR 2010/482). Signed consent was given by 20 consecutive patients who had an epidural catheter in place for analgesia after major gastrointestinal surgery. Our aim was to compare results from point-of-care and routine coagulation tests, hypothesizing that the whole blood assays ROTEM® and Multiplate® might give normal results despite moderately abnormal routine test results, which are run on plasma. This would of course be of interest since it is a common clinical scenario to be presented with a patient whose epidural catheter needs to be removed but whose routine coagulation parameters suggest a mild bleeding diathesis. We also compared preoperative routine coagulation results with postoperative results to confirm our clinical impression that the normal pattern of coagulation in these patients is a tendency towards coagulopathy as measured by PT-INR and aPTT.

Results

Included in the study were 20 patients with a thoracic epidural catheter in place, 13 men and seven women. Of the 20 patients, 15 had undergone major gastrointestinal surgery by laparotomy alone and the other five had also undergone thoracotomy. The mean age was 58 years (range 26 to 83). None were treated with platelet inhibitors. Mean blood loss during their operation was 415mL (standard deviation 315mL). All patients received 500mL or more of synthetic colloid as hydroxyethyl starch 130/0.42 (Venofundin®). All were treated with thrombosis prophylaxis in a standard once daily dose at 8 p.m. of 40mg enoxaparin irrespective of weight. The mean time between epidural catheterization and removal was 5.6 days (range 2 to 14 days, standard deviation 2.8 days). A small number of routine test results were missing (four preoperative PT-INR values, one preoperative Plc and one postoperative aPTT).

Routine test results in the 20 patients

Postoperative aPTT and PT-INR results were as expected significantly prolonged at the time of removal of epidural catheters in comparison to those taken preoperatively (aPTT: mean 39.5 seconds, SD 0.15 versus 32.2 seconds, SD 4.84; PT-INR: mean 1.08, SD 0.15 versus 1.01, SD 0.11; see Figures 1 and 2). There was also, unsurprisingly, a significant correlation ($p<0.05$; $r=0.76$) between the length of time after operation and Plc (see Figure 1). Normal values are shown in Table 1.

Multiplate® test results in the 20 patients

Median Multiplate® area under curve (AUC) was for all three tests within the reference ranges (see Table 2): after activation by adenosine diphosphate (ADP) it was 98U; after activation by collagen (COL) it was 104U and after activation with thrombin receptor activator (TRAP) it was 128U. AUC after activation with ADP correlated significantly to the length of time between operation and testing (see Figure 2) but AUC after activation with COL or TRAP did not (not shown).

ROTEM® test results in the 20 patients

Out of a total of 480 results, 18 (4%) from the 20 patients (24 parameters per patient) were not included: results were excluded in four patients due to desiccation artifacts, caused by the surface of the sample drying and clotting, or an inconsistent pattern of results suggesting that the wrong reagents had been used. Median results for each test's results were within the normal ranges (see Table 2). Median maximum clot firmness (MCF) for each of the assays was as follows: EXTEM: 71mm;

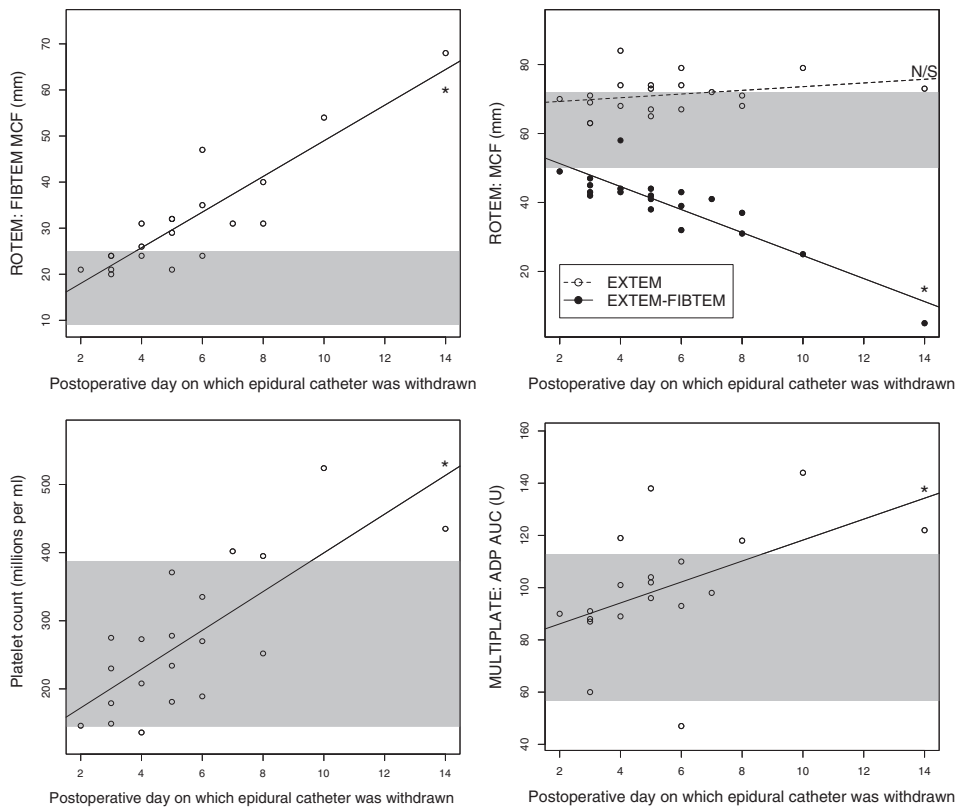

Figure 1 Results from assays related mainly to platelet count and function. These results give an overall impression of normo- or hypercoagulability. Shaded areas indicate the normal ranges. Platelet count, ROTEM®-FIBTEM®-maximum clot firmness (taken to be a quantitative measure of blood fibrinogen concentration) and Multiplate® adenosine diphosphate-area under curve (a measure of platelet activity) all correlated significantly to the length of time after surgery ($p<0.05$, $r=0.89$, 0.76 and 0.49 respectively). ROTEM®-EXTEM-maximum clot firmness, an overall measure of the extrinsic pathway, neither increased nor decreased with time after operation while ROTEM®-(EXTEM minus FIBTEM®)-maximum clot firmness significantly negatively correlated to time after operation ($p<0.05$, $r=-0.89$). The latter measure is often taken to be a measure of platelet function but here its decrease would appear to be due to increasing fibrinogenemia rather than weaning platelet function. *: significant correlation $p<0.05$. ADP: adenosine diphosphate. AUC: area under curve. MCF: maximum clot firmness. N/S: no significant correlation.

FIBTEM®: 30mm; INTEM: 68mm; HEPTEM®: 66mm; APTEM: 70mm; NATEM: 66mm. There were no significant differences between EXTEM and APTEM results, which would have indicated hyperfibrinolysis. Of the results, 61 (13%) indicated mild hypercoagulability and 16 of these were NATEM-clot formation time (CFT) measurements. Five results (1%) indicated hypocoagulability, but four of these were HEPTEM® results in patients whose INTEM results were normal, which ought to be impossible since HEPTEM® is identical to INTEM other than it contains heparinase, which would not be expected to inhibit coagulation. There was no significant difference between results from INTEM and HEPTEM®, which would have suggested an excessive heparin effect.

Case studies of the four patients with abnormal routine coagulation results

Case report 1: normal ROTEM® and Multiplate® despite a prothrombin time-international normalized ratio of 1.5

A previously healthy 26-year-old woman weighing 67kg and not on any medication other than aluminum oxide and antacids due to dyspepsia, presented with a 2-month history of jaundice which later

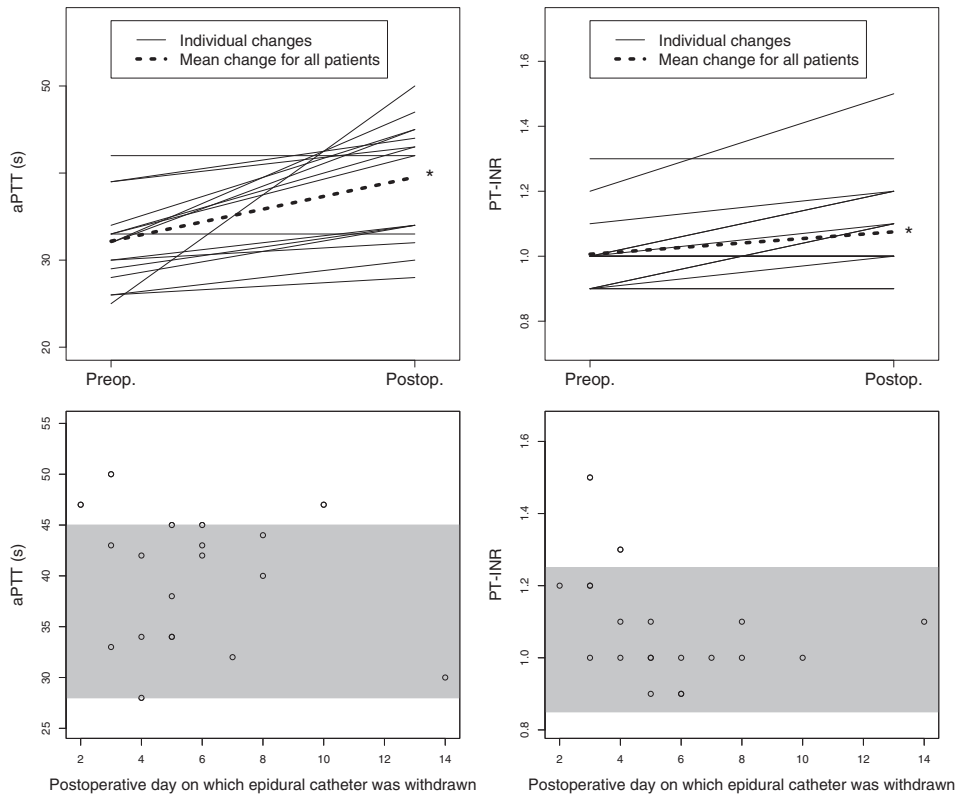

Figure 2 Perioperative dynamics of activated partial thromboplastin time and prothrombin time-international normalized ratio. These results give an overall impression of postoperative normo- or hypocoagulability. Shaded areas indicate the manufacturers' reference ranges. aPTT: activated partial thromboplastin time. PT-INR: prothrombin time-international normalized ratio. Preop: preoperative test results. Postop: test results taken on the day on which epidural catheters were removed. s: seconds. *: significant difference between pre- and postoperative results ($p<0.05$, Student's paired *t*-test).

proved to be due to chronic pancreatitis. Since magnet resonance imaging suggested pancreatic malignancy, Whipple's procedure was carried out, involving extensive pancreas resection. The operation was conducted under uncomplicated combined inhalational general and epidural anesthetic: the total perioperative hemorrhage was 700mL and in addition to crystalloid infusions (Ringer's acetate and glucose solution), this patient received 2000mL of hydroxyethyl starch (Venofundin® 60mg/mL) and 250mL of 5% human albumin. Routine coagulation tests on the first postoperative day indicated a coagulopathy (see Table 3). Thrombosis prophylaxis was omitted that evening but continued thereafter. On the morning of the fourth postoperative day, ROTEM® and Multiplate® results were within their reference ranges despite a PT-INR of 1.5 (see Table 3). The patient's epidural catheter was removed without complication 14 hours after the last dose of enoxaparin.

Case report 2: contamination by heparin demonstrated by ROTEM®

A 61-year-old man weighing 52kg but who had recently lost 50kg was admitted for resection of a lower esophageal tumor. He had previously received a subcutaneous venous port (Port-a-Cath™) for adjuvant chemotherapy and a percutaneous gastromy for nutrition. He had been prescribed oral esomeprazole, ondansetron, betamethasone, diazepam and mirtazapine in addition to

Table 1 Summary of assays performed

Test	Vial	Apparatus	Place of analysis	Normal range
ROTEM® using the following reagents: EXTEM, FIBTEM®, INTEM, APTEM, NATEM, HEPTEM®, recording the following results: CT (Clotting time), CFT (Clot formation time), Alpha-Angle (AA), MCF (Maximum Clot Firmness), ML (Maximum Lysis).	2.7mL citrate tubes for ROTEM® analysis (3.2% citrate, BD Vacutainer® Systems, Plymouth, UK)	ROTEM®, Pentapharm, Munich, Germany	Point-of-care laboratory, Intensive Care Unit	See Table 2.
Multiplate® using the following agonists: adenosine diphosphate, collagen, thrombin receptor activator, recording the following results for each agonist: area under curve (AUC), aggregation (AU), velocity (AU/min).	3.0mL Hirudin tubes. Dynabyte GmbH, Munich, Germany.	Multiplate®, Roche, Basel, Switzerland		See Table 2.
aPTT: activated partial thromboplastin time	2.7mL citrate tubes (3.2% citrate, BD Vacutainer® Systems, Plymouth, UK)	PTT-Automate, Stago (Asnière sur Seine, France)	Hospital's usual clinical chemistry laboratory	28 to 45 seconds at the time of the study
PT-INR: prothrombin time-international normalized ratio		Stago prothrombin complex assay, Stago calibrated by Equalis, (Uppsala, Sweden)		≤1.2
Plc: platelet count	3.0mL K_2EDTA tubes (BD Vacutainer® Systems, Plymouth UK)	Sysmex XE 5000 cell counter, Sysmex Corp., (Kobe, Japan).		165 to 387 million/mL and 145 to 387 million/mL for women and men respectively

Table 2 Reference ranges for ROTEM® (Manufacturer's information) and Multiplate® [6]

ROTEM®	CT (s)	CFT (s)	Alpha angle (°)	MCF (mm)
EXTEM	38–79	34–159	63–83	50–72
APTEM	38–79	34–149	63–83	50–72
INTEM	100–240	30–110	70–83	50–72
HEPTEM®	100–240	30–110	70–83	50–72
NATEM	300–1000	150–700	30–70	40–65
FIBTEM®	-	-	-	9–25
(EXTEM-MCF)-(FIBTEM®-MCF)	-	-	-	41–48
Multiplate®	**AUC (U)**	**Aggr (AU)**	**Vel (AU/min)**	
ADPtest	57–113	108–122	16–19	
COLtest	72–125	126–140	18–21	
TRAPtest	84–128	140–152	24–26	

CT: Clotting time. s: seconds. CFT: Clot formation time. MCF: Maximum clot firmness. AUC: Area under curve. Aggr: aggregation. Vel: velocity. U: units. AU: area units. min: minute.

transcutaneous fentanyl and subcutaneous ketobemidone. He received a combined general inhalational and epidural anesthetic and received 1000mL hydroxyethyl starch (Venofundin® 60mg/mL) during the operation. Blood loss during the operation was 300mL. It was considered clinically appropriate to remove his epidural catheter on the third postoperative day and coagulation tests were drawn from his heparinized Port-a-Cath™ (which has an internal volume of 1.3mL): the first 10mL blood was discarded. His aPTT was prolonged to 50 seconds and his Plc of 149 was slightly lower than expected.

ROTEM® results were all within the normal limits but there was a discrepancy between INTEM, a measure of the intrinsic pathway, and HEPTEM® which is identical other than it contains heparinase which removes any effect of heparin: clotting time (CT) was 100 seconds shorter for HEPTEM® than INTEM (154 seconds compared to 261 seconds). Multiplate® results were around the lower limits of the reference range (ADP-AUC was 60U). Routine tests the next day (see Table 4) sampled from a peripheral vein, were normalized despite the patient having received the same dose of low-molecular-weight heparin as on the previous days. The ROTEM® results show that the first sample taken was contaminated by heparin despite 10mL of dead space being withdrawn from the Port-a-Cath™ system which had an internal volume of 1.3mL. A repeat test could have being run on the same day, allowing earlier removal of this epidural catheter.

Case report 3: normal ROTEM® and Multiplate® results despite an activated partial thromboplastin time of 47 seconds and borderline prothrombin time-international normalized ratio at time of epidural removal

A previously healthy 52-year-old woman weighing 65kg received a combined general inhalational and epidural anesthetic for Whipple's procedure due to pancreas cancer. Perioperative hemorrhage was 200mL and she received 1500mL hydroxyethyl starch (Venofundin® 60mg/mL) in addition to crystalloid infusions. She received standard thrombosis prophylaxis postoperatively. Her epidural catheter unfortunately failed to give effective analgesia and the decision to remove the catheter was made on the second postoperative day. Routine coagulation tests showed that PT-INR had increased to 1.2 and aPTT was slightly elevated to 47 seconds (see Table 5). ROTEM® and Multiplate® results were all normal: EXTEM-MCF was 70mm and Multiplate®-AUC was 90U. Her epidural catheter was removed without complication. As many clinicians would have delayed manipulation of this patient's epidural given the slightly elevated aPTT and PT-INR at the upper end of normal, the ROTEM® and Multiplate® results might have contributed to the decision to withdraw the catheter without delay.

Table 3 Routine laboratory results for Case report 1

	Hb g/L	PT-INR	APTT seconds	Plc 10^6/mL	Creatinine umol/L	Albumin g/L
Preoperative	123	1.2	33	266	51	38
First postoperative day	103	2.5*	44	152	43	28
At time of epidural catheter removal, fourth postoperative day.	107	1.5*	33	179	44	29

APTT: activated partial thromboplastin time. Hb: hemoglobin. Plc: platelet count. PT-INR: prothrombin time-international normalized ratio. *: outside normal reference range.

Table 4 Routine laboratory results for Case report 2

	Hb g/L	PT-INR	APTT seconds	Plc 10^6/mL	Creatinine umol/L	Albumin g/L
Preoperative	142	1.0	25	230	49	36
On tenth postoperative day	107	1.0	50*	149	44	Not measured
At time of epidural catheter removal, 11th postoperative day.	113	1.0	31	178	45	Not measured

Summary of Case report 2's routine laboratory studies. APTT: activated partial thromboplastin time. Hb: hemoglobin. Plc: platelet count. PT-INR: prothrombin time-international normalized ratio. *: outside normal reference range.

Case report 4: hypercoagulant Multiplate® despite an activated partial thromboplastin time of 47 seconds at time of removal of epidural catheter

A previously healthy 49-year-old man weighing 93kg who had been a cigarette smoker for 30 years, and who took only omeprazole for chronic gastric regurgitation, presented with a 6-week history of weight loss and dysphagia which was diagnosed as a lower esophageal adenocarcinoma. He received a combined general inhalational and epidural anesthetic for esophageal resection and para-aortic lymph node dissection by laparotomy and thoracotomy: a total perioperative hemorrhage of 500mL was recorded and in addition to crystalloid infusions, this patient received 1500mL of hydroxyethyl starch (Venofundin® 60mg/mL) and 250mL of 5% human albumin. His postoperative recovery was uncomplicated and routine coagulation tests on postoperative day 8 were normal, although it may be noted that this patient's blood albumin and hemoglobin were low (25g/L and 96g/L respectively). (See Table 6). Tests on postoperative day 10 showed a slightly elevated aPTT of 47 seconds, a thrombocytosis and Multiplate® results indicated strong platelet aggregation: the ADP test showed an AUC of 144U, AGG (aggregation) 244AU and VEL (velocity) 39.7AU/minute (see Table 2 for reference ranges). ROTEM® results were within the reference intervals and the epidural catheter was removed without complication. Again, ROTEM® and Multiplate® might have contributed to the decision to remove the epidural catheter despite an aPTT suggesting mild coagulopathy.

Discussion

This pilot study constitutes a small collection of somewhat heterogeneous data but does bring to light several important topics concerning 'coagulative safety' in the context of postoperative epidural anesthesia. There may be a place for these tests in routine practice, although this is currently not realistic due to these tests' lack of validation in this setting, their operator-dependency and the fact that few hospital laboratories offer these tests for routine clinical use.

There appears to be a lack of concordance between whole blood viscoelastic tests and routine coagulation tests in the postoperative context, which brings current guidelines and the usefulness of both types of test in to question.

While PT-INR and aPTT would appear to indicate a trend towards postoperative coagulopathy in this and other studies [7], ROTEM® and Multiplate® suggested a trend towards postoperative hypercoagulation in this study.

Davignon *et al.* emphasize the importance of monitoring coagulation before removal of epidural catheters in case manipulation should disturb a clot and initiate an epidural hematoma, which they describe in a patient who received anticoagulation shortly after removal of an epidural catheter [8].

Current guidelines do not clearly describe what to do when there is a clinically pressing indication for removing an epidural catheter amidst laboratory tests indicating a coagulopathy: delaying removal of the epidural catheter in Case report 1 (in which PT-INR was 1.5) might possibly have delayed mobilization but there was no suspicion of local infection or sepsis, which would have made delayed withdrawal of the catheter potentially dangerous. The American Society of Regional Anesthesia and Pain Medicine recommends a PT-INR of 1.4 or lower but does not mention aPTT or Plc. That guidelines do not address aPTT is unfortunate since over half of the cases of spinal hematoma described by Miyazaki *et al.* were treated with anticoagulant therapy which would not necessarily be detected by the PT-INR alone [2]. Should anticoagulation be reversed, and if so how?

PT-INR is best validated for monitoring the effect of vitamin K antagonists such as warfarin, which was not something that we give patients undergoing major surgery. Viscoelastic tests are insensitive to increases in PT-INR: ROTEM®-CT is prolonged first when the

Table 5 Routine laboratory results for Case report 3

	Hb g/L	PT-INR	APTT seconds	Plc 10^6/mL	Creatinine umol/L	Albumin g/L
Preoperative	130	1.0	32	201	60	Not measured
At time of epidural catheter removal, 2nd postoperative day.	123	1.2	47*	146	54	Not measured

Summary of Case report 3's routine laboratory studies. APTT: activated partial thromboplastin time. Hb: hemoglobin. Plc: platelet count. PT-INR: prothrombin time-international normalized ratio. *: outside normal reference range.

Table 6 Routine laboratory results for Case report 4

	Hb g/L	PT-INR	APTT seconds	Plc 10^6/mL	Creatinine umol/L	Albumin g/L
Preoperative	148	Not measured		293	61	40
At time of epidural catheter removal, tenth postoperative day.	96	1.0	47*	524	71	25

Summary of Case report 4's routine laboratory studies. APTT: activated partial thromboplastin time. Hb: hemoglobin. Plc: platelet count. PT-INR: prothrombin time-international normalized ratio. *: outside normal reference range.

PT-INR is around 3.5 (manufacturer's information): neither TEG® nor ROTEM® are validated for reversal of vitamin K antagonism with prothrombin complex concentrate. Tissue factor can be used as an activator to give viscoelastic tests better sensitivity for PT, but this is at present not commercially available [9]. ROTEM® and Multiplate® nevertheless gave the clinician looking after the patient in Case report 1 the confidence to remove her epidural catheter [10].

There is a report by Hepner *et al*., of TEG®, technically similar to ROTEM®, being used to monitor coagulation at the time of removal of epidural catheters in 52 orthopedic patients treated with low-dose warfarin to give a PT-INR of up to 1.5 [11]. This is an attractive concept since the risk of spinal hematoma after epidural catheterization may be highest after such procedures and a point-of-care test might allow for more accurate prescription of warfarin [1]. TEG® was insensitive to warfarin's effects in Hepner and colleague's study which, like all studies in this area, was underpowered to draw any conclusion about TEG® and PT-INR's predictive value regarding the risk of spinal hematoma. There are several commercial point-of-care whole-blood PT assays available: Hemochron Junior®, iStat® and Coaguchek Pro®.

There is no conclusive evidence that it is unsafe to place an epidural catheter when the Plc is less than 100×10^6/mL, yet this is the generally accepted recommendation [4,12]. None of our patients had thrombocytopenia and it was of no surprise that both the number of platelets and Multiplate®-ADP-AUC increased with time after operation as part of the general postoperative inflammatory reaction. Being able to trust measures of platelet function in thrombocytopenic patients would be desirable, and being able to monitor the effect of attempted amelioration of platelet function with desmopressin for example, would be attractive since it might avoid unnecessary platelet transfusion.

Figure 1 shows three measures which correlate positively to length of time after operation, presumably as part of the inflammatory reaction. They are Plc, ROTEM®-EXTEM-MCF and Multiplate®-ADP-AUC. The difference between the MCF of ROTEM®-EXTEM and ROTEM®-FIBTEM® becomes smaller since the extrinsic pathway does not increase in activity as a whole despite hyperfibrinogenemia. ROTEM®-(EXTEM-FIBTEM®) would therefore not appear to be a useful measure of platelet function in the context of dynamic postoperative inflammation [13].

Meticulous sampling technique is paramount

Case report 2 demonstrates that preanalytical errors such as contamination with heparin can remove any potential benefit that a test might offer. An experienced clinician, however, ought to notice a difference in CT of 100 seconds between HEPTEM® and INTEM, consider sampling technique and ask for a repeat blood sample taken by venepuncture or from a non-heparinized line. Since results from ROTEM® are available in real time, it should be possible to obtain these even before initial routine coagulation results are obtained from the hospital laboratory. ROTEM®-HEPTEM® and INTEM were certainly useful in this patient, who had several factors that predisposed to coagulation defects: malnutrition, multiple medications, a large infusion of hydroxyethyl starch, major operative trauma and slightly low Plc. It is noteworthy that viscoelastic tests are not capable of monitoring thromboprophylactic dosages of low-molecular-weight heparin [14].

It is significant that 18 of the 480 ROTEM® results (4%) were excluded due to artifacts or suspicion that the wrong reagents had been used. Point-of-care tests have the limitation that they are often used by clinicians who are competent to interpret the results but who are neither trained to use nor experienced in using the equipment. Running ROTEM® and Multiplate®, for example, involves pipetting several different reagents. The operator has ample opportunity to use the wrong or contaminated materials, or even the wrong blood sample. Some of these sources of error are eliminated by those hospital laboratories which have introduced 'point-of-care' tests with telemetry: samples are sent to the laboratory and run by trained and experienced technicians. Results are displayed in real time on a monitor at the Intensive Care Unit or operating theatres.

Possible iatrogenic coagulopathy

It is troubling that patients 1, 3 and 4 had routine laboratory results suggesting a coagulopathy without our knowing for certain why. Lack of diagnosis precludes specific prevention and treatment. Patient 1's spontaneously transient but somewhat dramatic increase in PT-INR from 1.2 to 2.5 has several possible explanations:

dilutional coagulopathy and platelet inhibition by infusion of 2L of synthetic colloid and 250mL albumin; loss of coagulation factors by hemorrhage and possibly inability to synthetize new factors due to preoperative malnutrition and systemic inflammation [15]. It is also possible that our current practice of prescribing 40mg of enoxaparin as thrombosis prophylaxis to all patients regardless of weight leads to accumulation and coagulopathy in smaller patients who have decreased renal function. It is clearly of interest to prospectively and directly investigate coagulation factors and indicators of malnutrition in the perioperative period. We are currently running a study of this type.

The importance of being sensible

Since there is no optimal or validated method to predict the risk of epidural hematoma one must be vigilant for signs and symptoms of epidural hematoma not only after catheterization but also after removing an epidural catheter. Magnetic resonance imaging should be carried out early to enable surgical intervention to avoid neurological damage.

So long as we are not sure why a patient's coagulation tests indicate coagulopathy before removal of an epidural catheter, we cannot be sure how to treat them. Current strategies at our hospital include administration of between 10 and 30mg vitamin K per day and stopping enoxaparin, then taking new coagulation tests a day later. Transfusions of plasma have previously been given before the removal of catheters without further testing.

Conclusions

This pilot study is new in that it examines ROTEM® and Multiplate® at the time of epidural catheter removal. These point-of-care tests may have a role to play in this setting since they showed a normal or hypercoagulative signal in most patients despite aPTT and PT-INR showing a trend towards the hypocoagulable.

Normal values need to be defined for viscoelastic tests and platelet aggregometry after major surgery. ROTEM®-(EXTEM minus FIBTEM®)-MCF does not appear to be a suitable measurement of platelet function.

At present we do not know enough about the pathophysiology of postoperative coagulation defects: the causes of prolonged PT-INR and aPTT should be investigated further, including the effects of synthetic starches.

Table 7 Overview of ROTEM® parameters

	Trade name	Content	Action	Relevance
1	NATEM (= 'classic thromboelastometry')	Only recalcification agent.	None – coagulation is activated by contact with the surface of the measurement container.	Produces a curve representing ' whole blood coagulation'.
2	EXTEM	Tissue factor and phospholipids.	Activates the extrinsic pathway.	CT corresponds to PT. Curve represents clot formation, stability and fibrinolysis resulting from the extrinsic pathway.
3	INTEM	Ellagic acid and phospholipids.	Activates the intrinsic pathway.	CT corresponds to aPTT.
4	FIBTEM®	As EXTEM, plus cytochalasin D.	Cytochalasin D inhibits platelets.	Allows qualitative assessment of fibrinogen levels.
5	APTEM	As EXTEM, plus aprotinin.	Aprotinin inhibits plasmin and therefore fibrinolysis.	Comparing EXTEM and APTEM can rule in or out hyperfibrinolysis.
6	HEPTEM®	As INTEM, plus heparinase I.	Degrades heparin.	A comparison of HEPTEM® and INTEM indicates how much coagulation is affected by heparin.
CT (Clotting time)		Gives information about the kinetics of fibrin formation and clot development. Prolongation of CT may be a result of heparin or coagulation factor deficiency.		
CFT (Clotting formation time)		Gives information about the rate of clot formation. It is calculated as the interval between the onset of coagulation, arbitrarily defined as when the amplitude is 2mm, and the curve reaching an amplitude of 20mm. A prolonged CFT but normal MCF indicates a clot polymerization disorder.		
AA (Alpha angle)		Indicates the rate at which a solid clot forms. Both CFT and alpha angle are influenced by coagulation factors, platelet count and/or function and fXIII.		
MCF (Maximum clot firmness)		Shows the maximal strength and stability of the fibrin and platelet clot. A reduced MCF and normal CFT suggest lack of fibrinogen and/or platelets.		

Contents according to manufacturer's information (Pentapharm, Munich, Germany).

Methods

On the day on which patients' epidural catheters were to be removed, venous blood was sampled from indwelling peripheral or central venous catheters, which is routine practice at our hospital. ROTEM® and Multiplate® were run on this blood at the same time as the standard coagulation tests described in Table 1. Routine test results, both pre- and postoperative, were retrieved from the hospital's electronic notes system (Melior, Siemens Healthcare, Upplands Väsby, Sweden).

ROTEM®

Thromboelastometry was carried out using the ROTEM® (rotational thromboelastometry) apparatus (Pentapharm, Munich, Germany) according to the manufacturer's instructions. ROTEM® assays were run for 60 minutes.

Each sample was analyzed by ROTEM® using each of the six activators described in Table 7. Recalcification was carried out using 20μL of 0.2M calcium chloride (Star-TEM™). The following variables were registered: CT, CFT, alpha-angle (AA), and MCF.

Multiplate®

Impedance aggregometry was carried out using the Multiplate® analyzer (Roche, Basel, Switzerland) according to the manufacturer's instructions. Three platelet receptor agonists were applied: ADPtest, COLtest and TRAPtest, which activate coagulation with ADP, COL and thrombin receptor activating peptide 6 respectively. The following results were recorded for each agonist in each patient: area under curve, aggregation, and velocity. Normal values are described in Table 2.

Data was initially recorded on paper case report forms and later entered into an Excel sheet before statistical analysis using the statistical computing environment 'R' [16]. The significance of differences between pre- and postoperative test results were tested with Student's paired *t*-test. Pearson's product moment correlation test was used to define correlation coefficients.

Consent

Written informed consent was obtained from all 20 patients involved in this study for publication of this case series and accompanying images. Copies of the written consents are available for review by the Editor-in-Chief of this journal. This study was approved by The Swedish Central Ethical Review Board (Lund, DNR 2010/482).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

US and AG designed the study. AG collected the data. OT and US wrote the final manuscript, interpreted data and produced the figures and tables. US, OT, and AG were involved in producing an original manuscript which did not use case reports. All authors read and approved the final manuscript.

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Study II

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Correction of hypothermic and dilutional coagulopathy with concentrates of fibrinogen and factor XIII: an in vitro study with ROTEM

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Abstract

Background: Fibrinogen concentrate treatment can improve coagulation during massive traumatic bleeding. The aim of this in vitro study was to determine whether fibrinogen concentrate, or a combination of factor XIII and fibrinogen concentrates, could reverse a haemodilution-induced coagulopathy during hypothermia.

Methods: Citrated venous blood from 10 healthy volunteers was diluted in vitro by 33% with 130/0.42 hydroxyethyl starch (HES) or Ringer's acetate (RAc). The effects of fibrinogen concentrate corresponding to 4 gram per 70 kg, or a combination of the same dose of fibrinogen with factor XIII (20 IU per kg), were measured using rotational thromboelastometry (ROTEM). The blood was analysed at 33°C or 37°C with ROTEM EXTEM and FIBTEM reagents. Clotting time (CT), clot formation time (CFT), alpha angle (AA) and maximal clot formation (MCF) were recorded.

Results: Fibrinogen with or without factor XIII improved all ROTEM parameters in either solution irrespective of temperature, with the exception of EXTEM-AA and EXTEM-CFT in HES haemodilution. Fibrinogen increased FIBTEM-MCF more in the samples diluted with RAc than HES, particularly in presence of factor XIII.

Conclusions: Fibrinogen improved in vitro haemodilution-induced coagulopathy at both 33°C and 37°C, though more efficiently after crystalloid than HES haemodilution. Factor XIII had an additional effect on FIBTEM-MCF, but only after crystalloid dilution.

Keywords: Factor XIII, Fibrinogen, Hemodilution, Hypothermia, Hemostasis, Thrombelastography

Introduction

Haemodilution and hypothermia both contribute to coagulopathy and aggravate acute traumatic coagulopathy, which has been recognized as a significant cause of death in patients with traumatic injuries [1]. We therefore studied the efficacy of concentrates of fibrinogen and factor XIII (FXIII) in improving in vitro coagulopathy induced by haemodilution and hypothermia.

Hypothermia impairs coagulation mainly by platelet inhibition [2], whereas haemodilution principally impairs plasma coagulation [3]. In addition to the dilutional effects seen with crystalloids, synthetic colloid solutions impair fibrinogen polymerization and platelet function [3]. Hypothermia and haemodilution induced coagulopathy has been studied extensively with whole blood viscoelastic haemostatic assays (VHA) such as thromboelastometry (ROTEM), but there are few studies of simultaneous hypothermia and haemodilution [4,5]. In addition, in vitro correction of haemodilution-induced coagulopathy with fibrinogen concentrate has been studied extensively [6,7], but not during hypothermia. Previous in vitro studies indicate an additional effect of high doses of FXIII together with fibrinogen to correct haemodilution-induced coagulopathy, but this needs to be studied at clinically relevant dosages [7-9]. Therefore, the purpose of this study was to evaluate the in vitro effects of mild hypothermia in the context of coagulopathy induced by haemodilution with crystalloid or hydroxyethyl starch solutions; and the effects of fibrinogen concentrate, alone or in combination with factor XIII concentrate using ROTEM, a well-known VHA. The primary hypothesis was that fibrinogen concentrate, with or without factor XIII, reduce dilutional

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coagulopathy and secondly that hypothermia attenuates their corrective effects.

Material and Methods

Volunteers

Ten healthy individuals (one woman and nine men, age 27–65 years) gave their written informed consent to participate in this study. None of the subjects received any medication in the preceding 7 days and none had a history of coagulopathy. The Regional Ethical Review Board in Lund, Sweden, approved the study (DNR 2008:484).

Sampling

Blood was sampled into citrated plastic vacuum tubes (BD Vacutainer® Coagulation Tube, PET, 0.3 ml 0.109 M citrate). Venepuncture with minimal stasis was conducted, using a 21 Gauge needle, and the first tube was discarded. The samples were incubated at 33°C or 37°C for 30 minutes to ensure temperature equilibration and analysed within 4 hours of sampling.

Hypothermia and haemodilution

A digital thermometer submerged into a fluid-filled reference tube was used to check all blood sample temperatures. The ROTEM instruments were set at either 33°C or 37°C. The blood samples were diluted 2:1 volume by volume with either Ringer's acetated solution (RAc; Fresenius Kabi, Bad Homburg, Germany(G)) or 6% hydroxyethyl starch in saline (HES; MW 130 kDa, substitution ratio 0.42, Venofundin®, B.Braun, Melsungen, G) that is 33% haemodilution.

Fibrinogen and factor XIII

Human fibrinogen concentrate (Riastap®, CSL Behring Marburg, G) and factor XIII (FXIII) concentrate (Fibrogammin®; now registered as Cluvo®, CSL Behring) were dissolved according to the manufacturer's instructions, giving concentrations of 20 mg/ml and 62.5 IU/ml respectively. 120 μl of fibrinogen concentrate or 120 μl of fibrinogen +15 μl of FXIII were added to the respective blood sample, to a total sample volume of 3000 μl. These dosages correspond to 4 g of fibrinogen and 1550 IU of FXIII to a 70-kg man or 55 mg fibrinogen and 22 IU FXIII per kg of body weight. The FXIII dosage followed recommendations from the manufacturer on how to treat haemorrhage in congenital FXIII-deficient patients (10–20 IU/kg of body weight). The 4 g fibrinogen dosage is in line with current guidelines on treatment of massive bleeding.

ROTEM

Rotational thromboelastometry (ROTEM®; Pentapharm, Munich, Germany), a viscoelastic coagulation analysis instrument, was used according to the manufacturer's instructions. Two ROTEM assays, EXTEM and FIBTEM, were used. Coagulation was stimulated with tissue factor in the EXTEM test and the following parameters were recorded: clotting time (CT), clot formation time (CFT), alpha-angle (AA) and maximal clot formation (MCF). The following EXTEM parameters measure the clot velocity: CT shows how long clot initiation takes while CFT and AA reflect the clot amplification and propagation. EXTEM-MCF measures the maximal clot strength and is dependent on platelet count and function, as well as fibrin formation and polymerisation. The FIBTEM test is identical to the EXTEM test except for that cytochalacin D, a platelet inhibitor is added to the test reagent. This results in FIBTEM-MCF representing clot strength dependent on fibrin formation and polymerization alone. The only FIBTEM parameter recorded was FIBTEM-MCF. The last parameter EX-FIBTEM-MCF is a surrogate measure of platelet activity, which is calculated as FIBTEM-MCF subtracted from EXTEM-MCF.

Statistical analysis

For all statistical calculations the software package R, version 3.0.0, was used [10]. Repeated measures were analysed using univariate mixed models, using the package nlme ver. 3.1-109 [11]. Heterogeneous variances were evaluated with weighting. Post-hoc comparisons were made using the Multcomp package ver. 1.2-17 [12] using false discovery rate adjustment of p-values [13]. Data were analysed in two steps. The first step with haemodilution (control, RAc, HES) and two different temperatures (33°C, 37°C) sought to evaluate the potentially different effects of haemodilution with different solutions on normo- and hypothermic blood respectively. The second step used haemodilution with two different solutions (RAc, HES), two temperatures (33°C, 37°C) and the addition of coagulation factors (control, fibrinogen, fibrinogen + FXIII); this analysis evaluated the potential interaction between coagulation factors and the different solutions or different temperatures.

Results

Baseline values

In the undiluted samples, all EXTEM parameters and FIBTEM-MCF were within the normal range at 37°C [14].

Hypothermia and haemodilution

All clot velocity parameters were impaired by hypothermia of 33°C: in both undiluted and diluted blood CT and CFT were prolonged and AA was decreased (Figure 1). EXTEM-MCF decreased to a small extent during hypothermia, but only during concurrent haemodilution with either solution (Figure 2). Despite these hypothermic effects, the average values in undiluted samples were still mainly within the normal range at 33°C: a few samples' EXTEM-CFT exceeded the reference range and EXTEM-AA and

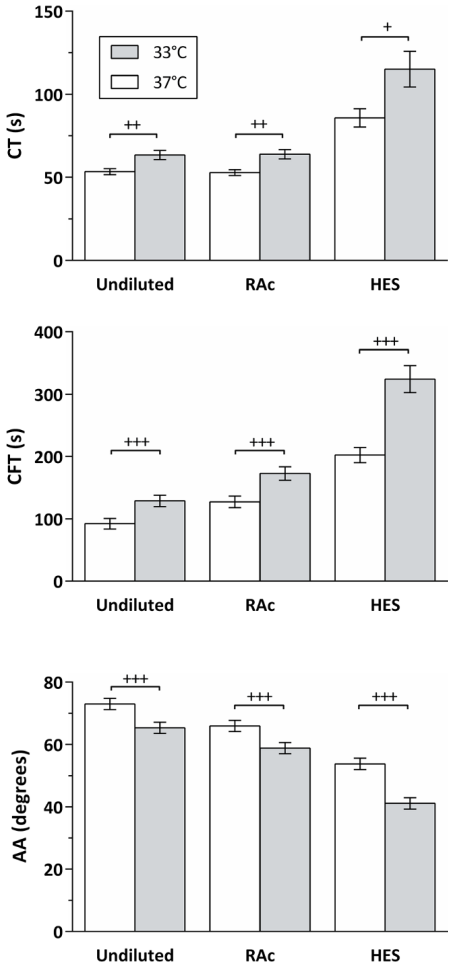

Figure 1 Effects of hypothermia (33° vs. 37 C) and haemodilution on the ROTEM EXTEM parameters clotting time (CT), clot formation time CFT) and alpha angle (AA). RAc: Ringer's acetate, HES: hydroxyethyl starch. All differences between haemodilution groups (Undiluted vs. RAc, Undiluted vs. HES, and RAc vs. HES) within each temperature were significant (P < 0.001), except the differences of CT between Undiluted and RAc at both temperatures, which were not significant. Brackets show significant differences between temperatures. (+: $P < 0.05$, ++: $P < 0.01$, +++: $P < 0.001$). Data are presented as mean values, error bars are 95% simultaneous confidence intervals. N = 10.

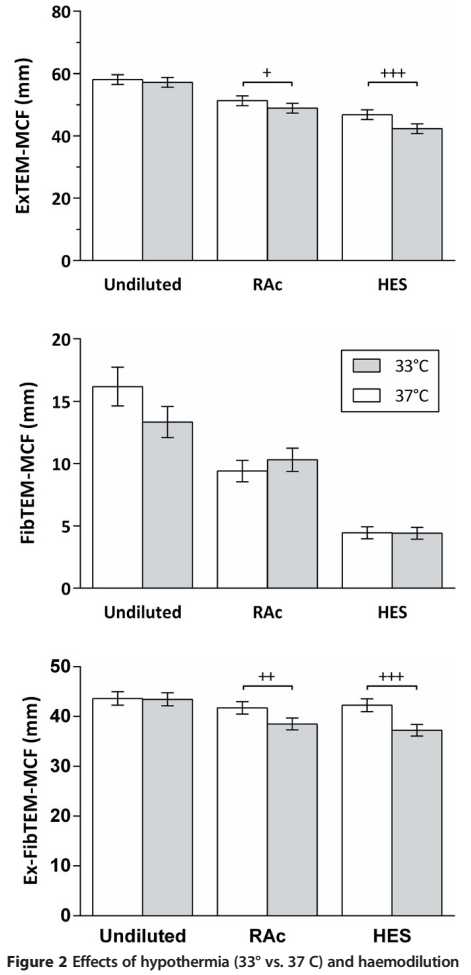

Figure 2 Effects of hypothermia (33° vs. 37 C) and haemodilution on the ROTEM parameters. EXTEM-MCF, FIBTEM-MCF and EX-FIBTEM-MCF; the latter calculated as FIBTEM-MCF subtracted from EXTEM-MCF. RAc: Ringer's acetat, HES: hydroxyethyl starch. All differences of EXTEM-MCF and FIBTEM-MCF between haemodilution groups (Undiluted vs. RAc, Undiluted vs. HES, and RAc vs. HES) within each temperature were significant ($P < 0.001$), except the difference of FIBTEM-MCF between Undiluted and RAc at 33°C. Differences of EX-FIBTEM-MCF were only significant ($P < 0.001$) between Undiluted and haemodilution with either RAc or HES, at 33°C. Brackets show significant differences between temperatures. (+: $P < 0.05$, ++: $P < 0.01$, +++: $P < 0.001$). Data are presented as mean values, error bars are 95% simultaneous confidence intervals. N = 10.

FIBTEM-MCF were below the reference range in a few of the samples.

At normothermia, haemodilution with HES significantly impaired all measured parameters, whereas RAc significantly impaired all parameters except CT. In addition, HES impaired ROTEM parameters significantly more than RAc (Figures 1 and 2). Moreover, the combination of

hypothermia and HES haemodilution interacted to impair CFT and AA in relation to undiluted samples (CFT: $P < 0.001$ and AA: $P < 0.05$), as well as in relation to RAc haemodiluted samples (CFT: $P < 0.001$ and AA: $P < 0.01$).

Addition of fibrinogen with or without factor XIII

Addition of fibrinogen concentrate to haemodiluted samples enhanced coagulation in general, except for CFT and AA in HES-haemodiluted samples. With FIBTEM-MCF and EXTEM-AA, there were significant interactions between fibrinogen concentrate and RAc haemodilution as compared to HES haemodilution, that is FIBTEM-MCF and AA were more effectively increased during RAc haemodilution than during HES haemodilution ($P < 0.05$ at both temperatures with FIBTEM-MCF and $P < 0.05$ at 37°C with AA) (Figures 3 and 4).

Almost all the parameters were improved to the same degree by fibrinogen combined with FXIII, as by fibrinogen alone. However, FXIII had additional effects to the fibrinogen effects during RAc haemodilution; AA increased more at 33°C and FIBTEM-MCF increased more at both 33° and 37°C after addition of fibrinogen + FXIII as compared with fibrinogen alone (Figures 3 and 4). The better effect of fibrinogen combined with FXIII on FIBTEM-MCF is also demonstrated by significant synergies between RAc haemodilution and fibrinogen + FXIII as compared to control ($P < 0.001$ at both temperatures) as well as compared to fibrinogen alone ($P < 0.05$ at both temperatures).

Platelet dependent clot strength (EX-FIBTEM-MCF)

The platelet dependent clot strength (EX-FIBTEM-MCF) was 72% and 76% of the EXTEM-MCF, at 37°C and 33°C respectively. Hypothermia at 33°C and haemodilution in combination decreased EX-FIBTEM-MCF, but hypothermia or haemodilution alone had no effect on EX-FIBTEM-MCF. HES and RAc affected EX-FIBTEM-MCF to the same extent (Figure 2). Addition of fibrinogen or fibrinogen + FXIII did not significantly change EX-FIBTEM-MCF with HES haemodilution, whereas EX-FIBTEM-MCF significantly *decreased* after adding fibrinogen + FXIII during RAc haemodilution (Figure 4).

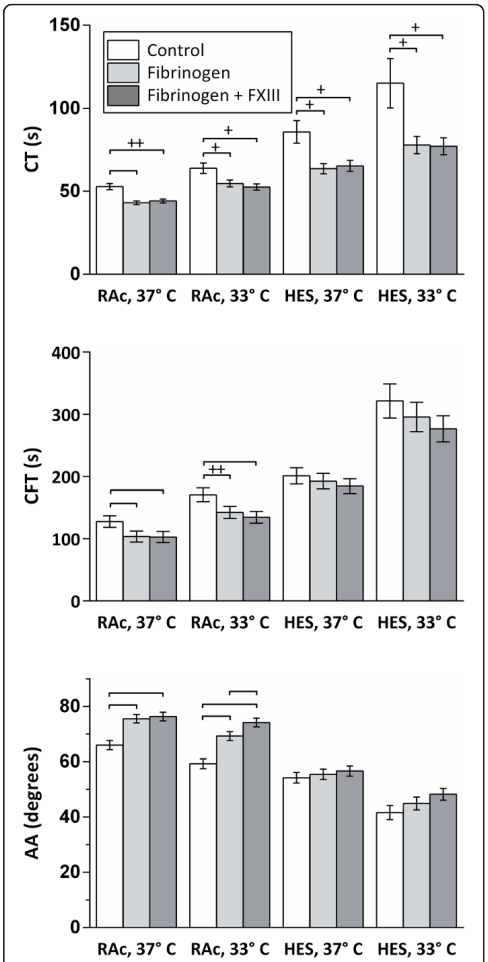

Figure 3 Effects of coagulation factor concentrate (Fibrinogen or Fibrinogen with factor XIII (FXIII)) at two different temperatures (33° vs. 37 C) during haemodilution with Ringer's acetate (RAc) or hydroxyethyl starch (HES). ROTEM EXTEM parameters shown are clotting time (CT), clot formation time CFT) and alpha angle (AA). Statistically significant differences are marked with brackets. All significances are $P < 0.001$, except where elsewise stated; +: $P < 0.05$, ++: $P < 0.01$. Data are presented as mean values, error bars are 95% simultaneous confidence intervals. N = 10.

Discussion

Principal findings

Our principal finding is that fibrinogen concentrate, with or without factor XIII concentrate, reduces dilutional coagulopathy irrespective of temperature (33° or 37°C) and that this effect was more pronounced with RAc than HES haemodilution. We also found that hypothermia affects coagulation more during haemodilution with HES than with RAc.

Hypothermia and haemodilution

The effects of cooling undiluted blood to 33°C on ROTEM parameters were small. Although significant impairments of clot initiation (CT) and propagation (CFT, AA) were seen, measures of clot strength (EXTEM-MCF and FIBTEM-MCF) were unaffected. These results

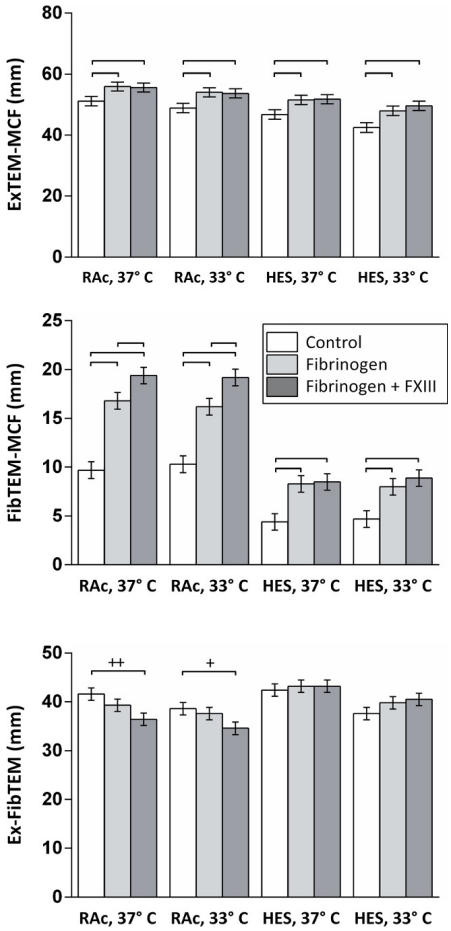

Figure 4 Effects of coagulation factor concentrate (Fibrinogen or Fibrinogen with factor XIII (FXIII)) at two different temperatures (33° vs. 37 C) during haemodilution with Ringer's acetate (RAc) or hydroxyethyl starch (HES). ROTEM parameters shown are EXTEM-MCF, FIBTEM-MCF and EX-FIBTEM-MCF; the latter calculated as FIBTEM-MCF subtracted from EXTEM-MCF. Statistically significant differences are marked with brackets. All significances are $P < 0.001$, except where elsewise stated; +: $P < 0.05$, ++: $P < 0.01$. Data are presented as mean values, error bars are 95% simultaneous confidence intervals. N = 10.

are in line with results from other in vitro studies with VHA's, which demonstrate mild to moderate hypothermia to decrease the rate of clot formation but not the clot strength [15,16], even though more severe hypothermia (<28°C) may decrease clot strength [17]. An in vitro study on the relative effects of hypothermia on platelets and plasma coagulation indicated an impaired platelet adhesion as the primary cause of coagulopathy during mild hypothermia [2]. This may explain our previous results with free oscillation rheometry (FOR), which measured a decreased clot velocity as well as clot strength during hypothermia [9]. ROTEM is possibly better at detecting plasma coagulation than platelet function whereas FOR is more sensitive to platelet function.

The observations that haemodilution impaired parameters reflecting both the rate of clot formation and clot strength, and that HES attenuated these parameters more than RAc, is in line with previous research [18,19]. Numerous studies with VHA's have shown a progressive decrease of initiation, amplification and propagation of coagulation as well as clot strength, where clot strength was more affected by synthetic colloids than crystalloids [9,20,21]. Synthetic colloids, such as gelatine and HES, interfere with fibrin network structure and consequently decrease clot strength more than explained by dilution of plasma proteins alone [22]. In contrast, some investigations have shown that haemodilution up to 50% with isotonic saline may induce a hypercoagulable state [23]. This has been suggested to be caused by decreased plasma antithrombin levels [24]. However, these studies used native non-activated blood, which may explain the difference from our results [25].

Hypothermia in combination with haemodilution mainly affected ROTEM parameters measuring clot velocity. Thus, HES haemodilution interacted with hypothermia to further impair CFT and AA. This is in line with our previous study with free oscillation rheometry (FOR) [9], but few other studies have addressed the combined effect of haemodilution and hypothermia. A study from 1994 measuring activated partial thromboplastin time found additional but not synergistic effects of hypothermia and haemodilution with a crystalloid [5]. Possible explanations for the observed synergy between hypothermia and HES, may be that both hypothermia [2,26] and HES [27,28] have direct effects on platelets, and that also plasma coagulation activity is decreased during both hypothermia [2] and haemodilution [29]. Platelet activation is very important for the thrombin burst associated with propagation of coagulation, which may explain why the ROTEM parameters CFT and AA were particularly affected by the combination of HES-haemodilution and hypothermia.

Addition of fibrinogen with or without factor XIII

Our results imply that fibrinogen concentrate can be used to improve coagulation at 33°C. This was also suggested, albeit not statistically significantly, in our previous study with FOR [9]. The results from the present study also imply that a clot weakened by RAc is easier to improve than a clot weakened by HES, which corroborates results

from previous studies [8,30,31]. Although fibrinogen also improved fibrinogen-dependent clot strength (FIBTEM-MCF) irrespective of fluid or temperature, this improvement was greater during RAc than HES haemodilution. The general view that hypothermia-induced coagulopathy is refractory to treatment with coagulation factor concentrates [32] is contradicted by our results, as well as by a previous study where fibrinogen concentrate increased whole blood coagulation at 32°C [33]. It is a principal finding of our study, that the clot stabilizing effect of fibrinogen concentrate on coagulopathy induced by RAc is as effective at 33°C as it is during normothermia. This implies that correction of hypothermia before substitution with fibrinogen concentrate is clinically not necessary.

Factor XIII improved fibrinogen's effect on FIBTEM-MCF during RAc-haemodilution but not during HES haemodilution. In line with our study, several previous in vitro haemodilution studies on healthy volunteers have shown that although FXIII alone does not improve dilutional coagulopathy, it can enhance fibrinogen's corrective effect [7-9,34]. These results contradict an in vitro study on blood from intensive care patients where supraphysiological doses of FXIII were used [35]. In this study, a high pre-existing concentration of fibrinogen in plasma may explain the corrective effect of FXIII alone. It is probably only worthwhile to administer factor XIII to correct a coagulopathy if normalizing plasma fibrinogen concentrations is cared for. Consequently, it may be of value to combine fibrinogen with factor XIII in order to improve clot stability during RAc-haemodilution.

Platelet dependent clot strength (EX-FIBTEM-MCF)

The derived parameter EX-FIBTEM-MCF is considered to reflect platelet dependent clot strength. EX-FIBTEM-MCF decreased both after haemodilution and mild hypothermia. This is in line with studies using the cone and platelet analyser, where haemodilution [18] and mild hypothermia [2] showed reduced platelet aggregation. In contrast, some studies have found hypothermia to increase platelet aggregation, as measured with impedance aggregometry [36,37]. Aggregation is a process where fibrinogen stick platelets to each other through the platelet receptor glycoprotein IIb/IIIa, and fibrinogen concentrate has previously been shown to counteract glycoprotein IIb/IIIa–directed platelet inhibition [38]. We therefore expected the hypothermia induced reduction of platelet dependent clot strenght to be reversed by fibrinogen concentrate, but instead, EX-FIBTEM-MCF *decreased* significantly after the addition of fibrinogen + FXIII concentrates to RAc diluted blood. A recent study found platelet aggregation to be reduced or unaffected by in vitro supplementation with fibrinogen concentrate, depending on which platelet activator being used [39]. However, we believe that the reduction of EX-FIBTEM-MCF should not be interpreted as a decreased platelet function but that the addition of fibrinogen + FXIII increased FIBTEM-MCF values more than EXTEM-MCF, and hence the so called platelet dependent clot strength (EX-FIBTEM-MCF) decreased regardless of actual platelet function. There are principally two possible explanations of this shortcoming of ROTEM: 1) the ROTEM instrument may be more sensitive to changes at low levels of clot strength (MCF) than at normal levels; or 2) platelets are not fully inhibited by the FIBTEM assay. ROTEM uses forced oscillation and an arbitrary clot strength scale with a theoretical maximum of 100 mm, which makes a limited sensitivity at the upper scale possible. Platelet inhibition with cytochalacin D has been shown to be improved if combined with abciximab, a fibrinogen receptor inhibitor [40]. In conclusion, we and others question the reliability of EX-FIBTEM-MCF as a platelet function parameter [41] and suggest that other methods may be more suitable.

Limitations

There are several methodological concerns when performing in vitro coagulation tests. For example, there is no activation of procoagulative or fibrinolytic responses secondary to tissue trauma [1]. Furthermore, shear strains applied with viscoelastic haemostatic assays such as ROTEM are substantially lower than those found in the human circulation, which may modify clot structure during measurements [42]. There are also concerns with in vitro haemodilution: acid–base regulation is not physiological and the decreased haematocrit may increase fibrin thread formation in the reaction chamber [43]. Therefore, we restricted dilution to 33%. Finally, there is much debate regarding the use of native whole blood versus citrated whole blood in VHAs. We used citrated blood, since it can be incubated and stored for up to four hours whereas native blood must be analysed within 4 minutes. However, incubation of citrated blood over 30 minutes has been shown to decrease thrombelastographic differences between native and citrate blood analyses [25].

Conclusion

In conclusion, our results show that fibrinogen with or without FXIII corrected in vitro dilutional coagulopathy also during hypothermia and that the corrective effects were weaker during haemodilution with HES, as compared to RAc. Factor XIII enhanced the corrective effects of fibrinogen, but only during RAc haemodilution. Finally, HES haemodilution interacted with hypothermia to impair coagulation.

Abbreviations

HES: Hydroxyethyl starch; RAc: Ringer's acetate; ROTEM: Rotational thromboelastometry; CT: Clotting time; CFT: Clot formation time; AA: Alpha

angle; MCF: Maximal clot formation; VHA: Viscoelastic haemostatic assays; FXIII: Factor XIII; FOR: Free oscillation rheometry.

Competing interests

U.S. received research grants from CSL Behring 2010 and lecture fees from CSL Behring 2014.
U.S. is a member of Guideline Committee for Critical Bleeding, Swedish Society of Thrombosis and Haemostasis, www.ssth.se.
None of the other authors has any competing interests to declare.

Authors' contributions

DW: study design, volunteer recruitment, laboratory work, main responsible for manuscript preparation; ODT: manuscript preparation and linguistic correction; FN: statistical analysis: KO: manuscript preparation; US: study design, manuscript preparation and providing laboratory facilities. All the authors have approved the final manuscript for submission.

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Study III

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Thromboelastometry versus free-oscillation rheometry and enoxaparin versus tinzaparin: an in-vitro study comparing two viscoelastic haemostatic tests' dose-responses to two low molecular weight heparins at the time of withdrawing epidural catheters from ten patients after major surgery

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Abstract

Background: Monitoring low molecular weight heparins (LMWH's) in the perioperative period is prudent in patients at high risk of coagulative complications, especially when the patient has an epidural catheter requiring withdrawal, which is associated with the risk of spinal haematoma. The aim of this study was to evaluate the *in vitro* dose-responses of two different LMWH's on two different viscoelastic haemostatic tests, using blood sampled from patients with normal routine coagulation parameters, on the day after major surgery when their epidural catheters were due to be withdrawn.

Methods: Enoxaparin or tinzaparin were added *in vitro* to blood from ten patients who had undergone oesophageal resection, to obtain plasma concentrations of approximately 0, 0.5, 1.0 and 1.5 IU/mL. Coagulation was monitored using thromboelastometry (ROTEM®) using the InTEM® activating reagent; and free oscillation rheometry (FOR: ReoRox®), activated using thromboplastin. Clot initiation was measured using ROTEM-CT, ReoRox-COT1 and ReoRox–COT2. Clot propagation was measured using ROTEM-CFT, ROTEM-Alpha Angle and ReoRox-Slope. Clot stability was measured using ROTEM-MCF and ReoRox-G'max, and clot lysis was measured using ROTEM-ML and ReoRox-ClotSR.

Results: Clot initiation time assessed by thromboelastometry and FOR was prolonged by increasing concentrations of both LMWH's ($P < 0.01$). Equivalent doses of tinzaparin in international units (anti-FXa units) per millilitre prolonged clot initiation more than enoxaparin ($P < 0.05$). There was significant inter-individual variation – the ranges of CT and COT1 at LMWH-concentrations of 0 and 1.5 IU/mL overlapped. None of the tests reflecting clot formation rate or stability showed a dose–response to either LMWH but clot lysis showed a tentative negative dose–response to the LMWH's.

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Conclusions: Clot initiation time's dose-dependent prolongation by LMWH's in this study agrees with previous research, as does tinzaparin's stronger anti-coagulative effect than enoxaparin at equivalent levels of anti-FXa activity. This casts doubt on the validity of using anti-FXa assays alone to guide dosage of LMWH's. The significant inter-individual variation in dose–response suggests that the relationship between dose and effect in the postoperative period is complicated. While both ROTEM and FOR may have some role in postoperative monitoring, more research is needed before any conclusion can be made about their clinical usefulness.

Keywords: Coagulation, Factor Xa, Thromboelastometry, Free-oscillation rheometry, Low molecular weight heparin, Postoperative, Enoxaparin, Tinzaparin, Epidural haematoma, Spinal haematoma

Background

When low molecular weight heparins (LMWH's) were first used in clinical practice, monitoring was considered unnecessary [1], but this has recently been questioned since major haemorrhagic complications are regularly reported in patients treated with LMWH and the optimal LMWH dose in the aged, patients with obesity and renal insufficiency is not well defined [2–4]. Hypercoagulative states are common in many settings: postoperative, critical illness, obstetrics, oncology and coronary care; such that ordinary doses of LMWH are insufficient, but overdosing of thrombosis prophylaxis is also dangerous since it predisposes to haemorrhagic complications such as spinal haemorrhage in conjunction with withdrawing an epidural catheter [4–8].

Low molecular weight heparins have more predictable pharmacokinetic and pharmacodynamic properties than unfractionated heparin (UFH), and have therefore become the gold standard in many clinical situations such as thromboprophylaxis, and treatment of deep vein thrombosis (DVT) and pulmonary embolism (PE). Variation in the anticoagulant potency of the numerous LMWH's that are available is the result of different degrees of inhibition of coagulation factors Xa and IIa. LMWH's with greater molecular weight are more similar to unfractionated heparin [1, 9, 10]. UFH (mean molecular weight, MW, 15 kDa) inhibits factor Xa and IIa equally whereas enoxaparin (mean MW 4.2 kDa) is a LMWH with a high anti-FXa/anti-FIIa ratio: it inhibits factor Xa four times as strongly as IIa. Tinzaparin (mean MW 6.8 kDa) is more similar to unfractionated heparin in that it inhibits factor Xa only twice as strongly as factor IIa [11].

Routine laboratory plasma based coagulation tests for monitoring heparinization, such as the activated partial thromboplastin time (aPTT), and the chromogenic anti-FXa test only detect changes in the initiation phase of coagulation and are not always rapidly available at all times of the day. It is possible to run viscoelastic haemostatic tests (VHT's) in 'patient-near' laboratories or even bedside at any time of the day, providing preliminary results within minutes and complete results within an hour of blood sampling. There are several commercially available VHT's which allow analysis not only of the propagation and amplification phases of whole blood coagulation, but also of fibrinolysis and clot structure, which depend upon fibrin polymerization and platelet activity [12, 13].

It would be advantageous to be able to titrate LMWH doses using viscoelastic tests to reduce complications caused by bleeding and thrombosis. However, there are few studies in this area and to our knowledge there are no studies concurrently comparing different LMWH's with different anti-FXa/anti-FIIa ratios, using different VHT's [14–16].

The aim of this study was to evaluate dose–response effects of enoxaparin and tinzaparin on ROTEM® and FOR: can these instruments be used to monitor LMWH's at and above levels used for thrombosis prophylaxis? Our hypothesis was that FOR would be more sensitive to LMWH's effects on clot formation and strength than thromboelastometry.

Methods

Study subjects and sampling

Ten patients who had undergone oesophageal resection were included in the study after informed and signed consent. The study was approved by the local ethics committee in Lund (DNR 2010/482-100).

Blood was sampled from each patient's indwelling central venous catheter on the day that their epidural catheter was removed, using 4.5 mL BD Vacutainer® citrate tubes. All patients had been routinely sampled the day before to assure normal renal function (creatinine, urea), routine coagulation parameters: activated partial thromboplastin time (aPTT), prothrombin time international normalized ratio (PT-INR) and platelet count (PLT). All patients had received standard thrombosis prophylaxis with enoxaparin 40 mg at 8 p.m. the evening before blood sampling, which took place between 10 a.m. and 2p.m, 14–18 h after the last dose of enoxaparin.

Titration of blood with LMWH

Enoxaparin (Klexane, Sanofi-Aventis, Guildford, UK) and tinzaparin (Innohep, Leo Pharma, Ballerup,

Denmark) were diluted with isotonic saline (9 mg/mL NaCl: Fresenius Kabi, Bad Homburg, Germany) to concentrations of 10, 20 and 30 IU/mL. 60 μL aliquots of saline containing 0, 10, 20 or 30 IU/mL enoxaparin or tinzaparin were then added to 2 mL portions of prewarmed (37 ° C) citrated blood from each patient to obtain plasma concentrations of 0, 0.5, 1.0 and 1.5 IU/mL of enoxaparin and tinzaparin, respectively, assuming that the blood samples had a haematocrit of 40 %. The samples were incubated for 10 min at 37 °C.

The concentrations of LMWH between 0 and 1.5 IU/ml in this study encompass both thromboprophylactic levels of 0.2-0.4 IU/mL, and higher anti-FXa levels that are above recommended levels [17].

Viscoelastic coagulation analysis

Clot formation and lysis was studied using thromboelastometry (ROTEM®, Pentapharm, Munich, Germany) and FOR (ReoRox G_2®, MediRox, Nyköping, Sweden). Analyses were run at 37 °C within 1 h of sampling.

Thromboelastometry

Technical details on ROTEM have been described previously [18, 19]. Briefly, the ROTEM® has a fixed sample cup with a pin suspended in the blood sample. The pin oscillates and the movement is registered in the coagulating sample [18]. Analysis of coagulation with ROTEM gives rise to a curve from which the clotting time (CT), clot formation time (CFT), alpha angle, maximum clot firmness (MCF) and maximum clot lysis (ML), which represents fibrinolysis, can be determined as shown in Fig. 1 [19].

After addition of 20 μL of 0.2 M $CaCl_2$ (the 'star-tem®' reagent) to 300 μL of each sample, coagulation was initiated in each sample by addition of 20 μL of the InTEM® reagent, which contains partial thromboplastin phospholipid and ellagic acid.

Free oscillation rheometry (FOR)

FOR was assessed with the ReoRox G_2 rheometer (MediRox AB, Nyköping, Sweden). The sample is added to a reaction chamber which consists of a gold-coated sample cup with a gold-coated cylinder suspended in the blood sample [20]. The sample cup oscillates and the changes in the frequency and damping of the oscillation in the coagulating sample are registered. Changes in damping give rise to a viscosity curve measured in Pascal-seconds (Pa.s) against time and changes in frequency give an elasticity curve measured in Pascals (Pa) against time, as shown in Fig. 1. The clotting time (COT) can be obtained from the viscosity curve: COT1 represents the time to initiation of clot formation and COT2 the time when clot formation is complete and elasticity starts developing. COT2 is equivalent to ROTEM's CT. The difference between COT2 and COT1 is a measure of clot progression. From the elasticity curve the slope, maximum elasticity (G'max; the maximum strength/stiffness of the clot) and clot strength reduction (Clot SR; fibrinolysis) can be determined. These correspond to ROTEM's alpha angle, MCF or MCE and ML, respectively.

After addition of 25 μL of 0.5 M $CaCl_2$ (MediRox AB) to 1000 μL of sample, coagulation was initiated with thromboplastin (the HepScreen1 reagent, MediRox AB). The FOR tracings analyzed were: COT1, COT2, slope, G'max and Clot SR.

Statistical analysis

The 'R' Statistical environment (version 3.1.3: www.r-project.org) was used for statistical calculation and to create diagrams. Correlations were tested using Spearman's test. The significances of differences between results for different levels of heparinization using the same LMWH and different LMWH's at the same level of heparinization were tested using the Wilcoxon signed

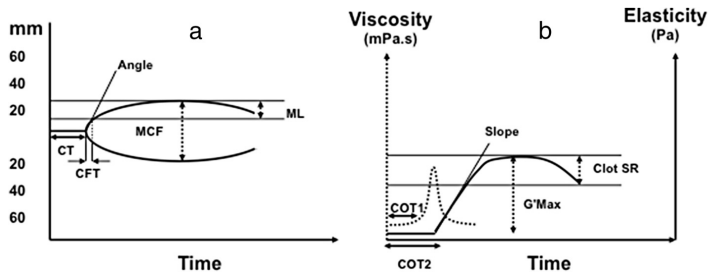

Fig. 1 Diagram showing the parameters recorded from rotational thromboelastometry (ROTEM) and free oscillation rheometry (FOR, ReoRox). (**a**) ROTEM. (**b**) ReoRox. A brief explanation of the parameters follows: measures of clot initiation: ROTEM-CT (clot time) and FOR-COT1 and -COT2. Measures of clot propagation: ROTEM-CFT and –alpha angle; and FOR-(COT2-COT1) and –Slope. Measures of clot structure: ROTEM-MCF and FOR-G'max. Measures of fibrinolysis: ROTEM-ML and FOR-Clot SR

rank test. A P-value of <0.05 was considered significant. Friedman's analysis of variance was used to detect significant differences in the distributions of results for enoxaparin and tinzaparin, taking into account inter-individual variation and differing concentrations of LMWH. Box and whisker diagrams were constructed using R's boxplot function. Boxes span the interquartile range and the whiskers encompass the data point furthest from the box yet within 1.5 times the length of the box from the box.

Results

Raw data of our results are available as a text file in 'Additional file 1'.

Measures of clot initiation were prolonged by increasing doses of LMWH

Measures of initiation of coagulation as assessed by ROTEM and FOR were significantly prolonged by increasing concentrations of both LMWH's (ROTEM-CT, FOR-COT1 and FOR-COT2: see Table 1 and Fig. 2a-c), with significant correlation coefficients (Spearman's Rho) of between 0.54 and 0.77, but there was a wide spread of results, with the lowest measured ROTEM-CT in the presence of 1.5 IU/mL tinzaparin being shorter than the longest ROTEM-CT in the control group (0 IU/mL tinzaparin). The two LMWH's values of ROTEM-CT correlated to each other significantly, as did their values of FOR-COT1 and FOR-COT2 (see Figs. 3a,b and d).

FOR-(COT2-COT1) was the only measure of clot propagation that showed a dose–response to LMWH

ROTEM-alpha angle, ROTEM-CFT and FOR-Slope were not affected by increasing concentrations of LMWH (see Table 1 and Fig. 2d and g). FOR-(COT2-COT1), which is the time delay between when viscosity starts to increase (COT1) and when elasticity starts to increase (COT2), was significantly prolonged by increasing doses of LMWH. The median (COT2-COT1) in the presence of 1.5 IU/mL tinzaparin and enoxaparin were 50 % and 30 % respectively longer than in the absence of added LMWH, giving correlation coefficients (Spearman's Rho) of 0.47 and 0.79 respectively ($P < 0.05$). Although the differences between (COT2-COT1) for the two LMWH's at each concentration were not significantly different, there was a significant whole-data difference in the results for tinzaparin and enoxaparin (see Table 1 and Fig. 2h). ROTEM-MCF and FOR-G'max for enoxaparin and tinzaparin showed good correlation (see Fig. 3e-f) but were not affected by increasing doses of either LMWH (see Table 1).

ROTEM and FOR's tests for clot lysis showed a tentative dose response

Neither our ROTEM-ML nor FOR-ClotSR results were outside the reference ranges for a normal level of fibrinolysis, and there were no significant differences between enoxaparin and tinzaparin at any concentration, or in ANOVA whole-data analysis. There was, however, a significant but weak negative correlation between the dose of enoxaparin and ROTEM-ML ($\sigma = -0.36$, $P < 0.05$); and the dose of tinzaparin and FOR-Clot SR ($\sigma = -0.41$, $P < 0.05$), but not between the dose of enoxaparin and FOR-Clot SR or tinzaparin and ROTEM-ML (see Table 1 and Fig. 2e and f).

Discussion

Miyazaki et al. estimated that around 70 % of spinal hematomas occurring at the time of withdrawing an epidural catheter were related to abnormal coagulation, which challenges the dogma that monitoring of prophylactic LMWH is unnecessary in this setting [7]. Due to the difficulty and expense involved in conducting prospective studies on rare complications, it is very unlikely that such a study will ever be able to show that viscoelastic tests are reliable predictors of spinal haematoma.

The most common and well-documented clinical viscoelastic tests are thrombelastography (TEG®) and rotational thromboelastometry (ROTEM®). Less well-documented are free oscillation rheometry (FOR, ReoRox®) and Sonoclot® [12, 13, 21]. Although these assays measure the same aspects of coagulation and can detect both hypocoagulation and hypercoagulation, they differ in their mechanisms [21–23].

LMWH's are a diverse group of antithrombotic molecules derived from unfractionated heparins (UFH) and have different structures and molecular weights (MW's), which results in varying pharmacological features [24]. In this study coagulation following treatment with LMWH's (enoxaparin and tinzaparin) was assessed using viscoelastic methods (thromboelastometry and FOR) to assess their potential to monitor treatment with LMWH's.

Previous studies have shown varying abilities of viscoelastic devices to monitor treatment with LMWH's [15, 16, 25–27] whereas UFH has been successfully monitored in healthy volunteers [27]. Louis et al. recently failed to show that the rate of deep vein thrombosis rate in trauma patients was reduced by using TEG tracings to titrate enoxaparin doses despite this leading to an increase in anti-FXa activity [28].

Both ROTEM and FOR show a linear relationship between measures of clot initiation and concentration of LMWH, albeit with great inter-individual variation

We found that both LMWH substances prolonged both instruments' measures of clot initiation in a significant

Table 1 Rotational thromboelastometry (ROTEM) and free-oscillation rheometry (FOR) results at varying concentrations of enoxaparin and tinzaparin

	Manufacturer's reference range	0 IU/mL	Enoxaparin 0.5 IU/mL	Enoxaparin 1.0 IU/mL	Enoxaparin 1.5 IU/mL	Tinzaparin 0.5 IU/mL	Tinzaparin 1.0 IU/mL	Tinzaparin 1.5 IU/mL	Enoxaparin vs Tinzaparin ANOVA❖	Enoxaparin Spearman (Rho, P)	Tinzaparin Spearman (Rho, P)
ROTEM											
CT (s)	100-240	178 ± 38	191 ± 89	214 ± 109	249 ± 94	223 ± 53	289 ± 84	326 ± 125	$P < 0.01$	0.62, $P < 0.01$	0.70, $P < 0.01$
CFT (s)	30-110	77 ± 23	87 ± 21	85 ± 16	83 ± 22	74 ± 27	84 ± 45	80 ± 21	$P < 0.05$	N/S	N/S
Angle (°)	70-83	75 ± 4	74 ± 4	75 ± 3	73 ± 4	75 ± 5	73 ± 5	73 ± 4	N/S	N/S	N/S
MCF (mm)	50-72	62 ± 5	61 ± 7	63 ± 5	63 ± 6	68 ± 7	64 ± 8	65 ± 6	N/S	N/S	N/S
ML (%)	<15	8 ± 4	6 ± 3	5 ± 4	2 ± 5	5 ± 4	6 ± 4	2 ± 4	N/S	−0.36, $P < 0.05$	N/S
FOR (ReoRox)											
COT1 (s)	20-35	30 ± 5	35 ± 7	39 ± 8	38 ± 11	36 ± 4	45 ± 9	48 ± 15	$P < 0.01$	0.58, $P < 0.01$	0.77, $P < 0.01$
COT2 (s)	30-90	65 ± 11	76 ± 13	82 ± 14	85 ± 20	74 ± 5	91 ± 16	108 ± 29	N/S	0.54, $P < 0.01$	0.84, $P < 0.01$
COT2-COT1 (s)	10-55	34 ± 13	41 ± 8	43 ± 9	46 ± 10	38 ± 4	46 ± 11	51 ± 15	$P < 0.05$	0.47, $P < 0.01$	0.79, $P < 0.01$
Slope (Pa/min)	45-145	99 ± 87	121 ± 82	126 ± 88	132 ± 76	118 ± 90	138 ± 94*	109 ± 88	$P < 0.01$	N/S	N/S
G'max (Pa)	770-2180	1629 ± 617	1777 ± 662	1831 ± 701	1612 ± 612	1656 ± 692	2069 ± 665	1615 ± 641	N/S	N/S	N/S
Clot SR (%)	10-25	17 ± 4	14 ± 5	16 ± 4	13 ± 6	15 ± 5	13 ± 6	11 ± 7	N/S	N/S	−0.41, $P < 0.05$

Results are presented as median ± SD. The significances of differences between individual concentrations, and between enoxaparin and tinzaparin at equal concentrations, are shown in Figs. 2 and 3, which display the results diagrammatically. ❖The significance of differences between results for enoxaparin and tinzaparin, corrected for concentration and individual, were assessed by Friedman's analysis of variance (ANOVA). A brief explanation of the above tests follows. Measures of clot initiation: ROTEM-CT (clot time) and FOR-COT1 and -COT2. Measures of clot propagation: ROTEM-CFT and –alpha angle; and FOR-(COT2-COT1) and –Slope. Measures of clot structure: ROTEM-MCF and FOR-G'max. Measures of fibrinolysis: ROTEM-ML and FOR-Clot SR

*indicates a significant inter-class difference with $p < 0.05$

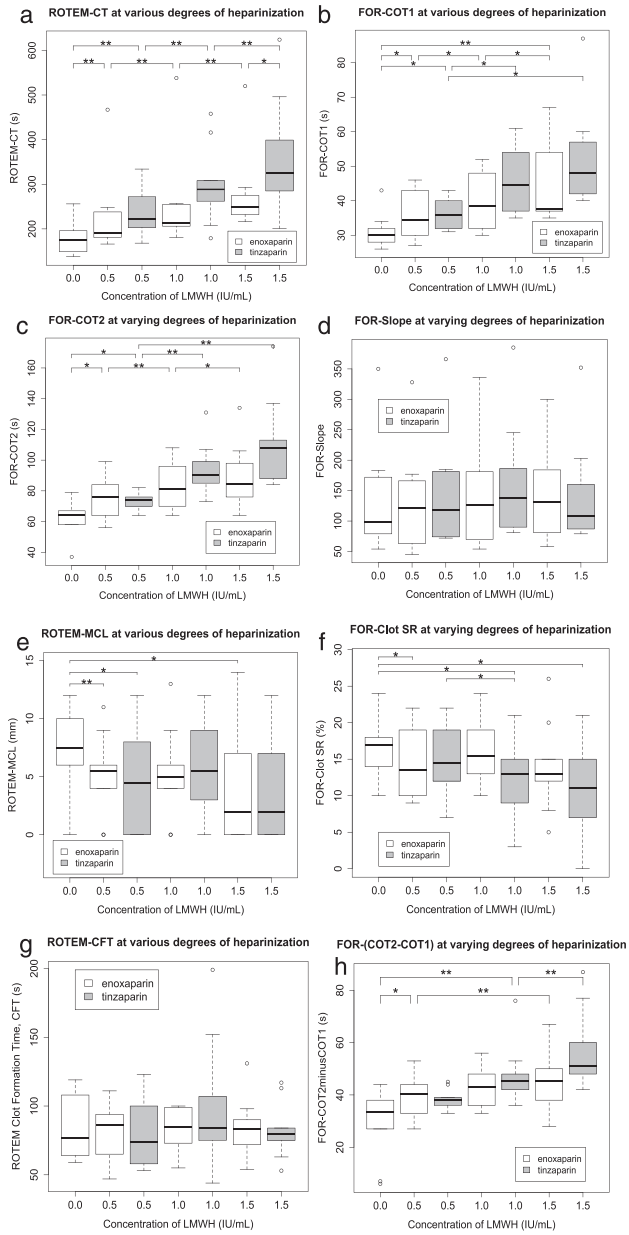

Fig. 2 (See legend on next page.)

(See figure on previous page.)
Fig. 2 Box and whisker plots showing rotational thromboelastometry (ROTEM) and free-oscillation rheometry (FOR) results for enoxaparin and tinzaparin at varying concentrations. A brief explanation of the parameters follows: measures of clot initiation: ROTEM-CT (clot time) (**a**) and FOR-COT1 (**b**) and -COT2 (**c**). Measures of clot propagation: ROTEM-CFT (**g**); and FOR-(COT2-COT1) (**h**) and –Slope (**d**). Measures of fibrinolysis:: ROTEM-MCL (**e**) and FOR-ClotSR (**f**). *indicates a significant difference with $p < 0.05$. **indicates a significant difference with $p < 0.01$. Panels inside the figures both reflect inter- and intra-group comparisons

dose-dependent manner, suggesting possible usefulness for postoperative monitoring. FOR measures both the time to initiation of increasing viscosity (COT1), reflecting early clot initiation and the time to increasing elasticity (COT2), which corresponds to ROTEM-CT. All these parameters increased significantly with increasing doses of LMWH's, which is in agreement with previous research [16].

Whether the great inter-individual variation that we observed precludes these techniques use in monitoring

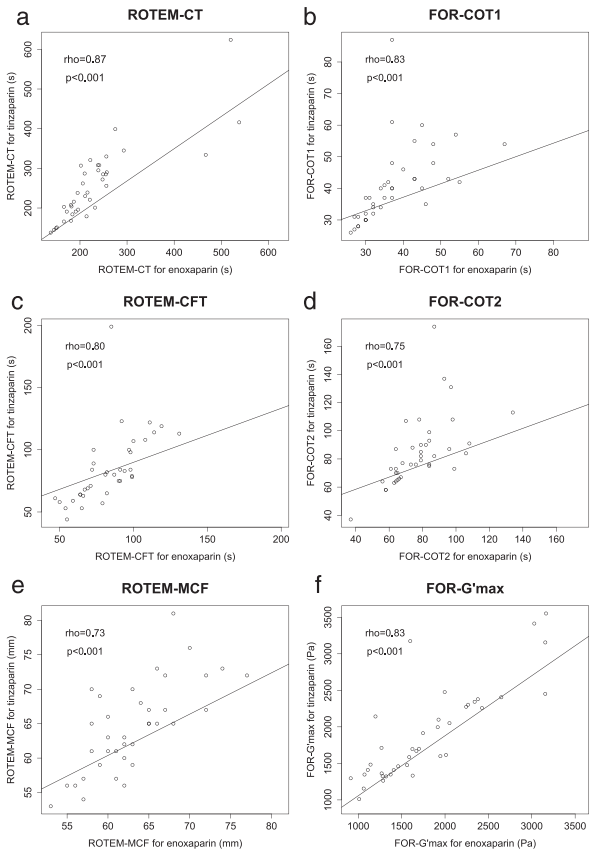

Fig. 3 Scatter plot comparing ROTEM and FOR results at corresponding doses of enoxaparin and tinzaparin in IU/mL. Results are tightly and significantly correlated but tinzaparin has a stronger anticoagulative effect than enoxaparin at any given concentration. This is due to tinzaparin having a lower anti-FXa/anti-FIIa ratio than enoxaparin: for each unit of anti-FXa activity, tinzaparin has more anti-FIIa effect than enoxaparin. Rho: Spearman's Rho: see methods section. A brief explanation of the parameters follows: measures of clot initiation: ROTEM-CT (clot time) (**a**) and FOR-COT1 (**b**) and -COT2 (**d**). Measures of clot propagation: ROTEM-CFT (**c**). Measures of clot structure: ROTEM-MCF (**e**) and FOR-G'max (**f**)

LMWH's depends on whether the results actually reflect the coagulation status of the patients or not: although methodological variation may account for some of the variation, the complex coagulation status occurring after major surgery is also likely to cause variation in patients' response to any given dose of LMWH and it is possible that viscoelastic tests have a place in identifying 'sensitive' patients for whom a 'normal dose' is actually an overdose: preoperative malnourishment results in a reduced capacity to produce vitamin K dependent coagulation factors, and a major inflammatory response to surgery can be expected to cause shifts in plasma levels of coagulation factors. Shifts in fluid balance in the aftermath of haemorrhage with or without excessive transfusion can cause unpredictable variations in renal function and thereby pharmakokinetics. The postoperative state generally predisposes to hypercoagulation [29]. It is tempting to attribute the great inter-individual variation detected in this study exclusively to varying 'postoperative factors', but the results are actually in agreement with results from healthy volunteers given a direct factor Xa inhibitor by Casutt et al. in 2012 [30]. This is clearly an under-researched area of perioperative medicine and deserves more attention.

Neither ROTEM nor FOR could detect that LMWH affected clot stability

We observed no significant correlation between the concentration of LMWH and maximum clot strength (ROTEM-MCF and CFT, and FOR-Slope and G'max), see Fig. 2 and Table 1. This confirms previous work by Feuring et al., who observed that ROTEM-MCF was only affected by supratherapeutic levels of dalteparin; but is in contrast to Gerotziafas et al. who found that therapeutic doses of enoxaparin did indeed affect TEG-MA (thrombelastography maximum amplitude, corresponds to ROTEM-MCF) in healthy volunteers [25, 31]. LMWH consistently impedes clot initiation as measured by viscoelastic tests but not clot propagation or structure, but this does not necessarily mean that LMWH does not affect clot propagation *in vivo* since both the *ex vivo* viscoelastic tests discussed in this article are flawed by the fact that they monitor coagulation in a stagnant container. *In vivo* coagulation takes place within or beside blood vessels in which there is blood flow. If clot initiation is too slow in a microenvironment where there is constant flow, the clot may be 'washed away' before it has even formed. In contrast, even a very slow-forming clot in a viscoelastic test container is able to contribute to the cell mediated positive-feedback loops that maintain propagation.

We had hypothesised that FOR might be more sensitive to LMWH's possible attenuations of clot propagation and maximum amplitude, but this could not be confirmed by our results. Our hypothesis was based on the knowledge that the shear forces applied by rotational thromboelastometry are known to exceed the linear viscoelastic properties of clots and may therefore in themselves weaken the developing clot [32]. FOR, however, does not apply shear force to the sample: it applies a short oscillation every 2.5 s instead, which allows measurement of both viscosity and elasticity, and should also disturb the clot less than ROTEM [33]. Other differences between the techniques that may lead to differing patterns of contact activation are that the reagents used to initiate coagulation are different: ROTEM® uses thromboplastin phospholipid and ellagic acid whereas ReoRox® uses thromboplastin alone, potentially resulting in different patterns of activation. The surfaces in the ROTEM® chamber are plastic while ReoRox® is gold-plated, which may affect initiation of coagulation and reduce the tendency of the clot to loosen from the cup wall giving a false impression of fibrinolysis.

The dose-effect observed on fibrinolysis was only tentative, and surprisingly suggested that increasing doses of LMWH's decreased fibrinolysis

Although the statistical significance of the dose-effect of LMWH's on measures of fibrinolysis were only tentative, Fig. 2e and f show a negative dose–response that may deserve further investigation. Previous findings suggest that LMWH's increase rather than decrease fibrinolysis [34]: it is an interesting hypothesis that the postoperative coagulative environment may provide conditions where the inverse is true.

Tinzaparin is more potent than enoxaparin and the two LMWH's measureable effects in this study are linearly correlated. We again question anti-FXa activity's 'gold standard status' for monitoring LMWH

We found significant correlations between tinzaparin and enoxaparin for several ROTEM and FOR parameters. However, all the parameters for which a dose–response could be demonstrated in this study showed that tinzaparin had a stronger anticoagulant effect than a corresponding dose of enoxaparin in international units per millilitre (see Fig. 2 and 3). This is in line with our previous findings where tinzaparin has been shown to prolong aPTT and impede thrombin generation to a greater degree than enoxaparin, and as explained previously is due to tinzaparin having a lower anti-FXa/anti-FIIa ratio than enoxaparin. If the two LMWH's are dosed in equal units of anti-FXa activity ('international units'), the tinzaparin will have a stronger overall anticoagulant effect due to the anti-IIa activity which accompanies each unit of anti-FXa activity [11]. There is also evidence that UFH and LMWH's with larger molecular weight (>2 kDa) exert an anticoagulant effect through plasma tissue factor pathway inhibitor [35].

At many institutions, including our own institution, the anti-FXa activity assay has become the clinical 'gold standard' for monitoring LMWH's. Although anti-FXa activity is likely a reliable measure of LMWH concentration [36], we would advise against relying on this assay alone to titrate the dose of LMWH: we suggest that several assays (anti-FXa, aPTT, antithrombin, viscoelastic tests, possibly thrombin generation) should be run concurrently and in series. Laboratory results should be combined with clinical judgement to dose LMWH's in patients at risk of thromboembolic or haemorrhagic complications, particularly in patients where haemorrhage could be catastrophic, such as those whose epidural catheter is due to be withdrawn. This is not particularly new: in 2009 Van *et al.* observed that thrombelastography was a better predictor of deep vein thrombosis than anti-FXa activity in trauma and surgical patients [14].

Limitations of this study

There are some limitations to this study: it is a small *in vitro* dose–response study and should thus be viewed as a pilot study with low specificity. Since all our patients are given LMWH to prevent postoperative thromboembolism, it was not possible to run tests on a control group that had been exposed to major surgery but not LMWH. While preoperative 'baseline' analyses could have been taken, they could potentially have been misleading since LMWH is only one of the factors affecting postoperative coagulation.

A criticism of the method could be that we did not test for the samples' haematocrits and adjust the doses of LMWH accordingly: a lower haematocrit means a greater fraction of plasma in the sample, and therefore a greater 'volume of distribution' for the LMWH that we added. We nevertheless decided to administer LMWH to our samples in standard doses because this is what happens in clinical practice: LMWH is either prescribed in standard doses or by weight, which are rarely adjusted for renal function or haematocrit.

Conclusions

Both ROTEM and FOR showed clot-initiation to be prolonged by increasing doses of both LMWH's albeit with significant inter-individual variation, which may preclude their use in monitoring LMWH in the postoperative period: it is unclear from this study whether the inter-individual variation was due to methodological variation or 'true' pharmacodynamic variation. The dose–response was, as expected, significantly greater for tinzaparin than enoxaparin at equivalent doses of anti-Xa activity. We could not confirm our hypothesis that FOR could measure LMWH's effects on other measures of coagulation more sensitively than rotational thromboelastometry. More research is needed before any conclusion can be made about the superiority of ROTEM or FOR in individualizing thromboprophylactic or therapeutic therapy with LMWH.

Additional file

Additional file 1: Raw data of our results. (TXT 3 kb)

Abbreviations

ANOVA: Analysis of variance; anti-FXa: Anti-factor Xa; aPTT: Activated partial thromboplastin time; CFT: Rotational thromboelastometry® clot formation time; ClotSR: ReoRox® clot strength Reduction; CT: Rotational thromboelastometry® clotting time; DVT: Deep vein thrombosis; FOR: Free-oscillation rheometry; G'max: ReoRox® maximum elasticity; IU: International Unit; LMWH: Low molecular weight heparin; MA: TEG® maximum amplitude; MCE: Rotational thromboelastometry® maximum clot elasticity (calculated as 100 × MCF÷(100-MCF).; MCF: Rotational thromboelastometry® maximum clot firmness; ML: ROTEM® maximum clot lysis; MW: Molecular weight; PE: Pulmonary embolism; PLT: Platelet count; PT-INR: Prothrombrin time international normalized ratio; ROTEM®: Rotational thromboelastometry; TEG®: Thrombelastography; UFH: Unfractionated heparin; VHT: Viscoelastic haemostatic test.

Competing interests

Dr Tynngård has previously been a part-time consultant to MediRox. None of the other authors have any potential competing interests.

Authors' contributions

Conceived and designed the experiments: US. Performed the experiments: AL US. Analyzed the data: OT NT US. Contributed reagents/materials/analysis tools: US. Wrote the paper: OT AL NT US. All authors read and approved the final manuscript.

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Study IV

PLOS ONE

RESEARCH ARTICLE

Monitoring Low Molecular Weight Heparins at Therapeutic Levels: Dose-Responses of, and Correlations and Differences between aPTT, Anti-Factor Xa and Thrombin Generation Assays

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Abstract

Background

Low molecular weight heparins (LMWH's) are used to prevent and treat thrombosis. Tests for monitoring LMWH's include anti-factor Xa (anti-FXa), activated partial thromboplastin time (aPTT) and thrombin generation. Anti-FXa is the current gold standard despite LMWH's varying affinities for FXa and thrombin.

Aim

To examine the effects of two different LMWH's on the results of 4 different aPTT-tests, anti-FXa activity and thrombin generation and to assess the tests' concordance.

Method

Enoxaparin and tinzaparin were added ex-vivo in concentrations of 0.0, 0.5, 1.0 and 1.5 anti-FXa international units (IU)/mL, to blood from 10 volunteers. aPTT was measured using two whole blood methods (Free oscillation rheometry (FOR) and Hemochron Jr (HCJ)) and an optical plasma method using two different reagents (ActinFSL and PTT-Automat). Anti-FXa activity was quantified using a chromogenic assay. Thrombin generation (Endogenous Thrombin Potential, ETP) was measured on a Ceveron Alpha instrument using the TGA RB and more tissue-factor rich TGA RC reagents.

Competing Interests: One of the authors of this manuscript has the following competing interest: N. Tynngård is a part-time consultant to MediRox. The other authors have declared that no competing interests exist. This does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

Results

Methods' mean aPTT at 1.0 IU/mL LMWH varied between 54s (SD 11) and 69s (SD 14) for enoxaparin and between 101s (SD 21) and 140s (SD 28) for tinzaparin. ActinFSL gave significantly shorter aPTT results. aPTT and anti-FXa generally correlated well. ETP as measured with the TGA RC reagent but not the TGA RB reagent showed an inverse exponential relationship to the concentration of LMWH. The HCJ-aPTT results had the weakest correlation to anti-FXa and thrombin generation (R_s0.62–0.87), whereas the other aPTT methods had similar correlation coefficients (R_s0.80–0.92).

Conclusions

aPTT displays a linear dose-respone to LMWH. There is variation between aPTT assays. Tinzaparin increases aPTT and decreases thrombin generation more than enoxaparin at any given level of anti-FXa activity, casting doubt on anti-FXa's present gold standard status. Thrombin generation with tissue factor-rich activator is a promising method for monitoring LMWH's.

Introduction

Low molecular weight heparins (LMWH's) are among the most-commonly used anticoagulants in modern healthcare, usually given in fixed doses once or twice daily. Although monitoring is not routine, it is recommended when accurate dosing is especially important, such as in the context of renal impairment, which incurs the risk of accumulation, or at extremes of weight or age [1].

Routine coagulation analysis includes activated partial thromboplastin time (aPTT), prothrombin time-international normalized ratio (PT-INR) and platelet count (Plc). aPTT is widely available but is generally considered suboptimal for monitoring agents other than unfractionated heparin (UFH) since it has low and varying sensitivity to anticoagulation caused by LMWH's, direct thrombin (factor IIa, FIIa) and anti-factor Xa (anti-FXa) inhibitors such as dabigatran and rivaroxaban respectively [2]. In contrast to PT-INR, which is internationally standardized such that all PT-INR measurements on a single sample ought always to be the same, variation in aPTT exists between laboratories and reagents, complicating the use of this assay for guiding dosage of LMWH [3,4].

While anti-FXa activity assays are reliable determinants of the concentration of LMWH in the blood [5] and are established as a gold standard, they do not necessarily correlate well to the actual effect of the drug in vivo: they describe pharmacokinetics rather than pharmacodynamics. Tests of global coagulation such as aPTT and PT-INR are different from anti-FXa activity tests in that they reflect LMWH's clinical effect [6,7].

Most of LMWH's anticoagulative effect is provided by indirect inhibition of FXa which cleaves FII to FIIa, but like UFH they also show some indirect inhibition of FIIa itself. The anticoagulative effect of UFH and LMWH also depends on numerous other factors both pharmacokinetic and pharmacodynamic. Among these factors are that these drugs release tissue factor pathway inhibitor, and there are interindividual variations in heparin-binding proteins [8,9].

LMWH's are produced through depolymerization of UFH resulting in shorter heparin fragments and a lower mean molecular weight. There are several brands of LMWH available,

Table 1. Molecular weight and anti-factor Xa and anti-factor IIa activities of heparin and commonly used low molecular weight heparins (LMWH).[a]

Agent	Trade name in Sweden	Mean molecular mass	Anti FXa/FIIa (anti-FXa IU/mg)	Anti FXa/FIIa ratio
Unfractionated heparin (UFH)	Heparin	15 kDa	193/193	1
Tinzaparin	Innohep	6.8 kDa	90/45	2.0
Dalteparin	Fragmin	6.0 kDa	130/52	2.5
Enoxaparin	Klexane	4.2 kDa	100/25	3.9
Fondaparinux	Arixtra	1.7 kDa	930/0	∞

[a]The shorter the LMWH fragments, the more specific the agent is for factor Xa [11].

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manufactured through different chemical methods. This results in various chemical structures in the saccharide fragments that are isolated [10]. LMWH's with longer fragments, such as tinzaparin, tend to inhibit FIIa more strongly, while LMWH's with shorter fragments, such as enoxaparin, exert more specific inhibition of FXa. The resultant pharmacodynamic differences are described by the FXa/FIIa ratio–see Table 1 [11,12].

In vivo studies have suggested that measurement of the area under the thrombin generation curve, the endogenous thrombin potential (ETP), could be superior to the aPTT in monitoring the effect of LMWH and UFH [9,13,14]. It measures the total and physiologically relevant amount of thrombin formed upon *in vitro* activation of coagulation, whereas global methods based on clot formation time measure are sensitive only when the amount of thrombin formed is small.

The aim of this study was to compare the correlation of anti-FXa activity and thrombin generation with aPTT using four different methods. This is relevant to our clinical practice where we are confronted with the simple question: which test should we order to ensure correct dosage of LMWH? The tests were run on both enoxaparin and tinzaparin, since they have different FXa/FIIa inhibition ratios. Our hypothesis was that increased concentrations of LMWH would give prolongations of the aPTT, increased anti-FXa activity and inhibit FIIa generation. Due to the higher FXa/FIIa inhibition ratio of enoxaparin we expected it to have a lesser effect on the aPTT and FIIa generation than tinzaparin at any given level of anti-FXa activity.

Materials and Methods

Approval was obtained from the Regional Ethical Review Board (Lund, Protocol DNR 2010/482) and signed consent given by the volunteers.

Blood sampling and preparation

Blood taken from 10 volunteers by venous puncture was collected in citrated tubes (BD Vacutainer, 4.5 ml, 0.109 M sodium citrate). The samples were diluted with enoxaparin or tinzaparin to concentrations of 0.0, 0.5, 1.0 and 1.5 anti-FXa international units (IU) per ml plasma by adding a volume of 30 µl LMWH (0, 10, 20 and 30 IU/ml in physiological saline) to each ml blood. This dilution assumed that plasma accounted for 60% of the blood volume. The samples were then incubated for 10 minutes at 37°C before those intended for plasma-based aPTT and thrombin generation tests were immediately centrifuged for 20 minutes (2000 rpm at −20°C). Plasma was separated and stored at −80°C for later analysis. Whole blood aPTT was measured using the free oscillation rheometry (FOR) and Hemochron Jr apparatuses within an hour of sampling.

The titrations of LMWH used were selected to cover therapeutic concentrations: the plasma level of LMWH for treatment of pulmonary embolism should be 0.5 to 1.1 IU/ml of anti-FXa activity for twice-daily dosing, or 0.8 and 1.6 IU/mL for once-daily dosing [15].

FOR–aPTT

FOR was performed using a ReoRox 4 instrument (MediRox AB, Nyköping Sweden). The reference interval in healthy individuals is 26–35 seconds (s). Two hundred μl of whole blood was added to a cylindrical plastic cup and incubated with 200 μl of aPTT reagent (MRX931, MediRox AB) for 5 minutes. The reaction was started by recalcification with 200 μl CaCl 2.5 mmol/ml. The sample was set into free oscillation and the frequency and damping of the sample measured over time.

Hemochron Jr–aPTT

Whole blood aPTT was analysed using the Hemochron Jr (International Technidyne Corporation, Edison, New Jersey) (HCJ) system with a reference interval of 26–36s. Seventy μl citrated blood was added to a disposable cuvette specifically for citrated blood. The instrument moves the blood back and forth within a test channel having mixed it with a reagent. Clotting is detected using a LED light source and optical detectors.

Plasma-aPTT

Plasma-aPTT was performed using a BCS-XP analyzer (Siemens Healthcare Diagnostics, Marburg, Germany) with two reagents, Actin FSL (Siemens) with a reference interval of 26–33s and PTT- Automat (Stago, Asnières, France) with a reference interval of 28–45s. For clinical reasons, aPTT's of more than 150s are recorded as '>150s'. Coagulation is initiated with a contact activator and phospholipids in the reagent and after recalcification with $CaCl_2$, the coagulation time is measured in seconds through optical detection.

Anti-FXa activity

Anti-FXa activity was analysed on a BCS-XP with Coamatic Heparin (Chromogenix, Instrumentation Laboratories, Bedford, USA). This method is based on the inhibition of FXa by its forming inactive complexes with the antithrombin. LMWH accelerates this process such that the concentration of LMWH can be determined by the addition of a substrate which releases a colour upon cleavage by the remaining FXa, which has not bound to antithrombin [16].

Thrombin generation

Thrombin generation was measured over time on a Ceveron Alpha (Technoclone, Vienna, Austria). The concentration of thrombin is measured with a fluorescent peptide substrate, which is cleaved by thrombin to release a fluorophore. Coagulation is initiated through the addition of tissue factor and phospholipids. The rate of thrombin generation is measured over time resulting in a thrombin formation curve. The area under the curve is calculated to give the 'endogenous thrombin potential' (ETP), which represents the total amount of thrombin generated.

We initially used the TGA RB (also manufactured by Technoclone) reagent, which contains 2pM human recombinant tissue factor. Due to the results from these analyses (see results section) we repeated the measurements using the TGA RC reagent, which contains a higher concentration of tissue factor (5pM).

Statistical analysis

All statistical analysis was performed using the R software (version 3.0.3, www.r-project.org). The Wilcoxon signed rank test was conducted on each pair of contiguous groups of LMWH concentrations to test whether there was a significant difference in aPTT between the two groups. It was chosen over the standard Student's t-test due to the relatively small number of samples in this study, where the Wilcoxon signed rank test has proven to be more powerful [13]. $P<0.05$ was considered statistically significant. Box-plots were drawn where the box represents the interquartile range and the whiskers represent the range excluding outliers, defined as being more than 1.5 times the interquartile range from the upper or low quartiles. Spearman's ranked correlation test was performed to quantify the correlation between each aPTT method and the anti-FXa activity. It was chosen instead of Pearson's product-moment correlation due to the samples of the full group not being normally distributed, which is a requirement for using the Pearson's product moment correlation.

Since the plasma-based methods (ActinFSL and PTT-Automat) gave numerical results for aPTT's of 150s or less, we registered results of '>150s' as 150s in our data set. Due to 8 of the 10 subjects having at least one plasma-aPTT of '>150s' in the presence of 1.5 IU/ml of tinzaparin, we excluded all data from this concentration of LMWH when calculating correlation factors.

Results

Ten volunteers were included, all healthy men with ages ranging from 23–62 years. None had a known coagulation disorder. Our results are available as raw data in S1 File.

aPTT exhibits a linear dose-response to LMWH; there is variation between assays.

Fig. 1 shows that within the range tested, there is a linear dose-response relationship for each LMWH measured with the four different aPTT tests. With every increase of 0.5 IU/ml in LMWH concentration there was a statistically significant increase in aPTT for all the tests.

There were some significant differences between different methods' aPTT results. The Hemochron Jr showed a tendency to give longer aPTT's but the only reagent which consistently gave different results compared to the other methods was the plasma-based ActinFSL, which gave the shortest mean aPTT for all concentrations of LMWH. Which significant differences were present between reagents at different concentrations is shown in S2 File.

Tinzaparin prolongs aPTT more than enoxaparin

Tinzaparin gave significantly longer aPTT's than enoxaparin at any given dosage of anti-FXa activity—see Figs. 1 and 2. Complete tables of this data in S3 File show that the mean aPTT at a concentration of 1 IU/mL of LMWH varied from between 54s (SD 11.4) and 69s (SD 13.8) for the enoxaparin samples to between 101s (SD 21.0) and 140s (SD 27.6) for the tinzaparin samples. The standard deviation of the aPTT results increases with increasing concentrations of LMWH.

APTT is linearly correlated to anti-FXa activity, which is strongly correlated to the concentration of LMWH

Anti-FXa activity showed a strong dose-response to both enoxaparin and tinzaparin—see Fig. 3. There is a linear relationship between the anti-FXa activity and aPTT with good correlations: see Fig. 4 and Table 2. Hemochron Jr had the lowest correlation coefficients (RS 0.67 and 0.85 for enoxaparin and tinzaparin respectively) while the other methods were correlated more strongly (RS between 0.80 and 0.91). ActinFSL's regression line was flatter than the other

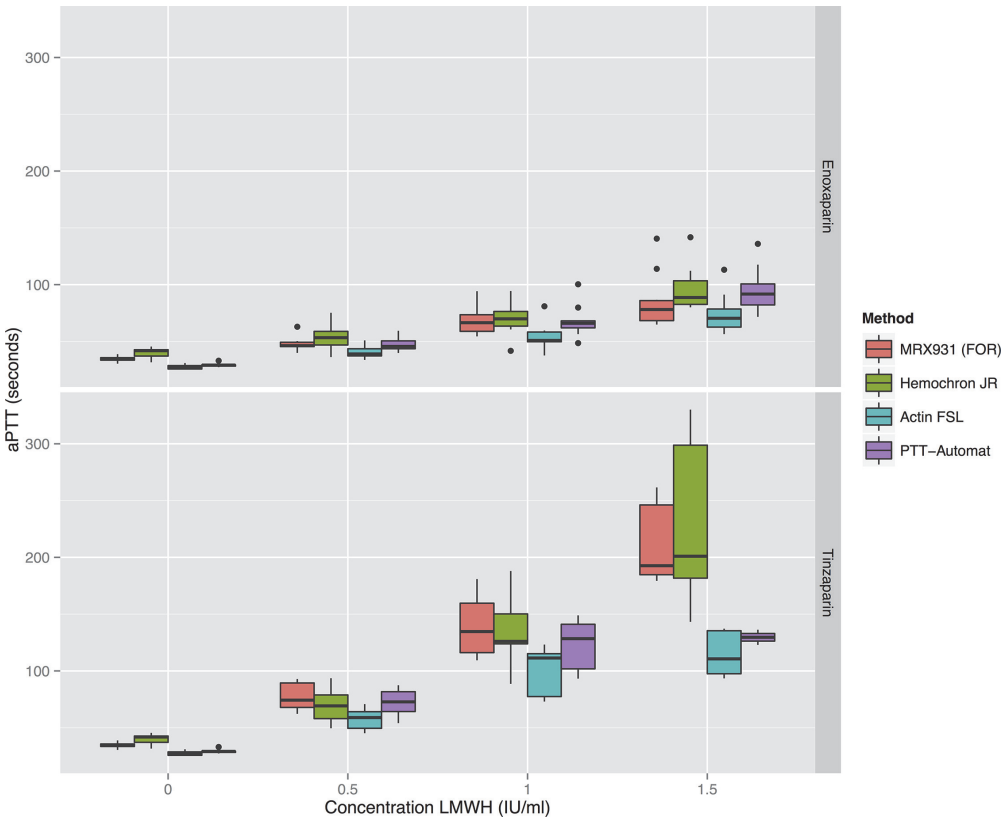

Figure 1. Boxplots of the four aPTT methods' results for enoxaparin and tinzaparin in three different concentrations. When comparing the contiguous groups with the same aPTT-method but different concentrations of LMWH, a significant difference in aPTT was found in all cases. Statistical significances between classes are shown in S1 File.

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methods, reflecting the fact that ActinFSL gives lower values of aPTT compared to the other methods.

Thrombin generation shows a negative exponential dose-response to increasing doses of LMWH

Thrombin generation measured using the TGA RB reagent was strongly inhibited by all concentrations of LMWH and showed an unacceptable degree of variation between results at each concentration—see diagram in S3 File.

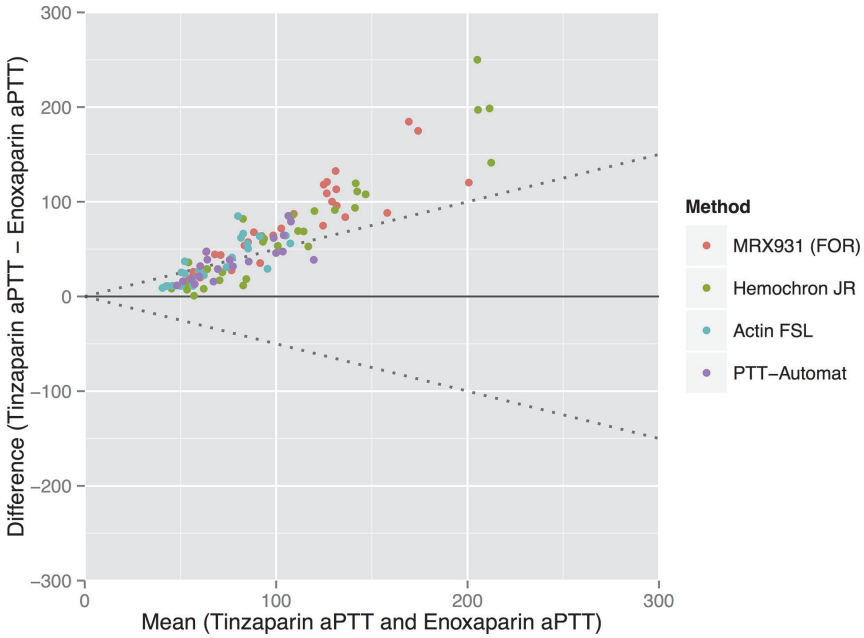

Figure 2. A comparison of enoxaparin and tinzaparin's relative effects on aPTT. Bland-Altman plot showing that the aPTT's induced by tinzaparin ranged from on average 49% more than enoxaparin (when measured using the PTT-Automat reagent) to 66% more than enoxaparin (when measured using the MRX931 reagent (FOR: free oscillation rheometry).

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Enough plasma remained to run the tests again using the TGA RC reagent on eight of the ten subjects' plasma. With this reagent the ETP showed a negative exponential relationship to the dose of LMWH, with tinzaparin exhibiting stronger inhibition of thrombin generation than enoxaparin: the mean ETP at a LMWH concentration of 1.0 anti-FXa IU/ml was 560 nM.min (SD 152) for enoxaparin and 206 nM.min (SD 104) for tinzaparin (see Fig. 5). Differences between the different concentrations as well as the two LMWH's were all statistically significant.

APTT shows a negative linear correlation to thrombin generation's logarithm

See Fig. 6 and Table 3. The logarithm of ETP measured using the TGA RC reagent showed a strong linear negative correlation to the aPTT, as measured using all apparatuses other than Hemochron Jr. For enoxaparin the correlation coefficient RS was between −0.80 and −0.88 while for tinzaparin RS was between −0.87 and −0.92. Due to HCJ's aPTT results being more variable at higher concentrations of LMWH, the correlation between HCJ-aPTT and $\log_{10}$(ETP) was weaker (Rs −0.62–0.85). In the whole blood assays (FOR and HCJ) there was a tendency for tinzaparin to elevate the aPTT more than enoxaparin at any given level of inhibition of thrombin generation.

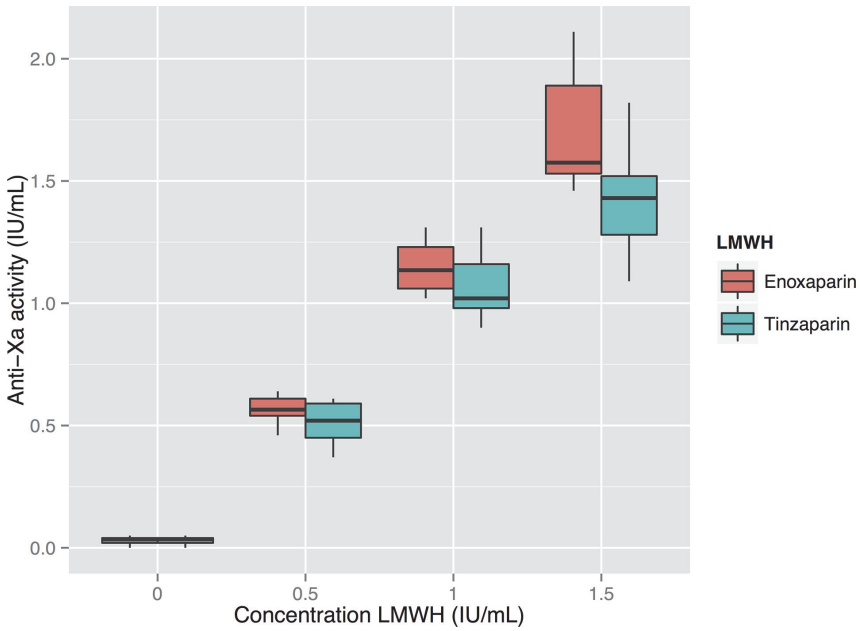

Figure 3. Relationship between measured anti-FXa and the concentration of low molecular weight heparin (LMWH). There is a strong linear correlation between anti-FXa results and the concentration of LMWH (dosed in anti-FXa units per ml). For enoxaparin and tinzaparin the correlation coefficients R_s are 0.97 and 0.96 respectively.

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aPTT: activated partial thromboplastin time. ETP: endogenous thrombin potential. FOR: Free-oscillation rheometry.

Anti-FXa activity shows a negative linear correlation to thrombin generation's logarithm

Correlations between the $\log_{10}$(ETP) and anti-FXa activity were also very strong for both LMWH's (RS −0.93 and −0.94 for enoxaparin and tinzaparin respectively)—see Fig. 7. In contrast to aPTT, which was elevated more by tinzaparin in the whole-blood samples, enoxaparin resulted in a somewhat more elevated anti-FXa for any given level of inhibition of thrombin generation, which is to be expected since it has an anti-FXa/FIIa ratio of 3.9 compared to tinzaparin's ratio of 2.0.

Discussion

Enoxaparin and tinzaparin vary in their pharmacological properties and the anticoagulative effect of LMWH may vary between patients

Our results support previous work showing that the anti-FXa assay is an excellent test for determining the concentration of LMWH in plasma [5]. It does not, however, reflect the absolute anticoagulative effect of the drugs it measures despite being strongly correlated to aPTT in this

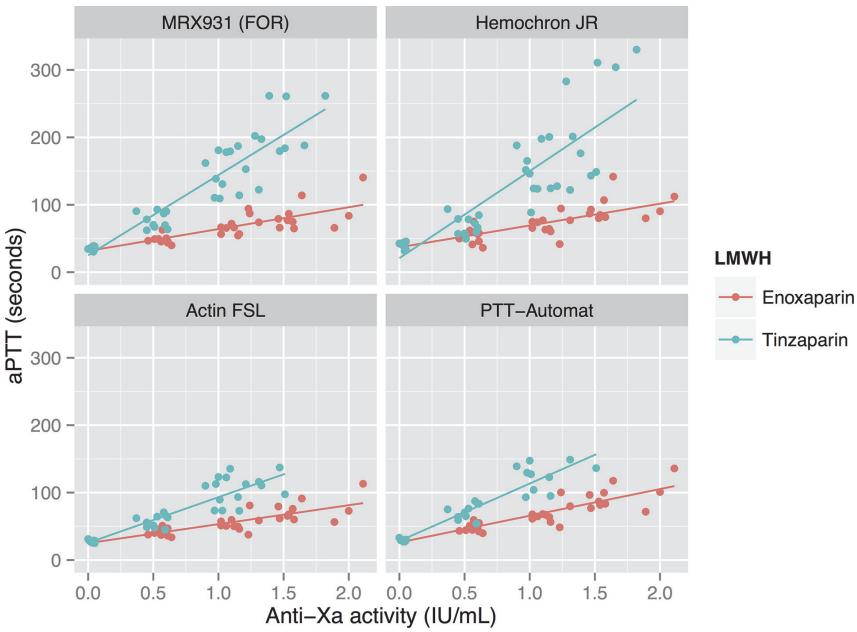

Figure 4. Correlations between aPTT and anti-FXa at varying concentrations of enoxaparin and tinzaparin, using various reagents. Anti-FXa activity reflects the concentration of LMWH and the aPTT correlated well to this measure. The correlation for Hemochron Jr is slightly weaker than for the other reagents (see Table 2).

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study (excluding Hemochron Jr: R_S = 0.80–0.91). One recent study conducted on 149 patients receiving unfractionated heparin for various indications found the correlation between the anti-FXa activity and aPTT to be much weaker (r = 0.61), which was attributed to variation in FII and FVIII [17]. We suggest that although the anti-FXa test should be used to dose LMWH's in patients with unpredictable pharmacokinetics: children, the obese, those in renal failure;

Table 2. Explanatory to Fig. 4: Correlation and regression between aPTT and Anti-FXa for enoxaparin and tinzaparin.

	Enoxaparin		Tinzaparin	
Reagent	R_S	Regression line	R_S	Regression line
MRX931 (FOR)	0.88	y = 32x + 32	0.88	y = 119x + 25
Hematochron Jr	0.67	y = 32x + 37	0.85	y = 129x + 21
ActinFSL	0.80	y = 28x + 25	0.89	y = 68x + 25
PTT-Automat	0.86	y = 39x + 26	0.91	y = 91x + 27

FOR: free-oscillation rheometry.

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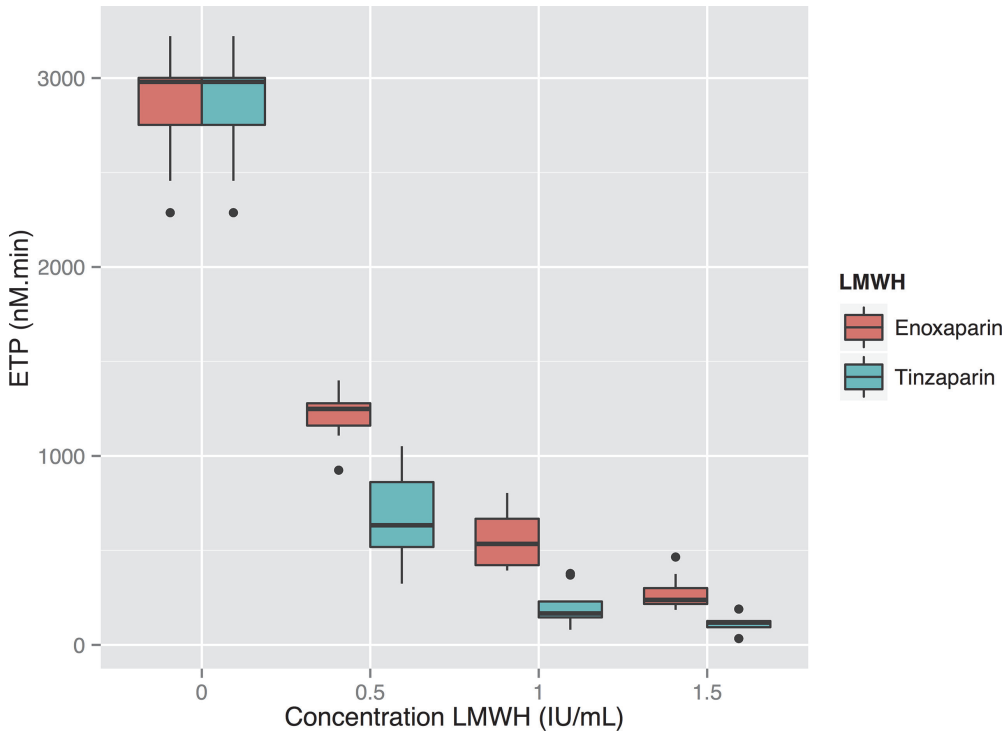

Figure 5. Thrombin generation at increasing concentrations of LMWHa, measured using the TGA RC reagent. Thrombin generation is inhibited in a negative exponential manner by increasing doses of LMWH. There is a significant difference between Endogenous Thrombin Potential (ETP) at all contiguous concentrations and between ETP for the two reagents at each individual concentration ($P << 0.05$). [a]Low Molecular Weight Heparin. [b]ETP: Endogenous Thrombin Potential.

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Table 3. Explanatory to Fig. 6.

Reagent	Enoxaparin		Tinzaparin	
	R_S	Regression line	R_S	Regression line
MRX931 (FOR)	-0.88	y = –0.017x + 80	-0.87	y = –0.042x + 147
Hematochron Jr	-0.62	y = –0.017x + 85	-0.87	y = –0.043x + 153
ActinFSL	-0.83	y = –0.015x + 66	-0.92	y = –0.027x + 100
PTT-Automat	-0.80	y = –0.021x + 85	-0.92	y = –0.035x + 125

FOR: free-oscillation rheometry.

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more functional tests of anticoagulation are required to monitor the actual effect of these drugs [18,19].

Since both enoxaparin and tinzaparin were dosed in anti-FXa units in our study, the anti-FXa effect of each drug at each concentration was the same—see Fig. 3. Tinzaparin attenuated the ETP more potently, and prolonged aPTT more than enoxaparin at equivalent levels of anti-FXa activity—see Figs. 1 and 5. This has been observed in previous studies and would appear to be due to tinzaparin's stronger inhibition of FIIa [20]. We would like to emphasize that it is illogical and confusing that despite tinzaparin's greater anti-FIIa effect, it is clinically administered in anti-FXa IU/mL/kg while enoxaparin is administered in mg/kg.

aPTT is prolonged by LMWH; there is variation between methods and reagents

Due to there being no standardisation of reagents used for aPTT, each laboratory has its own reference interval for their own specific reagent, instruments and population. This leads to clinical problems interpreting aPTT results in the clinical context, confounded by the fact that prolonged aPTT can be caused by factors other than coagulopathy *per se.* The time between sampling and analysing and the fasting state of the patient may influence the aPTT, as can a high haematocrit, which results in a lower plasma volume per ml blood taken at venepuncture such that the ratio of citrate to plasma is increased, prolonging the aPTT [21]. Our results demonstrate that there is significant variation between the different methods even when time to sampling and hydration state of each individual subjects was the same for all the tests.

The aPTT's produced using the ActinFSL reagent were lower than the values provided by the other methods (see Fig. 4). This does not mean that ActinFSL is a less useful assay than the other tests since it correlates just as strongly to the concentration of LMWH, aPTT and anti-FXa activity. Clinicians must, however, be aware that changing to ActinFSL from one of the other reagents will result in a shorter aPTT despite an unchanged level of anticoagulation.

Patient-near monitoring with aPTT

The two whole blood methods (FOR and Hemochron Jr: HCJ) can be used in the point-of-care setting. The Hemochron Jr apparatus in particular is portable, robust and easy to use. At lower concentrations of LMWH it has a similar profile to the FOR in terms of sensitivity and variance of results—see Fig. 1—and may therefore be suitable for monitoring thrombosis prophylaxis in patients at risk of accumulation of LMWH, for example in renal failure. The Hemochron Jr did, however, have a lower correlation coefficient to anti-FXa activity and ETP than the other aPTT methods in our study, and a greater variance than the FOR apparatus at higher concentrations—see Figs. 1 and 4. The more cumbersome FOR apparatus would therefore be more

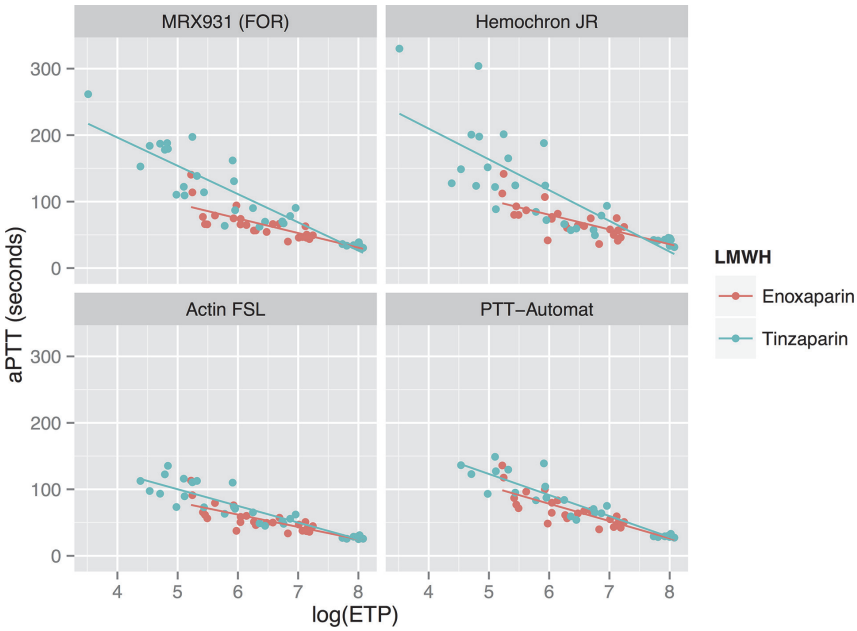

Figure 6. Relationship between aPTT measured using various reagents, and the logarithm of thrombin generation. See Table 3 for correlation factors and regression lines. There is a negative linear relationship between aPTT and $\log_{10}$(ETP). Tinzaparin results in prolonged aPTT results compared to enoxaparin in the whole blood analyses (FOR and Hemochron Jr) at any given level of thrombin generation.

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appropriate for monitoring higher concentrations of LMWH where the dose must be titrated to balance the risk of thrombosis and haemorrhage. It would be of clear clinical interest to compare FOR with the activated clotting time (ACT), which has shown some *in vitro* potential for monitoring LMWH [22].

Thrombin generation measures both anti-FXa and anti-FIIa activity and therefore has clinical potential

Generation of thrombin (FIIa) is a key event in the coagulation process, and is dependent upon FXa. Measurement of thrombin generation therefore reflects both LMWH's main effects: indirect inhibition of FXa and direct inhibition of thrombin itself.

The negative exponential relationship that we demonstrated between increasing concentration of LMWH and thrombin generation, and thrombin generation's very strong correlation to anti-FXa activity mean that it would also be useful for monitoring the effect of LMWH. When al Dieri et al. found that the ETP had a much higher sensitivity (43% for aPTT and 93% for ETP) for low doses of UFH, they attributed this to the exponential dose-response curve of the ETP resulting in a high reduction of the ETP already at very low doses of UFH, rather than a higher accuracy or more stable normal value than the aPTT [13].

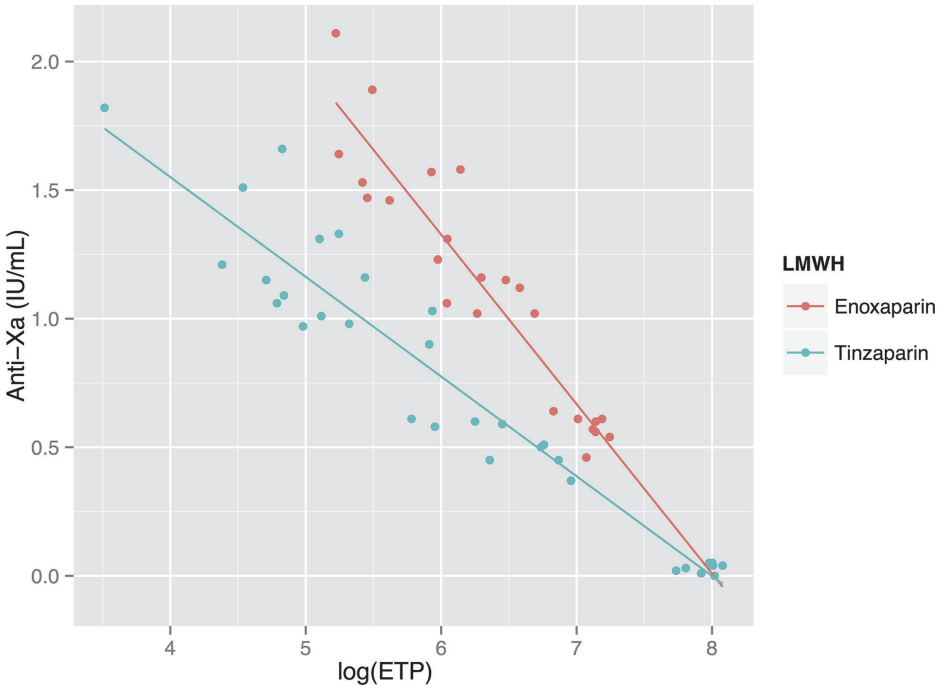

Figure 7. Relationship between anti-FXa activity and thrombin generation. Anti-FXa activity is strongly negatively correlated to the logarithm of ETP (Endogenous Thrombin Potential): R_s for enoxaparin and tinzaparin are −0.93 and −0.94 respectively. Anti-FXa activity is more prolonged by enoxaparin than tinzaparin at any given level of thrombin generation.

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Although thrombin generation appears to be a suitable method to estimate the anticoagulative effect of LMWH, it is noteworthy that the aPTT's produced by tinzaparin and enoxaparin at equivalent levels of thrombin generation differ (see Fig. 6). This supports the concept that the two LMWH's affect global coagulation by mechanisms other than pure thrombin-inhibition.

Strengths and weaknesses of the study

We are not aware of any previous study where several methods of aPTT measurement, anti-FXa activity and thrombin generation have simultaneously been tested with low molecular weight heparins with differing anti-FXa/anti-FIIa ratios. It is of particular clinical interest to bring to attention the finding that the anti-FXa is not a functional measure of anticoagulation.

Among the weaknesses of this study is that it is an *in vitro* study—enoxaparin and tinzaparin were administered directly into citrated blood samples rather than subcutanously into our volunteers. Another weakness is that all samples were taken from healthy men and not patients.

It is known that there is an interindividual variation in response to intravenous administration of unfractionated heparin to patients and after subcutaneous administration of LMWH to healthy volunteers [8,9]. We cannot in this study be sure whether the greatly increased standard deviation of aPTT results at higher levels of LMWH was due to the methods used to measure aPTT or to variation in the subjects' responses. We can, however, be sure that there was a significant difference between aPTT methods.

Conclusion

aPTT and anti-FXa display linear dose-responses to LMWH, although aPTT is less strongly correlated to the dose of LMWH than anti-FXa activity. There is some variation between aPTT assays. Tinzaparin increases aPTT and decreases thrombin generation more than enoxaparin at any given level of anti-FXa activity, which should lead to caution in interpreting clinical anti-FXa results. Thrombin generation with tissue factor-rich activator is a promising method for monitoring LMWH's.

Supporting Information

S1 File. Raw data as tabulated text file.
(TXT)

S2 File. Modification of Fig. 1 displaying which aPTT methods gave statistically different results at each concentration of low molecular weight heparin (LMWH). Horizontal bars indicate a significant difference in aPTT results given by reagents at each concentration of LMWH as tested by the Wilcoxon signed rank test ($P<0.05$). The aPTT results given by the ActinFSL reagent were significantly different from the other reagents at almost all concentrations.
(TIFF)

S3 File. Thrombin generation at increasing concentrations of LMWH[a], measured using the TGA RB reagent. ETP (Endogenous Thrombin Potential) is strongly inhibited by LMWH.
[a]Low Molecular Weight Heparin.
(TIFF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: US KS NT. Performed the experiments: EL OT US KS. Analyzed the data: OT EL US KS. Contributed reagents/materials/analysis tools: US KS. Wrote the paper: OT EL KS NT US.

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