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# Viscoelastic haemostatic assays and fibrinogen concentration tests during haemodilution

The effects of fibrinogen and factor XIII

Dag Winstedt



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DOCTORAL DISSERTATION

By due permission of the Faculty of Medicine, Lund University, Sweden.

To be defended at lecture hall F3 at Skånes Universitetssjukhus Lund  
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*Faculty opponent*

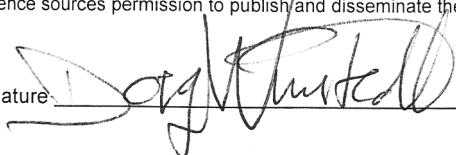
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<b>Title and subtitle</b> Viscoelastic haemostatic assays and fibrinogen concentration tests during haemodilution: The effects of fibrinogen and factor XIII		
<p><b>Background:</b> The efficacy of concentrates of fibrinogen and factor XIII in hypothermia and haemodilution has not yet been completely investigated. Clauss methods have not previously been evaluated perioperatively.</p> <p><b>Methods:</b> Coagulation was assessed with rotational thromboelastometry (ROTEM) or free oscillation rheometry (FOR) in vitro and different fibrinogen measurement methods in observational studies.</p> <p><b>Results:</b> Haemodilution with human albumin (HA) impaired clot strength (EXTEM MCF, maximal clot formation) less than the synthetic colloids hydroxyethyl starch (HES) or dextran (<math>p &lt; 0.001</math>), but equal with crystalloid fluids (normal saline, NA, or Ringer's acetated solution, RA). Addition of fibrinogen improved fibrinogen dependent clot strength (FIBTEM MCF) better after haemodilution with HA than synthetic colloids (<math>p &lt; 0.001</math>), but less than or equal with crystalloids. In haemodilution with HA, fibrinogen improved ROTEM parameters dose-dependently (<math>p &lt; 0.001</math>). Factor XIII (FXIII) had no effect alone, but an additional effect to that of fibrinogen on clot strength, FIBTEM-MCF (<math>p &lt; 0.02</math>). Hypothermia at 33°C and haemodilution with HES, but not RA, interacted to impair fibrinogen-dependent clot strength measured with FOR Fibscreen2 G'max (<math>p &lt; 0.001</math>), and clot velocity measured with FOR Fibscreen1 COT2 or ROTEM EXTEM CFT (<math>p = 0.035</math> and <math>p &lt; 0.001</math>). Fibrinogen (+/-FXIII) improved coagulation independently of temperature (33° or 37°C). After infusion of 1000 ml of HES to patients, fibrinogen measured with two Clauss methods and one immunological method decreased to the same extent (29, 27, and 31%), whereas fibrinogen-dependent clot strength (ROTEM FIBTEM MCF) decreased more (44%, <math>p &gt; 0.001</math>). Finally, when comparing seven different Clauss methods during cardiac surgery, within-method variability was low, but between-method variability was high (mean difference <math>&gt; 0.5</math> g/l). No differences between pre- and post-weaning from cardiopulmonary bypass was seen.</p> <p><b>Conclusion:</b> Fibrinogen concentrate improved coagulation better after haemodilution with HA than synthetic colloids, but equal with or less than after crystalloid haemodilution. FXIII had an additional effect to that of fibrinogen. Hypothermia and haemodilution with HES interacted to impair coagulation. Fibrinogen+/-FXIII improved coagulation also at 33°C. Clauss methods after in vivo HES haemodilution did not overestimate fibrinogen; however, fibrinogen-dependent clot strength decreased more than fibrinogen concentration. Clauss methods varied considerably between methods.</p> <p>These findings support the use of fibrinogen concentrate after resuscitation with HA, also at hypothermia, but question the use of colloids, especially HES, in resuscitation. Clauss fibrinogen methods need to improve.</p>		
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# Viscoelastic haemostatic assays and fibrinogen concentration tests during haemodilution

The effects of fibrinogen and factor XIII

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*To Lena*

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# List of original papers

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals.

- I. Winstedt D, Hanna J, Schott U. Albumin-induced coagulopathy is less severe and more effectively reversed with fibrinogen concentrate than is synthetic colloid-induced coagulopathy. *Scand J Clin Lab Invest* 2013; 73: 161-9.
- II. Hanna J, Winstedt D, Schott U. Fibrinogen and FXIII dose response effects on albumin-induced coagulopathy. *Scand J Clin Lab Invest* 2013; 73:553-62.
- III. Winstedt D, Tynngard N, Olanders K, Schott U. Free oscillation rheometry monitoring of haemodilution and hypothermia and correction with fibrinogen and factor XIII concentrates. *Scand J Trauma Resusc Emerg Med* 2013; 21: 20.
- IV. Winstedt D, Thomas O, Nilsson F, Olanders K, Schött U. Fibrinogen and factor XIII correct hypothermic and dilutional coagulopathy. *Under revision in Scand J Trauma Resusc Emerg Med*.
- V. Winstedt D, Solomon C, Hillarp A, Lundahl T, Schött U. Intraoperative hydroxyethyl starch in elective brain tumour surgery and its effects on different fibrinogen measurements. *In manuscript*.
- VI. Solomon C, Baryshnikova E, Tripodi A, Schlimp, C. J., Schochl, H., Cadamuro, J., Winstedt, D., Asmis, L., Ranucci, M. Fibrinogen measurement in cardiac surgery with cardiopulmonary bypass: Analysis of repeatability and agreement of Clauss method within and between six different laboratories. *Thromb Haemost* 2014; 112: 109-17.

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# Abbreviations

α2-AP	alpha 2-antiplasmin
AA	alpha angle (°)
ACoT	acute coagulopathy of trauma
APC	activated protein C
APTT	activated partial thromboplastin time
AT	antithrombin
ATC	acute traumatic coagulopathy
CFT	clot formation time (s)
COT 1 or 2	clotting time (s)
CPB	cardiopulmonary bypass
CT	clotting time (s)
DIC	disseminated intravascular coagulation
DS	degree of substitution
ELISA	enzyme-linked immunosorbent assay
EXTEM	ROTEM-assay with TF as activator
FV	factor V
FVa	activated factor V
FFP	fresh-frozen plasma
Fibscreen1	or Fib1: FOR-assay; TF as activator
Fibscreen2	or Fib2: FOR-assay; abciximab as PLT inhibitor
FIBTEM	ROTEM-assay with cytochalasin as PLT inhibitor
FOR	free oscillation rheometry
G'max	elastic modulus (Pa); definition: stress/strain
HA	human albumin in saline
HES	hydroxyethyl starch
ICC	intra-class correlations
INR	international normalized ratio
MCF	maximal clot formation
Mw	molecular weight
NS	normal saline; isotonic sodium chloride solution
PAI	plasminogen activator inhibitor
PC	platelet concentrate
PLT	platelets
PRBC	packed red blood cells

PT	prothrombin time
RA	Ringer's acetated solution
RCT	randomised controlled trial
RL	Ringer's lactated solution
ROTEM	rotational thromboelastometry
SCT	standard coagulation test
TAFI	thrombin activatable fibrinolysis inhibitor
TEG	thrombelastography
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TM	thrombomodulin
t-PA	tissue-type plasminogen activator
VHA	viscoelastic haemostatic assay
vWF	von Willebrand factor
WHO	World Health Organisation

# Introduction

Bleeding is the leading cause of preventable death in trauma.<sup>1</sup> Blood transfusions can be life-saving, but they can also increase the risk of, for example, infection and organ failure and subsequent death, also when given after less dramatic bleeding.<sup>2</sup> Thus, reducing bleeding and blood transfusions is very important.

Surgery is a cornerstone to reduce bleeding both in trauma and elective surgery, but successful surgery requires a well-functioning haemostasis. Hypothermia, acidosis and haemodilution are factors known to further decrease the activity of remaining coagulation factors and platelets.<sup>3</sup> After trauma, some patients develop an acute traumatic coagulopathy (ATC), which is probably triggered by tissue damage in combination with shock.<sup>4</sup> Therefore, managing resuscitation in traumatic bleeding is a balance between avoiding shock and excessive infusion with intravenous fluids. In addition, fluids containing colloidal substances (colloids), for example hydroxyethyl starch (HES), impair coagulation more than is explained by haemodilution.<sup>5</sup>

The first coagulation factor to reach critically low concentrations in bleeding is fibrinogen,<sup>6</sup> and mortality in trauma is reduced if fibrinogen is substituted.<sup>7</sup> However, a transfusion with only fresh-frozen plasma is not always enough to maintain sufficient fibrinogen concentrations, and substitution with fibrinogen concentrate is therefore recommended in major bleeding. To avoid both over- and under-treatment with fibrinogen concentrate, dosage is guided with either a measurement of fibrinogen concentration or with clot strength in platelet inhibited blood, which is considered to reflect functional fibrinogen.<sup>8</sup> Unfortunately, some colloids have been shown to interfere with both of these measurement methods.<sup>9,10</sup>

Factor XIII (FXIII) is crucial to the stability of blood clots, and it has recently been shown that reduced activity of FXIII is associated with bleeding in tumour surgery, for example.<sup>11</sup>

The work of this thesis aimed to elucidate the effect of different intravenous fluids on the coagulation and the efficacy of fibrinogen and FXIII concentrates to reverse coagulopathy induced by haemodilution and hypothermia. We also compared different methods to measure fibrinogen in two clinical settings.



# Background

## Bleeding

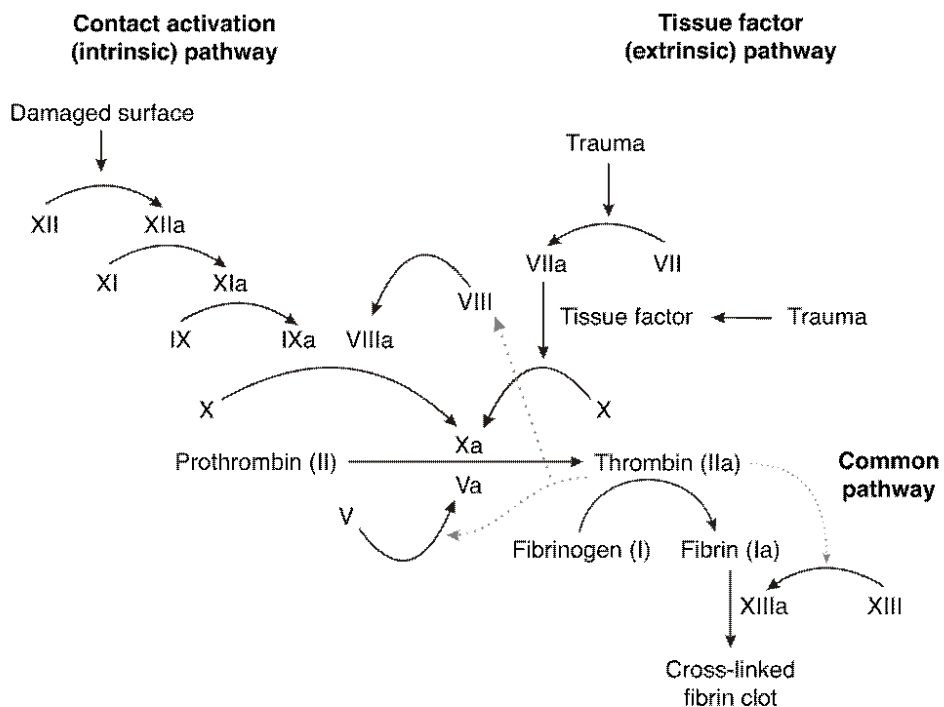
Bleeding, or haemorrhage, is traditionally classified in four stages of severity: from the patient with asymptomatic stage I bleeding with blood loss of less than 15% of the total blood volume, to the stage IV bleeding patient in severe shock with blood loss greater than 40% of the total blood volume. The acute loss of blood leading to death is conventionally called exsanguination. Massive blood loss is defined as the total loss of one blood volume within 24 hours or an acute 50% reduction of the total blood volume in less than 3 hours, or a rate of blood loss of 150 mL/min.<sup>12</sup> For practical reasons, and to guide blood product transfusion therapy, a massive transfusion is generally regarded to be a transfusion of more than 10 units of packed red blood cells (PRBC), or an equivalent of a patient's blood volume within a 24-hour period.<sup>13</sup>

According to the World Health Organization (WHO), injuries remain the leading global cause of death.<sup>14</sup> Among patients with severe trauma, massive bleeding is the leading cause of death — second only to central nervous system injuries — accounting for up to 40% of casualties, the vast majority occurring during the first 12 hours after the time of injury.<sup>15,16</sup> Post-traumatic bleeding is not only the direct cause of death, but a blood transfusion in trauma and surgery is also a well-known risk factor of immune disorder, multiple organ failure and death.<sup>2</sup> Thus, preventing bleeding and blood transfusions in trauma as well as in surgery is of great concern.

## Haemostasis

When animals evolved beyond a certain size it became necessary to have a circulation as a transporting system. Consequently, traumatic exsanguination became possible, and it is not surprising that a mechanism to reduce this risk evolved simultaneously. This mechanism — referred to as haemostasis (Greek "to stop bleeding") — is a complex interaction between endothelium, platelets and plasma coagulation to form a stable clot of fibrin and platelets (PLT). At the same time,

fibrinolysis and anticoagulant mechanisms work to avoid excessive thrombosis. Plasma coagulation is traditionally presented as a cascade of enzymes with the final step being the formation of a fibrin clot from fibrinogen through the cleavage by thrombin (activated prothrombin, factor IIa) (Figure 1). This classical coagulation cascade still serves as a valuable picture to understand the traditional coagulation tests, but the function of haemostasis is better understood as a cell-based model (Figure 2). This model, presented by the research team of Hoffman<sup>17</sup> in the 1990s, emphasises the interaction of plasma coagulation with platelets and tissue factor carrying cells. At the site of injury, exposed molecules in the subendothelial tissue direct and activate platelets and coagulation. However, the final goal with a strong clot consisting of platelets and cross-linked fibrin remains the same.



**Figure 1. The classical coagulation cascade.** Active enzymes in roman figures followed by an 'a'. The dotted arrow from Thrombin shows the positive feedback on the cofactors FV and FVIII, and on FXIII to promote clot stabilisation. Thrombin is also an important activator of platelets.  
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## Primary haemostasis

Damage of the blood vessel endothelium allows platelets (PLTs) to interact with subendothelial collagen and von Willebrand factor (vWF), and therefore to become partially activated and accumulate at the site of injury (adhesion). Following the collagen–PLT interaction, conformational changes switch the integrin glycoprotein IIb/IIIa (Gp IIb/IIIa) into a high affinity state and platelets bind to each other through the interactions of Gp IIb/IIIa with fibrinogen (aggregation), which also additionally activates platelets.<sup>18</sup> Assisted by vasoconstriction, this PLT plug produces an initial “primary haemostasis”, which is subsequently reinforced by a fibrin network, the “secondary haemostasis”.

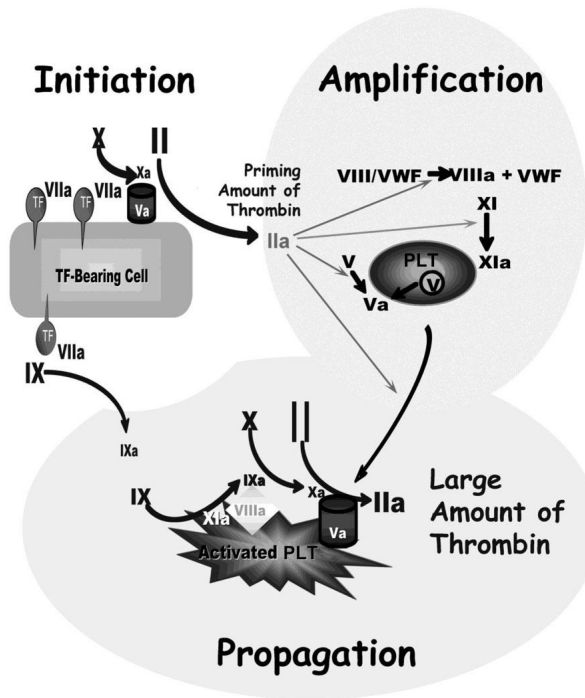
## Secondary haemostasis

The cell-based model (Figure 2) describes the coagulation as a stepwise process to assist our understanding of the process, but should be regarded as an overlapping continuum of events leading to clot formation.<sup>19</sup> Coagulation enzymes (factors) are abbreviated ‘F’ and numbered with roman figures, followed by an ‘a’ if activated.

**Initiation** The initiation step takes place on the surface of tissue factor-bearing cells, exposed at the site of injury. Tissue factor (TF) forms complex with factor VIIa (FVIIa) and the TF/FVIIa-complex then generates a small amount of thrombin, but also FIXa.

**Amplification** The initial thrombin forms the igniting spark to amplify further thrombin generation, through multiple positive feed-back loops. Thrombin is a very potent activator of PLTs, factor V and factor VIII.

**Propagation** The propagation phase is tightly linked to the surface of activated PLTs. The FIXa formed by the TF/FVIIa-complex diffuses to the platelets, and together with FVIIIa fuses with the platelet membrane to form active FIXa/FVIIIa complexes (tenase). The subsequently formed activated factor X (FXa) fuses with FVa to form prothrombinase, responsible for the thrombin burst necessary to produce a stable fibrin clot.



**Figure 2. The phases in a cell-based model of coagulation.** Initiation occurs on the TF-bearing cell as FX activated by FVIIa/TF combines with its cofactor, FVa, to activate small amounts of thrombin. This small amount of thrombin is important during the amplification phase, as it participates in multiple positive feedback loops by activating cofactors, FXI and PLTs. The propagation phase takes place on the PLT surface and is responsible for producing the large burst of thrombin that is required for effective haemostasis. PLT: platelet; TF: tissue factor; VWF: von Willebrand factor; roman figure: coagulation factor (followed by 'a' if activated). From "Practical coagulation for the blood banker" by Hoffman and Cichon, 2013, *Transfusion*, 53, p 1596. © John Wiley and Sons. Reprinted with permission.

## Clot stability

The strength of the final clot is not only dependent on sufficient amounts of functional platelets and fibrinogen, but also on the rate and amount of thrombin generated.<sup>20</sup> Fibrin clot formation begins very early during the burst of thrombin production, and in fact thrombin has other functions than to promote formation of fibrin from fibrinogen. For example, thrombin also stabilises the clot by activating the clot stabiliser FXIII and the antifibrinolytic enzyme thrombin activatable fibrinolysis inhibitor (TAFI), but also through cleavage of the platelet PAR-4-receptor.<sup>19</sup> The importance of FXIIIa to produce stable polymers of fibrin is described below.

## Anticlotting

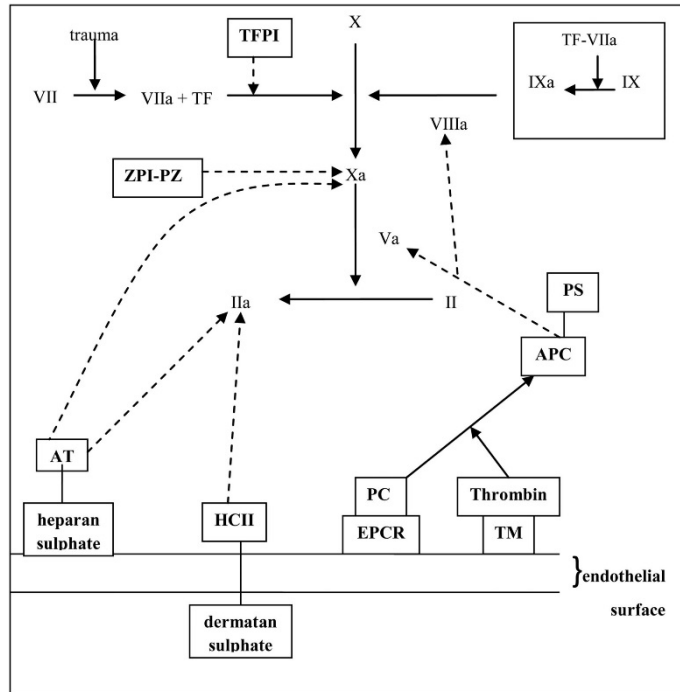
There are two main systems to restrict coagulation to the site of injury: 1) the anticoagulant or antithrombotic system that limits fibrin formation (Figure 3); and 2) the fibrinolytic system that dissolves already formed clots (Figure 4).<sup>21</sup> Coagulation is also limited with non-specific mechanisms: procoagulant factors are simply washed away and diluted beyond the site of injury and thrombin is bound to fibrin with a very high affinity.

### 1. Anticoagulant systems

**Antithrombin.** Antithrombin (AT) is the main inhibitor of thrombin (Figure 3). It is a serine protease inhibitor (SERPIN) produced in the liver and circulating in plasma. AT inhibits thrombin and FXa, and to a lesser extent FIXa, by forming complexes that are degraded in the liver. The activity of AT is enhanced more than 1000-fold by heparan sulphate, a glycoprotein belonging to the glycocalyx covering intact endothelial cells. It is important to understand that the effect of heparin is diminished in states of AT deficiency, for example in sepsis. High-dose substitution of AT in sepsis has been extensively studied, but shown to reduce mortality only in severe sepsis with disseminated intravascular coagulation (DIC) in patients not receiving heparin or low molecular weight heparins.<sup>22-25</sup>

**Protein C.** Thrombin in contact with intact endothelium receives anticoagulant properties (Figure 3). When thrombin is bound to thrombomodulin (TM), an endothelial trans-membrane receptor, thrombin effectively converts protein C into activated protein C (APC). APC is a potent anticoagulant by degrading FVa and FVIIIa. In addition, APC has anti-inflammatory and anti-apoptotic properties.

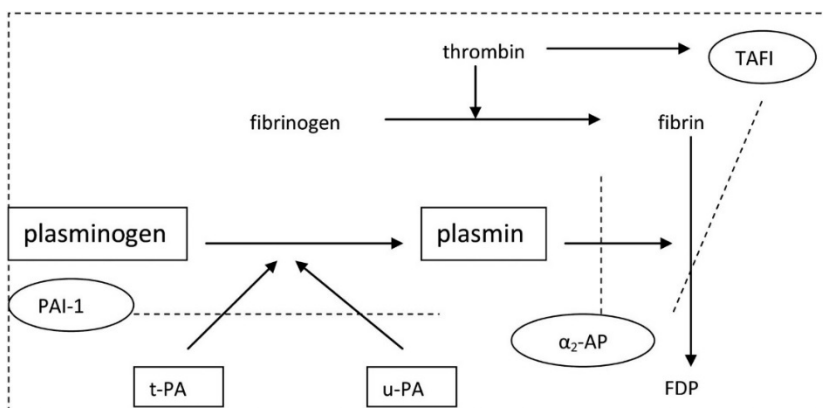
**Other anticoagulants.** The actions of tissue factor pathway inhibitor (TFPI) and protein Z-dependent inhibitor (ZPI) are outlined in Figure 3.



**Figure 3. Anticoagulant mechanisms:** activated protein C (APC), antithrombin (AT), and tissue factor pathway inhibitor (TFPI) are the most important. The importance of protein Z-dependent protease inhibitor (ZPI) with protein Z (PZ), and heparin cofactor II (HCII) is not known. Protein S (PS, cofactor of APC); endothelial protein C receptor (EPCR); thrombomodulin (TM). From “Anticlotting mechanisms 1: physiology and pathology”, by Ezihe-Ejiofor JA, Hutchinson N, 2013, *Continuing Education in Anaesthesia, Critical Care & Pain*, 13, p. 88. © Oxford University Press. Reprinted with permission.

## 2. The fibrinolytic system

Plasmin is the major enzyme of the fibrinolytic system and will dissolve already formed clots by degrading fibrin. The proenzyme of plasmin, plasminogen, is produced in the liver and circulating in plasma. It is activated to plasmin mainly by tissue-type plasminogen activator (t-PA). Both t-PA and plasminogen bind to fibrin, which is necessary for plasmin generation. Thus, fibrin is both a cofactor and substrate for plasmin. Fibrinolysis is tightly regulated by, for example, plasminogen activator inhibitor (PAI), alpha 2-antiplasmin ( $\alpha$ 2-AP, also known as plasmin inhibitor) and TAFI, which is outlined in Figure 4.



**Figure 4. Fibrin degradation by the fibrinolytic system.** Plasmin degrades fibrin into soluble fibrin degradation products (FDP) and is regulated by  $\alpha_2$ -antiplasmin ( $\alpha_2$ -AP), also called plasmin inhibitor. Plasminogen is activated by tissue-type plasminogen activator (t-PA) or urokinase-type plasminogen activator (u-PA). These enzymes are regulated by plasminogen activator inhibitor-1 (PAI-1). Thrombin not only converts fibrinogen into fibrin but also activates thrombin-activatable fibrinolysis inhibitor (TAFI), which inhibits fibrinolysis by modifying the fibrin substrate. From “Anticlotting mechanisms 1: physiology and pathology”, by Ezihe-Ejiofor and Hutchinson, 2013, *Continuing Education in Anaesthesia, Critical Care & Pain*, 13, p. 91. © Oxford University Press. Reprinted with permission.

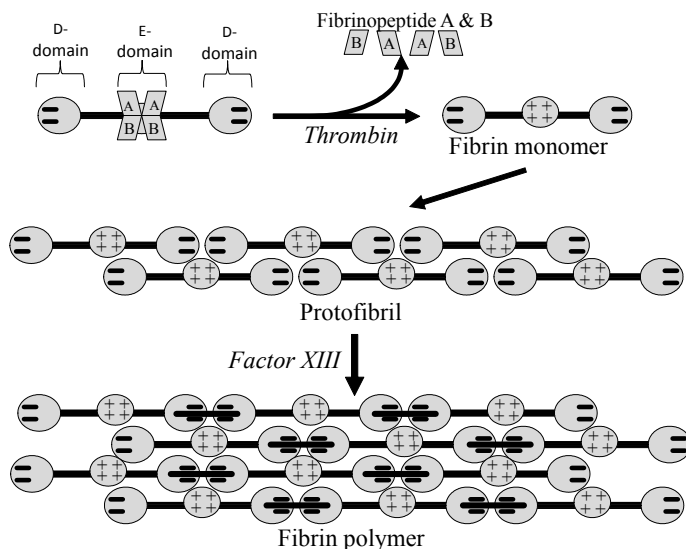
## Fibrinogen

### Structure and function

Second to albumin, fibrinogen is the most abundant protein in plasma, with a concentration of 2-4 g per litre. It is synthesised in the liver and is an acute phase protein. After bleeding, fibrinogen concentration is restored to baseline within approximately 12 hours.

Fibrinogen consists of three pairs of polypeptide chains: two  $A\alpha$ , two  $B\beta$  and two  $\gamma$  (Figure 5). These are linked together in such a way that N-terminal regions of the six chains meet to form a central E-domain. The C-terminal regions ( $A\alpha$ ,  $B\beta$  and  $\gamma$ ) form the D-domains, and these are joined by  $\alpha$ -helical ropes to the central E-domain to give the characteristic fibrinogen structure. Activation of fibrinogen by thrombin (IIa) cleaves two short peptides from the N-terminal regions — these peptides are known as fibrinopeptide A (FpA) and B (FpB), respectively. Removal of FpA and FpB reveals new N-terminal sequences within the E domain known as “knobs”. These knobs can interact spontaneously with the D-domain regions to form soluble fibrin protofibrils (Figure 5). FXIIIa insert covalent bindings between and within

these protofibrils to form cross-linked, insoluble fibrin polymers. The exact way that fibrin assembles into large polymers, and therefore how to explain the remarkable viscoelastic properties of fibrin, has been debated for many years.<sup>26</sup>



**Figure 5. Simplified picture of fibrin polymerisation from fibrinogen.** Thrombin cleaves off fibrinopeptide A and B, exposing regions on the E-domain (knobs) that spontaneously interact with regions on the D-domain (holes) to self-assemble into double-stranded protofibrils. Protofibrils aggregate laterally and eventually form thicker fibres. FXIII inserts covalent bindings between D-domains. Adapted from a picture by Ulf Schött.

## Fibrinogen in bleeding

Excessive bleeding has been reported at a fibrinogen concentration below 0.5-1.0 g/l,<sup>27</sup> but evidence is outlining that a concentrations of 1.5-2.0 g/l is required for sufficient fibrin clot polymerisation.<sup>28,29</sup> Fibrinogen is the first coagulation factor to reach critical concentrations in severe bleeding.<sup>6</sup> Several studies have shown that clot strength increases with fibrinogen concentrate treatment, both in vitro<sup>30-32</sup> and in an in vivo porcine model.<sup>33-35</sup>

A study from the Iraq war, published in 2008, showed improved survival the more fibrinogen per unit transfused PRBC that was given.<sup>7</sup> In that study, the source of fibrinogen was cryoprecipitate fresh-frozen plasma (FFP), and whole blood, but since then an increasing number of clinical studies on fibrinogen concentrate on bleeding patients have been presented. A human fibrinogen concentrate derived from human plasma has been on the European market since 1986, but until recently

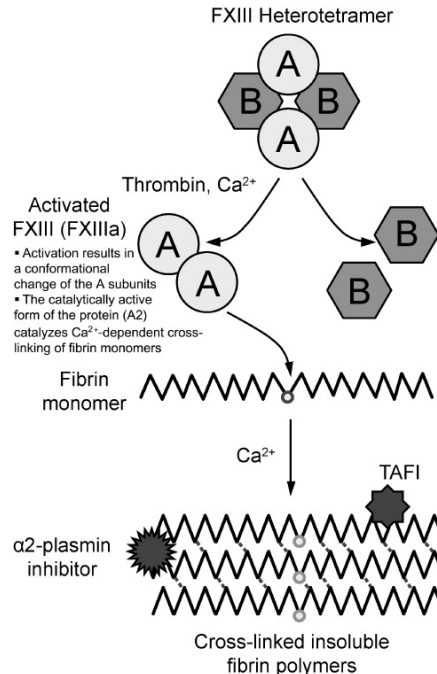
it was mainly used in congenital hypofibrinogenemia. Substitution with fibrinogen concentrate has been studied in a variety of surgeries, including orthopaedic surgery,<sup>36</sup> cardiac surgery,<sup>37-40</sup> aorta repair surgery,<sup>41-43</sup> craniosynostosis,<sup>44,45</sup> and radical cystectomy,<sup>46</sup> but also during obstetric bleeding<sup>47</sup> and in a massive transfusion of mixed causes (post-operative, trauma, gastrointestinal etc.).<sup>48-52</sup> However, only a few of these studies have been randomised studies.<sup>40,43,46,51,53-55</sup> Nevertheless, European guidelines for treatments of traumatic and perioperative bleeding recommend substitution with fibrinogen concentrate in severe traumatic or perioperative bleeding, if fibrinogen concentration is  $<1.5\text{-}2.0\text{ g/l}$ .<sup>56,57</sup>

## Factor XIII

### Structure and function

Factor XIII (FXIII) plays a crucial role in the terminal phase of the clotting cascade, and promotes the formation of cross-linked fibrin polymers and generation of a stable haemostatic plug, as described above.<sup>58</sup> The proenzyme FXIII circulates in plasma bound to fibrinogen. It is a tetramer consisting of two A and two B subunits. The B subunits are synthesised by hepatocytes, whereas the A subunits are mainly synthesised and excreted by platelets and other cells with their origin in the bone marrow.<sup>59</sup> In the presence of thrombin and calcium, the tetramer and proenzyme FXIII is split, whereupon the A and B subunits dissociates (Figure 6). Conformational changes of the A chains results in an active enzyme, FXIIIa, which remains attached to fibrin and starts to catalyse cross-linking of fibrin monomers into insoluble polymers. Another important haemostatic function of FXIIIa is to attach  $\alpha_2$ -AP to fibrin, which makes the fibrin polymer more resistant to fibrinolysis.<sup>58,59</sup>

In addition to its haemostatic functions, FXIIIa is very important for wound healing, angiogenesis and maintenance of pregnancy, but it also has a role in vascular permeability and in the physiology of cartilage and bone. The intracellular (B subunits) clearly has a role in the function of platelets and monocytes/macrophages, with implications for atherosclerosis.<sup>59</sup>



**Figure 6. Outline of structure, activation and catalytic function of FXIII.**

From “Biology of Factor XIII and clinical manifestations of Factor XIII deficiency”, by Levy JH, Greenberg C, 2013, *Transfusion*, 53, p 112. © John Wiley and Sons. Reprinted with permission.

## Factor XIII deficiency

Congenital deficiency of FXIII is very rare, with an approximate incidence of 1 in 3-5 million births. Those with a severe bleeding tendency typically have a plasma FXIII activity less than 5% of normal. Umbilical bleeding occurs in 80% of these cases, but it is also associated with, for example, recurrent ecchymosis and joint bleedings, intracranial haemorrhage and poor wound healing and recurrent miscarriages.<sup>58</sup> In Sweden, there are three patients alive right now (personal communication, Lilian Tengborn).

In a Japanese study, the incidence of acquired FXIII deficiency has been estimated to one case per million. Some of these cases are caused by FXIII inhibiting autoantibodies, which may occur in autoimmune diseases and cancer, or the inhibitors may be drug induced. In cases where no inhibitor is found, it is assumed to be caused by decreased biosynthesis or overconsumption, and these cases have been associated with, for instance, inflammatory bowel disease, cancer, atypical Henoch-Schönlein purpura, and myelodysplasia.<sup>60</sup>

## Factor XIII in surgery

Perioperative haemodilution is known to decrease FXIII activity in parallel with fibrinogen.<sup>61</sup> Perioperative FXIII deficiency differs from congenital, in that bleeding already increase at activity levels of <60%.<sup>62-64</sup> For example, the relative risk of postoperative haematoma after intracranial surgery was increased by 6.4 if postoperative FXIII was <60% and increased by 12 if there was a concomitant low fibrinogen level < 1.5 g/l.<sup>62</sup> It has also been shown that chest tube bleeding after cardiopulmonary bypass (CPB) correlated with perioperative values of FXIII activity,<sup>65,66</sup> and therapeutic interventions with concentrate of FXIII after CPB indicate a benefit when normalising FXIII activity, but not when achieving supranormal values.<sup>63</sup> Interestingly, patients suffering unexplained bleeding were found to have decreased availability of FXIII per unit thrombin generated before and during surgery, as well as an increased fibrin monomer plasma concentration throughout surgery.<sup>64</sup> FXIII concentrate early in surgery with cancer patients having these risk markers of bleeding was associated with reduced loss of clot firmness and reduced bleeding.<sup>67</sup> Thus far, substitution with FXIII concentrate has not been studied in major bleeding or trauma.

## Volume replacement therapy

Blood loss must be replaced in order to preserve tissue oxygenation, which is dependent on blood flow and oxygen carrying capacity. Since normal haemoglobin concentrations provide a large oxygen carrying capacity, maintaining intravascular volume in order to preserve cardiac output and organ perfusion takes priority over substituting haemoglobin concentrations. During perioperative bleeding, plasma substitutes such as crystalloid or colloid solutions are preferred,<sup>56</sup> whereas in a traumatic massive transfusion early transfusion with FFP and platelet concentrate (PC) has been suggested.<sup>13,68,69</sup> Intravenous volume therapy is probably one of the most debated and studied fields of medicine since its breakthrough during the Second World War. Below, the main characteristics of different intravenous fluids are described, whereas their coagulation effects are described in the section on Acquired coagulopathy.

### Crystalloids

Isotonic saline (sodium chloride, 154 mmol/l; NS) is the most used crystalloid solution in the U.S. To avoid hyperchloremia, different buffered solutions predominate in Europe, for example Ringer's acetated solution (RA) in Sweden. In

RA, some of the chloride has been replaced with acetate and the contents of cations have been adjusted to better mimic the extracellular fluid. Since sodium freely crosses the capillary wall but not the cellular membranes, crystalloid solutions are rapidly distributed within the total extracellular volume. The intravascular space makes up one third of the extracellular space and thus, only one third of the administered volume remains in the intravascular space after redistribution through the capillary wall.

## Colloids

A colloid is a substance in which microscopically dispersed insoluble particles are suspended throughout a crystalloid solution, usually saline. The word "colloid" may refer to the particles themselves, but usually, as in this presentation, refers to the fluid with its particles. Since colloids do not easily cross the capillary walls, most of the administered volume of colloid remains in the intravascular space. For this reason, colloids are sometimes recommended in order to avoid extracellular oedema. However, no convincing evidence of an advantage with colloids over crystalloids has been shown,<sup>70</sup> and the physiological concept behind the assumed avoidance of oedema has recently been questioned.<sup>71</sup>

### *Hydroxyethyl starch*

Hydroxyethyl starch derivatives (HES) [MESH term] are glucose polymers where a percentage of OH-groups have been substituted with 2-hydroxyethyl ether groups. HES is subclassified according to average molecular weight (Mw) and degree of substitution (DS), for example HES 130/0.42. For a full characterisation of HES, the number of hydroxyethyl groups in positions C2 and C6 (C2/C6-ratio) is also important, as a hydroxyethyl group in position C2 impairs hydrolysis more effectively than one in position C6. High C2/C6-ratios can lead to accumulation of HES in plasma. HES is eliminated only through the kidneys, as the products of hydrolysis cannot be oxidatively metabolised.

In June 2013, the European Medicines Agency (EMA) concluded that the benefits of HES solutions no longer outweighed their risks and recommended that the marketing authorisations for these medicines be withdrawn. This was based mainly on three randomised trials comparing HES with crystalloids in critical care, which showed a greater risk of kidney injury<sup>72-74</sup> and one even showed a higher 90 day mortality.<sup>72</sup> The current recommendation from the Swedish Medical Products Agency is that HES infusion should not be given to patients with sepsis, kidney failure, intracranial haemorrhage, serious liver failure or to patients during intensive care or the critically ill patient.<sup>75</sup>

### *Human albumin*

Albumin is the most common protein in human blood plasma and serum, with a plasma concentration of 35-50 g/l in healthy individuals. The protein is a monomer with a molecular weight of 66.5 kDa, which is synthesised and secreted by the liver. Albumin is primarily responsible for 75-80% of the normal colloid oncotic pressure of plasma, but serves other important functions as well: acting as a transport protein (different hormones, fatty acids, bilirubin, and certain drugs), free radical scavenger and acid-base buffer. There are growing evidence of other important functions, for example anti-inflammatory effects and maintenance of a normal capillary permeability.<sup>76</sup>

Human albumin in saline (HA) is produced by fractionation of blood plasma and further pasteurisation to prevent transmission of pathogens. It is provided in Sweden as iso-oncotic solutions of 40 or 50 g/l and a hyperoncotic solution of 200 g/l. It was first used in a large scale during the Pearl Harbour attack in 1941.<sup>70</sup> A review published in 1998 comparing HA with crystalloid solution in resuscitation concluded that administration of HA was associated with an increased mortality.<sup>77</sup> This meta-analysis was heavily criticised, and in 2004 the results of a large, blinded, randomised, controlled trial to compare the safety of saline and HA was published (SAFE study).<sup>78</sup> The SAFE study showed no difference between saline or HA with respect to neither the rate of death nor the development of new organ failure. Subgroup analyses show that HA may be associated with an increase of death among patients with traumatic brain injury,<sup>79</sup> whereas it was associated with a decrease of death rate in patients with severe sepsis.<sup>80</sup>

### *Dextran*

Dextran is a complex, branched glucose polymer manufactured from fermentation of sucrose, but also naturally occurring in dental plaques, for example. Dextran solutions for fluid therapy with different average molecular sizes (40-70 kDa) are available in Sweden. Dextran is excreted by the kidneys or degraded to CO<sub>2</sub> and water by endogenous dextranase.<sup>81</sup> The plasma expanding properties of dextran depends on molecular weight and concentration and, for instance, infusion of 6% dextran 70 increases plasma volume more than both 5% HA or 6% HES 130/0.4.<sup>82</sup>

## Acquired coagulopathy

Acquired coagulopathy has long been a dreaded condition in major bleeding, classically described to be a consequence of the so called "lethal triad": haemodilution, hypothermia and acidosis. Wittingly, this has been described as a "bloody viscous circle" of fluid therapy aggravating coagulopathy, more bleeding,

further demand of fluid therapy, and so on.<sup>83</sup> In addition, trauma victims may develop an acute traumatic coagulopathy (ATC) independently of the lethal triad.<sup>84</sup>

## Haemodilution

Mild to moderate in vitro haemodilution with both crystalloids and colloids, induce a hypercoagulability as measured with viscoelastic haemostatic assays (VHAs).<sup>85</sup> It is unclear whether this is an artefact produced in native whole blood or if it has any clinical relevance,<sup>86</sup> but the phenomenon has been suggested to be caused by decreased antithrombin activity.<sup>87</sup> It is undoubtable, that further haemodilution induces a hypocoagulable state related to non-specific dilutional effects and specific effects of colloidal macromolecules.<sup>88</sup>

### *Crystalloids*

Crystalloids exert dilutional effects on the haemostasis: decreased platelet function<sup>89</sup> and impaired thrombin generation,<sup>90</sup> but reduced clot formation,<sup>91</sup> and reduced clot strength<sup>9,32,92</sup> are also the effects of haemodilution with crystalloids. Changes of haemostasis is comparable between normal saline and balanced solutions both in pigs<sup>93</sup> and in vivo,<sup>94</sup> but several investigations comparing balanced and unbalanced solutions by J. Boldt have been retracted due to scientific misconduct. Interestingly, a recent subgroup analysis of previous studies comparing normal saline with Ringer's lactate (RL) show a possible increased blood loss volume among patients with a high risk of bleeding who received normal saline.<sup>95</sup>

### *HES*

Clinical bleeding problems have been described after HES 470/0.7 and HES 200/0.5<sup>96</sup>. Modern HES with lower Mw (130 kDa) and DS (0.4) have been suggested to affect coagulation to a lesser degree.<sup>88</sup> Some studies have implicated that the degree of substitution is more important than the molecular weight when comparing coagulation effects.<sup>97,98</sup>

HES can reduce vWF concentration<sup>99</sup> and slowly degradable HES also decrease platelet activity,<sup>100</sup> but perhaps most important HES impair fibrin polymerisation.<sup>92,101-104</sup> After the retraction of a number of articles on HES and coagulation due to scientific misconduct, a thorough review of the influence of HES on haemostasis was published in 2011.<sup>5</sup> Nineteen of the 24 reviewed articles confirmed that HES infusion resulted in a weaker clot with a less stable fibrin network than does infusion with crystalloid or human albumin.

## **HA**

Human albumin is generally suggested not to affect platelets<sup>88</sup> but has still been shown to inhibit collagen-induced aggregometry.<sup>105</sup> The effect of HA on whole blood haemostasis as measured with VHAs indicate that haemodilution with HA causes less hypocoagulation than different HES solutions<sup>106-108</sup> and is less<sup>106,109</sup> or comparable with gelatine.<sup>110,111</sup> The effect is probably comparable with that of haemodilution with crystalloid solutions.<sup>108,112</sup> Small clinical studies comparing HA with HES or gelatine have found no significant differences in bleeding.<sup>105,107,113,114</sup> A recent randomised trial comparing HA, HES or RL in 24 patients undergoing cardiac surgery found no difference in blood loss. However, the RL group had less transfusions than both the HA and HES group, probably due to more haemodilution in the colloid groups.<sup>115</sup>

## **Dextran**

Dextran affects the haemostasis in several ways. Like all synthetic colloids, it has a von Willebrand factor lowering potency<sup>116</sup> and its antiplatelet aggregation effects have been used to increase perfusion in microvascular surgery and arterial disease.<sup>117</sup> Dextran is effective as an antithrombotic,<sup>118</sup> and 500 ml dextran 70 was comparable with 20 mg enoxaparin to reduce the incidence of postoperative venous thromboembolism.<sup>119</sup> Enhanced fibrinolysis in combination with reduced platelet adhesion to vWF and reduced platelet activation by thrombin are possible mechanisms for dextran's antithrombotic properties.<sup>120</sup> Several studies evaluate the effect of dextran haemodilution on whole blood coagulation as measured with thrombelastography. Haemodilution with dextran 40 induces more hypocoagulation than dextran 70<sup>121</sup> or HES 200 and gelatine.<sup>122</sup> Dextran 70 induced more<sup>30,104</sup> or equal<sup>121</sup> hypocoagulation as compared with HES of different molecular weights, and more hypocoagulation than gelatine,<sup>121</sup> saline<sup>30</sup> or albumin.<sup>104</sup> Perioperative infusion of dextran 70 has been shown to increase bleeding in revision hip arthroplasty as compared to human albumin,<sup>123</sup> and also to increase bleeding following prostatectomy as compared with HA or RL.<sup>112</sup>

## **Hypothermia and acidosis**

In the Pennsylvania Trauma Outcome Study, patients with a hypothermia of  $\leq 35^{\circ}\text{C}$  at admission had a more than threefold increased odds of death, even when adjusted for confounding factors.<sup>124</sup> Blood loss during trauma laparotomy was greater in hypothermic patients and independent of injury severity.<sup>125</sup>

The plasma coagulation enzyme reactions, as measured with activated partial thromboplastin time (APTT) and prothrombin time (PT), are strongly inhibited by hypothermia.<sup>126</sup> In normal plasma, hypothermia  $< 33^{\circ}\text{C}$  produces a coagulopathy

equivalent to what is seen in factor deficient plasma (< 50% of normal activity) at normothermia.<sup>127</sup> Hypothermia also impairs platelet function and has been shown to reduce platelet activators<sup>128</sup> and reduce the response of platelets to vWF.<sup>129</sup> 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, but at temperatures below 33°C both reduced platelet function and enzyme activity contributed to the coagulopathy.<sup>130</sup> In vitro studies with VHAs such as thrombelastography (TEG) or rotational thromboelastometry (ROTEM) show that mild to moderate hypothermia mainly decreases the rate of clot formation,<sup>131</sup> whereas severe hypothermia (<28°C) also decreases clot strength.<sup>132</sup>

Acidosis affects coagulation with different mechanisms as compared with hypothermia. While hypothermia primarily inhibits the initiation phase of thrombin generation kinetics, acidosis severely inhibits the propagation phase of thrombin generation.<sup>133</sup> This is in line with an in vitro study with ROTEM that shows that acidosis impairs primarily clot formation time,<sup>134</sup> which corresponds with the propagation phase of thrombin generation. Similarly, hypothermia inhibits fibrinogen synthesis, whereas acidosis accelerates fibrinogen degradation, leading to a potential deficit in fibrinogen availability.<sup>135,136</sup> In addition, coagulation complications caused by acidosis cannot be immediately corrected by pH neutralisation alone.<sup>137</sup> Since hypothermia and acidosis affect coagulation with different mechanisms, the combination synergistically impair coagulation.<sup>131,133</sup>

## **Acute traumatic coagulopathy**

It has been known since the 1980s that trauma itself may induce a very early coagulopathy of trauma.<sup>138</sup> This coagulopathy used to be referred to as DIC with fibrinolytic (haemorrhagic) phenotype, later changing into a DIC with antifibrinolytic (thrombotic) phenotype.<sup>139</sup> In 2003, Brohi<sup>84</sup> and MacLeod<sup>140</sup> confirmed that trauma itself is the main cause of severe coagulopathy after trauma, now referred to as acute traumatic coagulopathy (ATC), but has also been called acute coagulopathy of trauma (ACoT). A combination of severe tissue trauma and hypoperfusion may induce ATC,<sup>4,141</sup> probably through an increased activation of protein C, shedding of endothelial glycocalyx with a subsequent autoheparinisation, and hyperfibrinolysis. ATC has recently been excellently reviewed.<sup>142</sup>

# Aims of the studies

The specific aims were:

- I. to study the in vitro effects of fibrinogen concentrate on in vitro dilutional coagulopathies induced by 5% albumin, 3% dextran 60, 6% dextran 70, buffered and un-buffered HES 130/0.4, saline, and Ringer's acetate, with thromboelastometry (ROTEM).
- II. to evaluate in vitro the ability of fibrinogen and/or of FXIII concentrate to reverse an in vitro albumin-induced dilutional coagulopathy, monitored by ROTEM plus a point-of-care device measuring whole blood prothrombin time (PT) and activated partial thromboplastin time (APTT). We also evaluated the therapeutic cost of different dose combinations of these factor concentrates.
- III. to investigate in vitro the combined effect of hypothermia- and haemodilution-induced coagulopathy, and to what extent this coagulopathy could be reversed with fibrinogen concentrate alone or in combination with factor XIII (FXIII) with the less known viscoelastic haemostatic assay: free oscillation rheometry (FOR).
- IV. to study the in vitro effects of hypothermia during haemodilution-induced coagulopathy, and the effects of fibrinogen concentrate alone or in combination with factor XIII (FXIII) using thromboelastometry (ROTEM).
- V. to compare in an in vivo clinical setting the effects of HES on two fibrinogen tests based on the Clauss method, an enzyme-linked immunosorbent assay (ELISA), and thromboelastometry (ROTEM).
- VI. to evaluate in a clinical setting the performance and repeatability of different fibrinogen assays.



# Methods

## Laboratory methods

### Standard coagulation tests

Standard coagulation tests (SCT), such as APTT and PT, measure the time to formation of a clot after adding an activator in the presence of calcium ions and phospholipids. They are both measured in platelet poor plasma and therefore not affected by platelet count or platelet activity. Originally, clot formation was detected visually, but mechanical or optical detection of the clot has the advantage of being able to automate. Today, photo-optical detection is the most common method.<sup>143</sup>

The PT test is activated with TF of varying origin, but standardisation to an international normalised ratio (INR) makes comparison between laboratories possible. The PT test used in Scandinavia (Owren) is sensitive to the activity of FVII, FX, and prothrombin, whereas the original method (Quick) is also sensitive to factor V and fibrinogen.<sup>144</sup> The PT is used to monitor the effect of oral vitamin K antagonists (e.g., warfarin) and liver function in advanced liver disease.

The APTT test is activated with a negatively charged contact activator (kaolin, celite, micronised silica or ellagic acid), but in contrast to PT, APTT has not been possible to standardise to enable easy comparison between laboratories. The APTT test is sensitive to all of the factors except FVII in the coagulation cascade, that is to FXI, FX with cofactor FV, FIX with cofactor FVIII, prothrombin, and finally to fibrinogen. In addition, APTT is sensitive to the contact activators FXII, high molecular weight kininogen and prekallikrein, but deficiencies of any of those enzymes is not linked to bleeding diathesis.<sup>143</sup> The APTT is used to monitor treatment with unfractionated heparin and screen for various clotting factors, including congenital deficiencies of factor VIII (haemophilia A) and factor IX (haemophilia B), but also to detect acquired clotting factor inhibitors, including FVIII- and FIX-inhibitors. In addition, APTT may become prolonged during therapy with new oral anticoagulants inhibiting FXa or thrombin.

## Fibrinogen assays

The most used method to measure plasma fibrinogen concentration is the Clauss method, first described in 1957.<sup>145</sup> It is a functional assay based on the time for fibrin clot formation after addition of a reagent with a high concentration of thrombin to ensure that the results are independent of thrombin generation and hence of the coagulation factors except from fibrinogen. The measured clotting time is compared with a calibration curve prepared by clotting a series of dilutions of a reference plasma sample of known fibrinogen concentration, and a result g/l is obtained.<sup>146</sup> Two principles of clotting time instrument are used: mechanical endpoint techniques depend on the tensile strength of the clot, and photo-optical techniques depend on the change of optical density resulting from fibrin formation. Fibrinogen measurements can be affected by a number of factors such as the type of instrument, reagent and calibrator used.<sup>147</sup> The Clauss method is also affected by heparin and fibrinogen degradation products, which may be a problem in for example cardiac surgery or DIC.<sup>148</sup> In addition, dextran and hydroxyethyl starch may interfere with photometric fibrinogen assays<sup>10,149</sup> and lead to overestimation of fibrinogen concentrations, assumed to be caused by the increased turbidity from addition of a colloid solution.

After the addition of a potent platelet inhibitor, maximal clot strength as measured with thrombelastography has been shown to correlate with fibrinogen concentrations.<sup>150,151</sup> The two thrombelastographs available (TEG and ROTEM), with their proprietary assays to measure clot strength during platelet inhibition, are not completely comparable.<sup>152,153</sup> Unlike the Clauss method, which measures fibrinogen concentration as a function of the initial formation of fibrin protofibrils or monomers in platelet poor plasma, thrombelastography measures clot strength of whole blood with a platelet inhibitor, which is additionally dependent on fibrin polymerisation by FXIII. It is not clear whether thrombelastography or fibrinogen concentration better predict massive bleeding.<sup>154,155</sup> Nevertheless, ROTEM has been used in various studies to guide treatment with fibrinogen concentrate, for instance a randomised controlled trial (RCT) during major aorta surgery showed a decrease of transfusion needs after therapy with fibrinogen concentrate,<sup>43</sup> but further RCTs are needed to conclude if fibrinogen concentrate should be used routinely in severe bleeding.<sup>156</sup>

## Viscoelastic haemostatic assays

Viscoelastic haemostatic assays (VHAs), or viscoelastic tests (VETs), provide dynamic profiles of the entire clot formation process in whole blood, which is in contrast to the SCTs, that only measure the time to beginning of clot formation in

platelet poor plasma. These are the probable reasons why the VHAs correlate better with bleeding than PT or APTT.<sup>157,158</sup> In addition, the VHAs are point-of-care systems and can decrease turnaround times as compared with SCTs. There are several VHAs available on the market, for example the well-established thrombelastography (TEG) and rotational thromboelastometry (ROTEM),<sup>159</sup> but also the less investigated free oscillation rheometry (FOR) instrument ReoRox.

### *Thrombelastography (TEG and ROTEM)*

Thrombelastography was first developed in Germany by Hellmut Hartert in late 1940s and early 50s<sup>160</sup> to measure the mechanical properties of blood clot formation. There is considerable confusion with the nomenclature of thrombelastography, which is a medical subheading (MeSH) term in the medical article database Medline/Pubmed, but thrombelastography and TEG are also registered trademarks of Haemonetics (USA). The alternative instrumentation, called rotational thromboelastometry and ROTEM, are registered trademarks of Tem International (Germany). There are numerous studies with both TEG and ROTEM and they are often regarded as equivalent instruments, though several studies show that their measurements are not completely interchangeable.<sup>153,161-164</sup> In Table 1, characteristics of TEG and ROTEM are summarised.

<b>Characteristic</b>	<b>TEG</b>	<b>ROTEM</b>	<b>FOR</b>
<b>Pipetting</b>	Manual	Semi-automated	Manual
<b>Rotation of</b>	Cup	Pin	Cup
<b>Angle of rotation</b>	4.75°	4.75°	3.5°
<b>Forced oscillation</b>	Every 5 sec.	Every 6 sec.	Every 2.5 sec.
<b>Free oscillation</b>	No	No	Yes
<b>Measurement</b>	Mechanical	Optical	Optical
<b>Physical quantity measured</b>	Transduction of pin	Impedance of rotation	Amplitude and frequency of free oscillation
<b>Cup and pin material</b>	Cryolite (acrylic polymer)	Polymethylmethacrylate	Gold coated surface
<b>Cup surface</b>	Smooth	Ridged (0.6 – 0.9 mm)	Smooth

**Table 1.**  
Comparative characteristics of three VHAs: TEG, ROTEM and FOR.

With ROTEM, a pin is submerged into a small cup with a blood sample and the pin is slowly rotated. Coagulation is usually induced by addition of an activator. As the clot forms, the fibrin strands couple the pin to the cup and hence the torsional stress is transmitted through the pin. The increased impedance of pin rotation decreases the amplitude of rotation, which is detected optically and the ROTEM software converts the rotational amplitude of the axis into a graphical recording with the clot

firmness. A clot firmness of 0 mm means unobstructed axis rotation, while a clot firmness of 100 mm (theoretical) would correspond to a complete blocking of the rotation. From the recording, several parameters can be extracted such as time to beginning of clot formation and rate of clotting, which are thought to correlate with thrombin generation,<sup>165</sup> and an arbitrary measure of clot strength<sup>159</sup> (see Table 2).

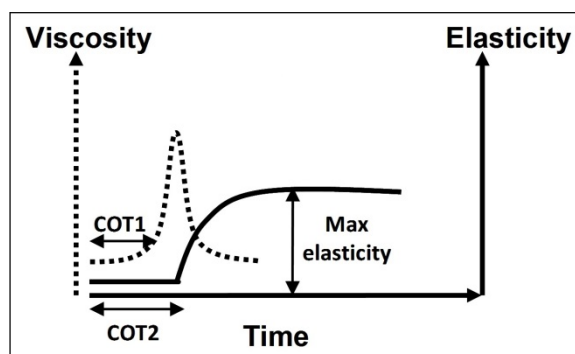
	Clot velocity			
	Clot initiation		Amplifikation and Propagation	Clot strength
ROTEM		CT	CFT & AA	MCF
FOR	COT 1	COT 2	$\alpha$	G' max

**Table 2.**

Comparison of clot velocity and clot strength parameters measured with ROTEM and FOR, and how clot velocity parameters relate with temporal progression of thrombin generation.<sup>165</sup> CT: clotting time (s); CFT: clot formation time (s); AA: alpha angle (°); MCF: maximum clot formation; COT: clotting time (s);  $\alpha$ -slope (°); G' max: elastic shear modulus (Pa).

### Free oscillation rheometry (FOR)

In the free oscillation rheometry (FOR) a cup with the blood sample is attached to a torsion wire, where the cup is rotated along its longitudinal axis every 2.5 seconds (forced oscillation) then released and set into free oscillation. A gold plated cylinder (bob) is submerged into the blood sample. The amplitude and frequency of the free oscillation is optically detected and changes as the clot attaches the cup to the bob. The damping of the amplitude correlates with the viscosity, which rapidly increases as clot formation is beginning. When the viscous blood has turned into a solid clot and coupled the bob to the cup, the damping and viscosity returns to baseline and the frequency of the oscillation starts to increase, which correlates with an increasing clot elasticity (Figure 7).<sup>166</sup> In Table 1 and 2, the characteristics of FOR and its measures are compared with TEG and ROTEM.



**Figure 7. FOR-curve.**

Damping of oscillation amplitude correlates with viscosity (dotted axis and line), and the increasing oscillation frequency correlates with elasticity (solid line and axis). From Paper III. Open Access.

## ROTEM versus FOR

There are some important differences between the ROTEM and the FOR. Firstly, the forced oscillation and arbitrary strain amplitude applied by the ROTEM is maintained throughout the coagulation process, whereas the FOR apply a transient forced oscillation followed by free oscillation during which measuring take place. It is well-known that shear applied during clotting substantially weakens clots in blood and plasma,<sup>167</sup> and that the shear applied by TEG exceeds the linear viscoelastic properties of clots,<sup>168</sup> which can delay clot formation and modify clot structure. In addition, strain amplitude applied by the ROTEM decreases progressively during coagulation. Secondly, the FOR measures the physical changes of the clot during free oscillation, which allows it to measure viscosity and elasticity in the SI-units Pascal-second (Pa·s) and Pascal (Pa), respectively. Thirdly, the FOR cup has a gold coating whereas the ROTEM cup is made of plastic which may lead to different contact activating effects.<sup>169 162</sup> Finally, differences in proprietary coagulation activators and platelet inhibitors (Table 3) affect coagulation measurements differently when comparing ROTEM with TEG,<sup>162</sup> and this is probably also the case when comparing ROTEM and FOR. The EXTEM-assay of ROTEM and FibScreen 1-assay of FOR both contain recombinant human tissue factor, but little information is provided about their exact contents. Their platelet inhibitors are very different: the ROTEM FIBTEM-assay contains cytochalacin D, a potent platelet cytoskeleton inhibitor, whereas FOR's FibScreen 2-assay contains abciximab, a potent inhibitor of the fibrinogen receptor (GPIIb/IIIa receptor) on platelets, and these inhibitors yield very different results.<sup>152,170</sup>

Instrument	ROTEM		FOR	
Assay	EXTEM	FIBTEM	Fibscreen 1	Fibscreen 2
Coagulation activator	recombinant human TF	recombinant human TF	recombinant human TF	recombinant human TF
Platelet inhibitor	x	Cytochalacin D	x	Abciximab

**Table 3.**

Comparison of assays activated with tissue factor, with or without platelet inhibitor. Both ROTEM and FOR use recombinant human TF, but they are from different manufacturers and are likely to have different properties.

# Study protocols

All studies were approved by the Regional Ethical Review Board in Lund, Sweden. All volunteers and patients gave their written informed consent to participate in the study. Study protocols and objectives are described in detail in each paper (see attachments).

## ***Paper I***

Blood samples were collected from 10 healthy volunteers. Coagulopathy was induced by 50% haemodilution in vitro with seven different solutions: normal saline (NS); Ringer's acetated solution (RA); 6% HES 130/0.4 in saline (HES); 6% HES 130/0.4 in buffered solution (buffered HES); 6% dextran 70 in saline; and 3% dextran 60 in buffered solution; and 5% human albumin (HA). The efficacy of in vitro added fibrinogen concentrate to reduce this coagulopathy was compared and studied with ROTEM ExTEM- and FibTEM-assays. The amount of fibrinogen concentrate added in vitro corresponded to an 8-gram dose to a medium-sized adult.

## ***Paper II***

Blood samples were collected from 10 healthy volunteers. The effects of adding factor concentrate of fibrinogen and/or factor XIII (FXIII) to blood haemodiluted 50% with 5% human albumin (HA) were evaluated with ROTEM ExTEM- and FibTEM-assays. Two different dosages of fibrinogen or factor XIII were added, corresponding with 5 or 10 g fibrinogen or 1500 or 3000 IU factor XIII to a medium-sized adult. Each dose of fibrinogen was combined with each dose of FXIII, that is, four combinations.

## ***Paper III***

Blood samples were collected from 10 healthy volunteers. The in vitro effect of hypothermia (33°C) and/or 33% haemodilution (Ringer's acetated solution (RA) or HES 130/0.4) and the corrective effects of adding fibrinogen or fibrinogen with factor XIII (FXIII) were evaluated with a new thrombelastograph: free oscillation rheometer (FOR; ReoRox™). Two assays, Fibscreen 1 and Fibscreen 2, were used as described above. The amount of added coagulation factor corresponds to 4 g of fibrinogen and 1150 IU of FXIII to a medium-sized adult.

## ***Paper IV***

Blood samples were collected from 10 healthy volunteers. The in vitro corrective effects of fibrinogen or fibrinogen with factor XIII (FXIII) during hypothermia (33°C) and/or 33% haemodilution (Ringer's acetated solution (RA) or HES 130/0.4) were evaluated with rotational thromboelastometry (ROTEM). Two assays, ExTEM

and FibTEM, were used as described above. The amount of added coagulation factor corresponds to 4 g of fibrinogen and 1150 IU of FXIII to a medium-sized adult.

### ***Paper V***

Blood samples were collected from patients during intracranial tumour resection surgery: before the start of surgery (baseline); after infusion of 1000 ml of HES; and also at 3, 6 and 12 hours after the end of surgery. The concentrations of fibrinogen were measured with an immunological method (ELISA), one photo-optical and one electromechanical Clauss method, and in addition ROTEM FIBTEM MCF was measured.

### ***Paper VI***

Samples were collected from 30 patients during cardiopulmonary bypass (CPB): 1) at start of surgery; 2) approximately 20 minutes before weaning from CPB; and 3) immediately after weaning from CPB and neutralisation of heparin with Protamin. Platelet poor plasma was prepared and sent to six different centres. A total of five photometric, two mechanical and two antigen assays were used for fibrinogen concentration analysis.



# Results

In the following presentation, increased clotting times (CT and CFT with ROTEM, and COT1 or COT2 with FOR), decreased ROTEM AA or decreased clot strength (MCF with ROTEM and G'max with FOR) are regarded as impaired coagulation, in other words coagulopathy.

## Study I

### ***Haemodilution***

All EXTEM parameters were impaired by haemodilution with all fluids (MCF:  $p<0.001$ , CFT, AA:  $p<0.05$ ). However, the prolongations of CT were not significant. EXTEM parameters were more seriously affected by haemodilution with synthetic colloids as compared with HA (CFT, AA, MCF:  $p<0.001$ ), but not more with crystalloids than with HA.

FIBTEM MCF decreased significantly after haemodilution with synthetic colloids or HA, ( $p<0.02$ ). In contrast to EXTEM-parameters, the decrease of FIBTEM MCF was comparable with synthetic colloids and HA.

### ***Addition of fibrinogen concentrate***

Addition of fibrinogen partially or completely reversed the haemodilution effects of all ROTEM parameters. The EXTEM parameters CFT decreased and AA and MCF (clot strength) increased, but were still significantly less reversed that is CFT longer and AA and MCF lower (all  $p\leq 0.001$ ) in samples with a synthetic colloid as compared with HA. Instead, CFT were shorter and AA and EXTEM MCF larger after addition of fibrinogen to undiluted or crystalloid diluted samples as compared with HA (e.g.,  $p<0.001$  with AA).

The addition of fibrinogen increased FIBTEM MCF in all samples, and was larger in HA samples than samples with synthetic colloid ( $p<0.001$ ), while FIBTEM MCF was larger in undiluted or crystalloid samples ( $p<0.001$  undiluted and NS), as compared with HA diluted samples. Interestingly, FIBTEM MCF was far above

baseline values after addition of fibrinogen to undiluted, crystalloid and HA samples.

### ***Summary of study I***

In conclusion, 50% haemodilution with HA induced a coagulopathy comparable with crystalloid solutions, but less pronounced than that seen after haemodilution with synthetic colloid solutions. After the addition of fibrinogen, coagulation was better in HA diluted samples as compared with samples diluted with synthetic colloids and coagulation in undiluted and crystalloid diluted samples was better or comparable, as compared with HA diluted samples.

## Study II

### ***Fibrinogen or FXIII alone***

High dose fibrinogen improved all ROTEM parameters ( $p<0.001$ ), whereas low dose fibrinogen improved all but EXTEM-AA ( $p<0.02$ ). High dose fibrinogen improved all parameters ( $p<0.001$ ) more effectively than low-dose fibrinogen, except EXTEM-CT. FXIII did not improve ROTEM-parameters, and low-dose was comparable with high-dose FXIII.

### ***Fibrinogen and FXIII in combination***

All combinations of fibrinogen and FXIII significantly improved all ROTEM parameters ( $p<0.001$ ).

ROTEM	Lo fib vs. Lo fib + Hi FXIII	Hi fib vs. Hi fib + Lo FXIII	Hi fib vs. Hi fib + Hi FXIII	Hi FXIII vs. Hi fib + Hi FXIII
EXTEM CT	ns.	ns.	ns.	$p<0.001$
EXTEM CFT	ns.	ns.	ns.	$p<0.001$
EXTEM AA	ns.	ns.	ns.	$p<0.001$
EXTEM MCF	ns.	$p<0.001$	ns. ( $p=0.05$ )	$p<0.001$
FIBTEM MCF	$p=0.01$	$p=0.02$	$p=0.005$	$p<0.001$

**Table 4.**

The combinations of factor concentrate statistically compared

Factor XIII had an additional effect on clot strength: combining either low- or high-dose FXIII with high-dose fibrinogen additionally increased EXTEM MCF as well as FIBTEM MCF, which also increased when combining high-dose FXIII with low-dose fibrinogen (Table 4). FXIII had no additional effect on clot velocity parameters (CT, CFT, and AA).

### ***Summary of study II***

Fibrinogen improved all ROTEM parameters dose-dependently. Factor XIII alone has no or minimal effect. Factor XIII had an additional effect to the effect of fibrinogen on clot strength in whole blood (EXTEM MCF), as well as in platelet-inhibited whole blood (FIBTEM MCF).

## Study III

### ***Hypothermia and haemodilution***

All FOR parameters were impaired at 33°C as compared with 37°C ( $p<0.01$ ). Haemodilution with RA or HES impaired all parameters ( $p<0.01$ ), and the effects were more pronounced with HES than with RA ( $p<0.05$  except for COT1). There were additional effects of hypothermia and haemodilution: the largest effects for all parameters were seen at 33°C with HES haemodilution (Figure X). Haemodilution with HES and hypothermia interacted (=synergy), to increase further COT2 and decrease Fib2 G'max as compared with undiluted blood at 37°C ( $p=0.035$  and  $p<0.001$ , respectively), and to further decrease Fib2 G'max as compared with RA haemodilution at 37°C ( $p=0.003$ ).

Fibscreen1-Fibscreen2 G'max decreased both by hypothermia ( $p<0.01$ ) or haemodilution: more with HES than RA (all  $p<0.001$ ).

### ***Addition of fibrinogen or fibrinogen with factor XIII***

Fibrinogen or fibrinogen with FXIII improved all parameters ( $p<0.01$ ) except COT1, which was reduced significantly only after the addition of fibrinogen with FXIII ( $p<0.01$ ). Factor XIII had an additional effect only on Fib2 G'max ( $p<0.01$ ). Interaction analysis shows that fibrinogen (+/-FXIII) decreased COT2 significantly more in blood diluted with HES than with RA ( $p<0.002$  as compared with control), whereas FibScreen 2 G'max increased more in blood diluted with RA than HES (fibrinogen+/-FXIII vs. control and fibrinogen+FXIII vs. fibrinogen, all  $p<0.001$ ). Fibscreen1-Fibscreen2 G'max increased with fibrinogen+/-FXIII ( $p<0.001$ ).

There were no significant interactions between temperature and addition of factor concentrates.

### ***Summary of study III***

FOR coagulation parameters were impaired by hypothermia (33°C) or 33% haemodilution (HES more than RA). HES and hypothermia interacted to further impair Fib2 G'max and COT2. Fibrinogen (with or without FXIII) improved coagulation and FXIII had an additional effect on Fib2 G'max. Factor concentrate improved COT2 better in HES haemodilution, but Fib2 G'max better in RA haemodilution.

The effect of factor concentrate were as effective at 33°C as at 37°C, as shown by the absence of interaction between temperature and coagulation factor.

## Study IV

### ***Hypothermia and haemodilution***

All ROTEM EXTEM clot velocity parameters (CT, CFT, and AA) were significantly impaired at 33°C, while clot strength (EXTEM- and FIBTEM-MCF) decreased very little at 33°. At both temperatures all parameters were significantly impaired by haemodilution ( $p < 0.001$ ) except EXTEM-CT and FIBTEM-MCF, which were not impaired by RA haemodilution. HES impaired all ROTEM parameters more than RA ( $p < 0.001$ ). In addition, HES and hypothermia at 33°C interacted to further impair CFT and AA ( $p < 0.05$  vs. undiluted).

EX-FIBTEM MCF decreased after haemodilution, but only at 33°C.

### ***Addition of fibrinogen or fibrinogen with factor XIII***

The addition of fibrinogen or fibrinogen with FXIII at either temperature significantly improved all EXTEM and FIBTEM parameters except EXTEM CFT and AA in HES haemodilution. FXIII had an additional effect to FIBTEM-MCF in RA haemodilution at both temperatures.

EXTEM-FIBTEM MCF was *decreased* by fibrinogen with FXIII in RA haemodilution and were unchanged in HES haemodilution.

### ***Summary of study IV***

Fibrinogen improved coagulation better in RA than HES haemodilution, and independently of temperature at 33°C or 37°C. FXIII had an additional effect only on FIBTEM MCF, also independent of chosen temperature but not in HES haemodilution.

## Study V

After administration of 1000 ml of HES (25 patients), the average haemodilution as calculated from haemoglobin values before surgery and after 1000 ml HES was 16%. Fibrinogen concentrations as measured with the three methods, and ROTEM FIBTEM MCF, are summarised in Table 5.

		Claus method		
	ELISA	Photometric	Electromechanical	FIBTEM MCF
<b>Baseline</b>	2.24 g/l	2.31 g/l	2.6 g/l	12.0
<b>After 1000 ml HES</b>	1.56 g/l	1.65 g/l	1.9 g/l	7.0
<b>Change</b>	-0.68 g/l	-0.66 g/l	-0.7 g/l	-5.0
<b>Relative change</b>	-31%	-29%	-27%	-44%

**Table 5.**

The electromechanical Claus yielded significantly higher fibrinogen than the other two fibrinogen methods (0.36 g/l and 0.34 g/l,  $p < 0.001$ ), while the absolute and relative change of fibrinogen concentrations were of the same magnitude as measured with the three different methods. However, the relative change of FIBTEM MCF was significantly higher than the relative change of fibrinogen concentration ( $p < 0.001$ ).

### **Summary of study V**

The main finding is that the relative decrease of FIBTEM MCF was significantly higher than that of fibrinogen concentration as measured with any of the three methods. The electromechanical method yielded higher values than the other two; however, the three fibrinogen concentration methods decrease to the same extent after infusion of 1000 ml of HES.

# Study VI

## ***Within-centre variability***

Differences between paired analyses of the same samples were generally low, with Bland-Altman mean differences  $\leq 0.05$  g/l at all three time points for most centres, and narrow (95%) limits of agreement. Two centres had a greater variability: at one centre mean differences were 0.15 g/l at one time point, and at two centres the limits of agreement were  $\pm 0.9$  g/l or more at one or two time points. Variability was also expressed as intraclass correlations (ICC): these were close to 1.0 at most time points, but  $< 0.8$  at one to two time points at two centres.

## ***Between-centre variability***

Differences between paired centres with similar methods were generally high. Although mean differences typically were less than 0.15 g/l, some were above 0.5 g/l, and limits of agreement were usually wide and typically more than  $\pm 1.0$  g/l. This is also shown with ICC, being  $< 0.5$  for the five photometric methods, and  $< 0.7$  for the two mechanical methods. Between-centre variability was least with the two antigen methods ( $ICC \geq 0.75$ ).

## ***Comparison of pre-and post-weaning from CPB***

The agreements between time points 2 (pre-weaning from CPB) and 3 (post-weaning from CPB) were good: only one method/centre found a significant difference, which was only 0.13 g/l.

## ***Fibrinogen standard***

Most methods differed considerably from the 1st WHO fibrinogen standard, which contains a total of 15 mg fibrinogen and 10.4 mg functional (“clottable”) fibrinogen. For example, one antigen method measured (total fibrinogen) values as low as 4.5 g/l, and all photometric methods measured (functional fibrinogen) values close to 7.8 g/l.

## ***Summary of study VI***

Within-centre variability was generally low, and agreement between pre- and post-CPB was very good or good with all methods. Between-centre variability was generally high with wide limits of agreement typically  $\geq 1.0$  g/l. The performance to correctly measure the content of the 1st WHO fibrinogen standard was generally poor.



# Discussion

## Main findings

With viscoelastic haemostatic assays (VHAs) on in vitro haemodilution-induced coagulopathy, we have found that albumin causes less coagulopathy than synthetic colloids, and that high-dose in vitro fibrinogen concentrate better reversed coagulopathy induced by albumin or crystalloid solutions than if induced by synthetic colloids. Factor XIII concentrate has no effect on in vitro haemodilution-induced coagulopathy, but has an additional effect to that of fibrinogen. Haemodilution and hypothermia (33°C) partly interacted to impair in vitro coagulation, but fibrinogen +/- factor XIII improved haemodilution-induced coagulopathy irrespective of temperature. In an in vivo clinical setting of HES haemodilution, we have found that the relative reduction of ROTEM FIBTEM MCF was greater than that of three methods measuring fibrinogen concentration. Finally, we have found that the variability of fibrinogen methods was low within methods but considerable between methods, and that calibration and standardisation of fibrinogen concentration methods need to be improved.

## Haemodilution and high-dose fibrinogen

Our finding in Paper I that HA affects coagulation less than synthetic colloids is corroborated by both previous and later studies. Comparisons of haemodilution with human albumin with different HES solutions,<sup>106,107</sup> gelatine<sup>106</sup> or dextran<sup>108</sup>, showed less effect on coagulation as measured with thrombelastography. We also concluded that the effect of HA is comparable with that of crystalloid solutions, which has also been shown previously.<sup>108,112</sup> However, no previous study has simultaneously compared HA with such an extensive panel of resuscitation fluids, including crystalloids, a modern low molecular weight HES, both dextran 60 and 70, and buffered and unbuffered variants of these solutions. Our selection of fluids better reflect the contemporary tradition of fluids used in Scandinavia.

Our finding in Paper I, that high-dose fibrinogen concentrate partly reverses haemodilution-induced coagulopathy, is in line with previous findings from both in

vitro studies<sup>30-32</sup> and a porcine model.<sup>33-35</sup> Two of these studies found fibrinogen to be more effective in crystalloid than colloid haemodilution,<sup>30,32</sup> but none of those used human albumin. Indeed, our study was the first investigation where the effect of fibrinogen on coagulopathy induced by human albumin was compared with other colloids, as well as crystalloids, at the same time. In our study, we also found that crystalloid-induced coagulopathy was completely restored by the addition of fibrinogen corresponding with a dose of approximately 120 mg/kg. This is in contrast with a dose finding study, which found that only doses  $\geq 200$  mg/kg restore ROTEM parameters.<sup>171</sup> However, they used a more severe in vitro haemodilution with coagulation factors and platelet count reduced by 80%.

## Factor XIII and fibrinogen dosage

After these findings on albumin-induced coagulopathy and reversal with high-dose fibrinogen, we decided to study dose effects of fibrinogen in albumin haemodilution, and if this could be further improved by adding factor XIII. In Paper II, we show that FXIII alone has no effect on any ROTEM parameters, but FXIII has an additional effect to that of fibrinogen but only on FIBTEM MCF. Fibrinogen alone improves coagulation dose dependently.

This corroborates other studies, showing no or minimal effects on ROTEM parameters after the addition of FXIII, but an additional effect in combination with fibrinogen.<sup>31,172</sup> In contrast, in an in vitro study on blood from patients after major surgery, factor XIII alone improved FIBTEM MCF.<sup>173</sup> The authors of that study suggested that these patients had a relative deficit of FXIII in relation to their fibrinogen concentrations, since FIBTEM MCF increased more the higher the basal fibrinogen.

## Hypothermia during haemodilution and effects of factor concentrates

In the next two studies (III and IV), we investigated the effects of hypothermia in haemodilution-induced coagulopathies to the reversing effects of fibrinogen or fibrinogen with factor XIII. Since the FOR used in study III is not very well-known and scantily evaluated, we decided to repeat the protocol with the well-evaluated ROTEM in study IV.

In both of these studies, the effect of fibrinogen or fibrinogen with FXIII was independent of temperature. This is of interest since the role of fibrinogen concentrate in hypothermia has not previously been studied. Guidelines state that hypothermia should be corrected before administration of activated factor VIIa to correct severe coagulopathy,<sup>57</sup> but our results indicate that this is not necessary when reversing coagulopathy with fibrinogen concentrate.

Hypothermia at 33°C reduced clot velocity parameters and whole blood clot strength with both FOR and ROTEM, whereas the fibrinogen dependent clot strength was impaired only when measured with FOR. This is in line with the results of other studies using ROTEM or TEG: mild to moderate hypothermia decreased the rate of clot formation but not the clot strength,<sup>131</sup> though more severe hypothermia (<28° C) may also decrease clot strength.<sup>132</sup> However, our results with the FOR where clot strength already decreased at 33°C may indicate that clot strength parameters as measured with FOR are more sensitive to hypothermia than the parameters of TEG or ROTEM. In addition to these effects, we found in both studies that hypothermia and HES haemodilution interact to further impair clot velocity. Interaction of hypothermia and haemodilution has not previously been very well studied: an older study using APTT was in line with these findings.<sup>174</sup>

Haemodilution with either fluid (RA or HES) impaired all parameters in both studies, and HES was worse than RA. Fibrinogen improved all clot strength parameters but not all clot velocity parameters (i.e., COT 1 (FOR) or CFT and AA (ROTEM) in HES haemodilution). Factor XIII had an additional effect to fibrinogen dependent clot strength with both FOR and ROTEM but only in RA haemodiluted blood.

The effect of haemodilution with RA or HES is corroborated by several other studies, including Paper I, which also shows a greater effect of HES than crystalloid fluids.<sup>9,32,92</sup> The reversal effects of fibrinogen is also well studied and corroborated by other studies, including Paper I: the effect is most obvious on clot strength parameters and more so with crystalloid than HES haemodilution as measured with ROTEM.<sup>30,32</sup> Electron microscope pictures show more dense clots after the addition of fibrinogen in haemodilution with saline as compared with HES.<sup>9</sup> The additional effect of FXIII on fibrinogen dependent clot strength is seen only in RA haemodilution, not in HES haemodilution. This is also confirmed by a study where fibrinogen with FXIII better improved FIBTEM MCF in haemodilution with saline, albumin and gelatine, but not HES.<sup>175</sup> The poor effect of FXIII in HES haemodilution supports the suggestion that HES reduce clot strength through an impaired interaction between fibrin monomers and FXIII.<sup>176</sup>

## Fibrinogen measurement and FIBTEM MCF in vivo

Different variants of the Clauss fibrinogen measurement method have been shown to overestimate fibrinogen concentration in the presence of colloids such as HES,<sup>10,149</sup> but this is not corroborated by our study V. Our main findings are that in vivo haemodilution with HES reduced fibrinogen concentration as measured with two Clauss method variants to the same extent as an immunological assay (ELISA), but that the relative decrease of FIBTEM MCF was significantly higher than seen with any of the fibrinogen concentration methods.

These findings are not in conflict with previous in vitro studies. The decrease of fibrinogen analysed with an immunological assay closely followed actual values calculated from in vitro haemodilution with crystalloid fluid.<sup>149,177</sup> The Dade Thrombin Reagent with a photometric readout were comparable with calculated values and immunological assays both in HES and crystalloid haemodilution,<sup>149</sup> and mechanical readouts were also more reliable than photometric readouts.<sup>149,177</sup> Those where the methods used in Paper V. Our finding that FIBTEM MCF is reduced more than fibrinogen concentration is in line with an in vitro study, where FIBTEM MCF was reduced disproportionately more than calculated or measured fibrinogen concentrations after haemodilution with HES as compared with saline.<sup>177</sup> Finally, the higher concentration with the mechanical Clauss method was probably caused by different calibrations.<sup>146</sup>

## Fibrinogen measurement methods in vivo

The known issue with different fibrinogen measurement methods was further investigated in Paper VI. The main findings were that variation was low with repeated measures with the same method, but that variation was high between different methods.

There are at least 50 different fibrinogen measurement methods defined as a specific combination of reagent and instrument, most of them of the Clauss type.<sup>147</sup> Instrument type has the greatest effect on precision, but nevertheless most instruments have a high precision.<sup>147</sup> In our study, this is reflected by low within-method variability. However, trueness and precision of results that is accuracy (as defined by ISO 5725) is affected by reagent type and instrument, but also by type of calibrator used. We used six different Clauss methods as defined by five instruments, five reagents and six calibrators, and we found a remarkable variation of between-methods results that are likely to affect decisions whether to administer

fibrinogen concentrate or not. The Multifibren U thrombin reagent is used with an undiluted plasma sample, which is known to be more sensitive to confounding substances in the sample such as heparin, colloids and fibrinogen degradation products, which are all likely to be present in for example cardiac surgery. This highlights the need for standardisation of perioperative fibrinogen measurements. Dutch and Japanese investigators have proposed better calibrators to improve between-methods agreement.<sup>178,179</sup>

## Limitations and strengths

Our studies have limitations shared by many others. In Papers I-IV, we study coagulation in vitro with blood from healthy individuals. This may not very well reflect the situation in disease. Surgical or traumatic disruption of endothelium exposes tissue factor and collagen to the blood stream, and releases substances and particles that activate coagulation and fibrinolysis.<sup>180</sup> The pathological hypocoagulation and hyperfibrinolysis seen in ATC is probably caused by a combination of increased activation of protein C<sup>181</sup> and shedding of the endothelial glycocalyx that releases heparin-like substances.<sup>182</sup> APC inactivates the co-factors V and VIII, and also plasminogen activator inhibitor (PAI). In patients with severe inflammatory activity such as septic inflammatory response, the opposite is often seen: hypercoagulation and impaired fibrinolysis.<sup>183</sup> None of these clinical situations is reflected with blood from healthy volunteers.

Though VHAs possibly better reflect haemostasis than traditional coagulation tests, there are still some drawbacks, specifically they do not measure coagulation during physiological shear stress. For example, in a study using ROTEM and a flow-chamber assay, the replacement of vWF accelerated thrombus formation more at higher flow rates, whereas fibrinogen was more efficient at the lower flow rate or when coagulation was evaluated with ROTEM.<sup>91,184</sup> Measuring coagulation at one particular frequency, as with ROTEM or FOR, is probably inadequate. A comparison with Fourier Transform Mechanical Spectroscopy measuring at several frequencies found that both the TEG and the FOR are unable to correctly define the incipient clot that is the transition from a viscous fluid into an elastic solid.<sup>185</sup>

The ability of VHAs to improve clinical outcome as compared to standard coagulation tests, has been evaluated in cardiac surgery. Some randomised controlled trials (RCT) show a reduction in transfusion of packed red blood cells (PRBC), platelet concentrate (PC) and FFP, but not fibrinogen concentrate.<sup>186,187</sup> VHAs have also been shown to better predict post-operative bleeding than APTT or PT, but not to improve mortality.<sup>187</sup> The conclusion, however, is that VHAs are

worthwhile and important to further evaluate both in vitro but above all in vivo. The strengths of Paper V and VI are that they both evaluate fibrinogen assays (and ROTEM) in clinical settings.

In the first paper, we studied seven different fluids. We found it valuable to compare albumin with most other fluids used in Sweden at the same time. On the other hand, this complicated statistical analysis and we only compared our results in relation to undiluted or albumin diluted samples. One can also argue that the studied dose of fibrinogen does not correspond with what is recommended in current guidelines. This is also an issue in the second paper, where the high dose of fibrinogen corresponds to more than twice the dose recommended in major trauma.<sup>57</sup> However, another in vitro study found even higher dosages to be necessary to completely reverse haemodilution-induced coagulopathy.<sup>171</sup> The dosage of FXIII used in Paper II is in line with recommendations by the manufacturer.

The protocols of Paper III and IV are more complicated than necessary to answer the question: is hypothermia interfering with the ability of fibrinogen and factor XIII to reverse haemodilution-induced coagulopathy? For that purpose, one could have considered omitting one of the fluids and studying one coagulation concentrate at a time. Instead, it would have been valuable to study several temperature points.

Paper V has the limitation of not comparing the effect of other solutions than HES. However, an interventional (or clinical) trial is far more expensive and time-consuming to perform than our observational study. Instead, the ELISA served as the control.

Paper VI is a multi-centre unit study comparing fibrinogen measurement in Austria, Germany and Italy, but all the same it reflects the various methods used in Swedish clinical laboratories.

## Implications

The finding that albumin is equivalent with crystalloids with regard to its in vitro effect on coagulation supports the use of any of those fluids in a situation of continuous or imminent bleeding, while the use of synthetic colloids seem unjustified and unnecessary. Other studies have also found that HES, dextran and gelatine affect coagulation more than crystalloids and albumin.<sup>5,106,110</sup> A recent Cochrane study concludes that the effect of different colloids on the amount of transfusion needed is not properly evaluated in previous clinical studies.<sup>188</sup> However, dextran as compared to human albumin has been shown to increase bleeding in revision hip arthroplasty<sup>123</sup> and during prostatectomy,<sup>112</sup> and HES has

been found to increase bleeding as compared with albumin in cardiopulmonary bypass surgery.<sup>96</sup>

The benefits of albumin and crystalloids, as compared with synthetic colloids, is further supported by the finding that in vitro correction of coagulopathy with fibrinogen concentrate is just as efficient whether albumin or a crystalloid solution has been used as resuscitation fluid. Another recent study also shows that fibrinogen restore coagulation after haemodilution with albumin, gelatine and saline, but not with HES,<sup>175</sup> while others found crystalloid-induced coagulopathy to be better enhanced by fibrinogen than after haemodilution with dextran<sup>30</sup> or HES.<sup>32</sup> In our study, dosages of fibrinogen to sufficiently restore the coagulation were higher than those recommended in current guidelines, and this may also be necessary according to clinical practice and a porcine model.<sup>189,190</sup>

The preserved effect of fibrinogen concentrate also at 33°C implies that hypothermia is not necessary to reverse before administration of fibrinogen. The availability of fibrinogen decreases in hypothermia,<sup>135</sup> but others have found that hypothermia, in contrast to acidosis, does not need to be corrected before the administration of fibrinogen.<sup>191</sup>

The in vitro additional effect of FXIII warrants further in vivo investigations, but not yet the use of FXIII concentrate in clinical practice.

The in vivo profound effect of HES on FIBTEM MCF as compared with fibrinogen concentration adds further evidence that HES should be avoided in bleeding or in situations with risk for bleeding.<sup>5</sup> It is also evident that fibrinogen dependent clot strength yields valuable extra information on the clotting process, which is not measured with fibrinogen concentration methods. However, FIBTEM MCF is not superior to fibrinogen concentrate to predict bleeding or transfusion needs in trauma, but FIBTEM MCF is more quickly obtained.<sup>192</sup> Therefore, ROTEM should be regarded as a valuable coagulation screening test when trauma patients arrive to the hospital.

The poor between-method agreement of different Clauss fibrinogen methods indicates a need to compare the various methods used in Swedish laboratories and to improve standardisation of perioperative fibrinogen testing. However, good within-method agreement allows us to trust changes of fibrinogen concentration in a particular patient measured with the same method.



# Conclusions

In conclusion, this thesis outlines that albumin and crystalloids impair coagulation less than synthetic colloids, and that haemodilution induced by crystalloids or albumin is more readily restored by fibrinogen. It also shows that the effect of fibrinogen is unaffected at a temperature of 33°C and that factor XIII may add extra clot strength. ROTEM FIBTEM MCF gives additional information as compared to fibrinogen concentration, and measurement methods of fibrinogen need to be better standardised.



# Future aspects

In a broader perspective, all in vitro studies must be followed by randomised controlled trials (RCT) with appropriate endpoints, ultimately morbidity and mortality. For example, we now know from in vitro studies that albumin is no worse than crystalloids to induce coagulopathy, but does this mean that albumin should have a role in resuscitation? Colloids including albumin have recently been abandoned in a Cochrane Study, but there may be benefits with albumin in subgroups, for example in septic patients.<sup>80</sup> This needs to be properly investigated.

Fibrinogen concentrate effectively improve in vitro haemodilution-induced coagulopathy, as was shown in this thesis. However, few RCTs on fibrinogen concentrate have been published and none of them in trauma.<sup>156</sup> Some of these RCTs show a reduced transfusion need in the fibrinogen concentrate group, but they did not have enough power to conclude anything about mortality. We need a large RCT to answer this question.

It is currently advised to give fibrinogen concentrate only in ongoing bleeding, but fibrinogen concentrate as prophylaxis to patients before cardiac surgery has been shown to reduce bleeding.<sup>40</sup> In our clinical studies, we could see that occasional patients with an intracranial tumour had very low preoperative FIBTEM MCF and fibrinogen concentration. It would be interesting to investigate the effect of giving fibrinogen concentrate preoperatively to reduce bleeding in these patients.

The knowledge about acquired deficiency of FXIII is scarce, and FXIII concentrate is currently not recommended in guidelines on the management of trauma<sup>57</sup> but in certain cases of severe perioperative bleeding.<sup>56</sup> Recent studies show that FXIII is decreased in severe traumatic brain injury<sup>193</sup> and FXIII may decrease endothelial permeability.<sup>59</sup> FXIII may also be reduced in some patients with tumours.<sup>194</sup> It is tempting to investigate these findings further, but interventional studies probably need to await better laboratory methods.

ROTEM and TEG have been shown to reduce transfusion needs in cardiac surgery, and there is currently an ongoing study comparing traditional coagulation tests with TEG to guide transfusion therapy (study NCT01536496 on <http://clinicaltrials.gov>). A logical next step would be to compare different goal levels of fibrinogen, be it

concluded that it is better guided with TEG or fibrinogen concentration. TEG and ROTEM are not completely comparable and a similar study with ROTEM would be eligible.

The knowledge of FOR is scarce and it needs to be further evaluated. A shortcut is to compare FOR directly with well-studied VHAs, for example ROTEM, both in vitro and in clinical settings. There is a potential advantage of FOR over ROTEM and TEG: our data indicate that FOR may be more sensitive to normal or high clot strength. A comparison of FOR with other coagulation tests to predict post-operative thromboembolic disease seems justified. It is noteworthy that more patients die from thromboembolic events than from exsanguination.

# Summary in Swedish

Skador genom yttre våld är den vanligaste dödsorsaken enligt statistik från WHO. Vid genomgång av dödsfall kan man i efterhand konstatera att blödning som dödsorsak ofta skulle ha gått att undvika. Blödning är emellertid inte bara en direkt dödsorsak, utan också en indirekt dödsorsak eftersom blodtransfusion ökar risken för dödliga komplikationer t.ex. infektion. Kirurgi av hög kvalitet är den viktigaste åtgärden för att stoppa blödningar, men det krävs också att kroppens egen förmåga att stoppa blödningar (hemostas) är välfungerande. Det är till och med så att vi idag undviker att operera patienter med blödande leverskador och i första hand låter kroppen självt stoppa blödningen.

Hemostas (grekiska: haimó: blod stasis: stoppa) är kroppens förmåga att stoppa blödning, huvudsakligen genom att vid en kärlskada låta blodet omvandlas till ett gel-liknande och så småningom fast koagel (levrat blod). Ett koagel består av blodplättar, som hålls samman av ett nätverk av fibrin-trådar. Fibrin är sista steget i vad som brukar kallas koagulationskaskaden: en serie proteiner (eller koagulationsfaktorer) som likt katten-på-rättan-principen reglerar bildningen av trombin, vilket i sin tur klyver fibrinogen till fibrin. Slutligen förstärks fibrinet till tjocka trådar av koagulationsfaktor XIII. Koagulationsprocessen är hårt reglerad: antikoagulationssystem förhindrar koagel från att bildas i oskadade blodkärl och fibrinolys (grekiska: lysis: lösning) löser upp redan bildade koagel efterhand som kärlskadan läks.

För att upprätthålla tillräcklig transport av syre till kroppens celler krävs ett bra blodflöde och en tillräcklig mängd av hemoglobin som binder syret i röda blodkroppar. Som tidigare nämnts är blodtransfusioner i sig farliga, och flera undersökningar visar att det är till fördel att acceptera låga blodvärden hos patienter. Det är viktigare att blodflödet är bra och avgörande för ett bra blodflöde är att blodbanan innehåller en tillräcklig volym vätska. Blödning ersätts därför i första hand med infusion av vätskor innehållande koksalt och andra salter. Dessa vätskor kallas gemensamt för kristalloider. Kristalloider har den ogynnsamma egenskapen att delvis läcka ut även genom friska blodkärlsväggar och skapa ödem (vätska i vävnaden). Detta kan undvikas med en annan typ av infusionsvätskor, så kallade kolloida lösningar, till exempel albumin (framställt från humant blod) och syntetiska kolloider t.ex. stärkelse (HES), dextran eller gelatin. Alla dessa vätskor späder

förstås ut blodet, vilket bidrar till försämrade koagulationsförmåga, så kallad koagulopati. Vätsketerapi vid blödning är därför en avvägning mellan att ge tillräckligt med vätska för att upprätthålla blodcirkulationen och att undvika biverkningar i form av ödem, koagulopati eller ett alltför lågt blodvärde. Till detta kommer att även nedkylning (hypotermi), som är vanligt i samband med olyckor, bidrar till koagulopatin.

Den koagulationsfaktor som först når kritiskt låga nivåer är fibrinogen, och viss forskning tyder på ökad överlevnad om man ser till att upprätthålla fibrinogennivån vid blödning. I dag är rekommendationen att ge fibrinogenkoncentrat (renframställt fibrinogen) vid svår blödning med låg fibrinogennivå. Den klassiska metoden att mäta koncentrationen av fibrinogen i plasma, Clauss-metoden, har flera brister: dels är den för långsam, dels kan flera Clauss-varianter ge felvärden om blodet innehåller t.ex. kolloider. Viskoelastiska hemostatiska mätmetoder (VHA), till exempel trombelastografi (TEG), rotations-tromboelastometri (ROTEM) eller en nyare variant, fri oscillations-reometri (FOR), kan ge mer information och är dessutom en snabbare metod än fibrinogennivå enligt Clauss eller andra traditionellt använda mätmetoder av koagulationsförmågan. VHA ger olika mått på koagulationens hastighet och det slutliga koaglets hållfasthet i helblod med eller utan hämning av blodplättar.

Det övergripande målet med avhandlingen har varit att belysa den koagulationshämmande effekten av olika infusionsvätskor och förmågan att med koncentrat av fibrinogen och/eller faktor XIII kunna behandla koagulopati orsakad av utspädning med olika vätskor och/eller hypotermi. Dessutom har olika metoder att mäta fibrinogen i samband med kirurgi jämförts.

I studie I till IV undersöktes blod från friska frivilliga försökspersoner, så kallade in vitro försök (läkemedel tillsattes i blodprovet och inte till patienten), med hjälp av rotations-tromboelastometri (ROTEM) eller fri oscillations-reometri (FOR). I studie V och VI togs blod från patienter under och efter kirurgi, så kallade observationsstudier. I dessa analyserades fibrin i plasma med olika metoder och i studie V dessutom med ROTEM.

I korthet har följande fynd gjorts:

- Syntetiska kolloider försämrar koagulationen mer än kristalloider eller humant albumin.
- Albumin försämrar koagulationen lika mycket som kristalloider.
- Fibrinogen förbättrar koagulationen efter utspädning, sannolikt effektivare efter utspädning med albumin eller kristalloid än efter utspädning med syntetiska kolloider
- Fibrinogen förbättrar koagulationen bättre ju högre dosen är.
- Faktor XIII har ensamt ingen effekt på koagulationen efter utspädning, men förbättrar fibrinogenets koagelstärkande effekt
- Kombinationen hypotermi och utspädning med HES har extra negativ inverkan på koagulationen jämfört med dessa faktorer var för sig
- Fibrinogen+/-faktorXIII förbättrar koagulopatin orsakad av utspädning även vid hypotermi
- Fibrinogenberoende koagelhållfasthet sjunker mer än fibrinogen hos patienter som har fått HES
- Olika mätmetoder för fibrinogen varierar ibland sinsemellan kraftigt men variationen inom respektive metod är låg



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xxx

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