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Plasma coagulation in cardiac surgery

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Ineko Intellecta

Abstract

Background: Cardiac surgery using cardiopulmonary bypass is a complex procedure, sometimes accompanied with excessive bleeding. The nature and pathophysiology of this bleeding is multifactorial and not completely understood. Fibrinogen is a final step in the coagulation cascade and its substitution can alter postoperative hemorrhage.

Aims: To investigate different aspects of plasma coagulation in cardiac surgery. Firstly, to assess potential changes in coagulation factor levels during surgery and its relation to bleeding. Secondly, to study plasma's potential to generate thrombin after cardiac surgery. Thirdly, to compare two different protocols to dose anticoagulant heparin in regard to thrombin building capacity. Fourthly, to investigate effects of recombinant human fibrinogen *ex vivo*.

Material and methods: In study I, coagulation factor activities were measured before and after surgery in a cohort of 57 patients undergoing first time elective coronary artery bypass grafting (CABG). Study II comprises the same cohort, now measuring thrombin generation potential using calibrated automated thrombography (CAT). Study III is a prospective trial, randomizing 60 elective CABG or valve replacement surgery patients to either anticoagulation with weight-based heparin dosing or using heparin and protamine titration with a bedside device. In study IV, plasma of 10 cardiac surgery patients was spiked with various concentrations of human plasma derived fibrinogen or recombinant human fibrinogen. *Ex vivo* clot formation was assessed by rotational thromboelastometry.

Results: There is pronounced variation in level of individual coagulation factors after surgery. Concentration of fibrinogen and FXIII two hours after surgery showed a weak correlation to total bleeding volume. Pronounced deterioration of thrombin generation capacity after surgery was found, possibly caused by persistent heparin effect and/or heparin rebound. Different heparin dosing protocol had no effect on pre- and postoperative plasma's thrombin generation capacity or on bleeding volume. *Ex vivo*, there is no difference between the new recombinant human fibrinogen concentrate and plasma derived regarding clot formation ability.

Conclusions: Postoperative decline (or rise) of individual coagulation factors does not seem to affect the postoperative bleeding volume, with reservation for fibrinogen and FXIII. Heparin effect is still present at 2 and 4 hours after surgery, affecting thrombin generation. More precise heparin and protamine dosing protocols does not influence this phenomenon. Recently manufactured recombinant human fibrinogen concentrate is able to generate a clot of similar viscoelastic properties as the one plasma derived.

Sammanfattning på svenska

Hjärtkirurgi med användning av hjärtlungmaskin är en tämligen vanlig procedur idag. Drygt sex tusen ingrepp görs i Sverige varje år. Heparin används för att motverka blodproppsbildning under själva kirurgin. Komplikationer under och efter hjärtkirurgi är inte så vanliga men förekommer. En av de vanligaste är ökad blödning, och omkring fem procent av patienterna måste opereras på nytt på grund av blödningen. Blödningsorsakerna är mångfaktoriella och till en stor del fortfarande oklara. Huvudsyftet med vårt projekt var att studera blodets koagulation (levringsförmåga) efter att den initiala trombocytpluggen hade bildats. Vi har funnit att aktiviteten av koagulationfaktorer varierar kraftigt efter kirurgi och att låga nivåer av fibrinogen och faktor XIII är kopplade till ökad blödningsbenägenhet. Vidare har vi noterat att efter operationerna har patienterna sänkt förmåga att bilda aktiverad faktor II (trombin), en faktor av särskild betydelse för fullgod koagulation. Detta var orsakat av kvarstående heparineffekt. En annan metod att dosera heparin med hjälp av ett patientnära instrument har också testats, men utan att kunna minska heparinets negativa effekt på blodplasmas trombinbildande potential. Fibrinogen har också tidigare visats vara särskilt viktigt för adekvat koagulation. En del patienter som drabbas av stora blödningar behandlas därför idag med fibrinogenkoncentrat. Dessa utvinns ur lagrad plasma, samlad från många blodgivare. Sådant förfarande innebär en teoretisk smittorisk i fall att en ny, okänd infektiös agens uppstår. Vår grupp har fått möjlighet att testa fibrinogen som är framställt med rekombinant teknik (fritt från mänskliga proteiner i samtliga steg av tillverkningsprocessen). I testförsök utanför kroppen har det rekombinanta fibrinogenet visat sig kunna forma blodkoagler av lika bra kvalité som fibrinogen framställt från human plasma.

Original papers

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

- I) Ternström L, Radulovic V, Karlsson M, Baghaei F, Hyllner M, Bylock A, Hansson KM, Jeppsson A
Plasma activity of individual coagulation factors, hemodilution and blood loss after cardiac surgery
Thromb Res 2010; 126: e128-e133
- II) Radulovic V, Hyllner M, Ternström L, Karlsson M, Bylock A, Hansson KM, Baghaei F, Jeppsson A
Sustained heparin effect contributes to reduced plasma thrombin generation capacity early after cardiac surgery
Thromb Res 2012; 130(5): 769-74
- III) Radulovic V, Laffin A, Hansson KM, Backlund E, Baghaei F, Jeppsson A
Heparin and protamine titration does not improve thrombin generation capacity after cardiac surgery: a prospective randomized study
Manuscript
- IV) Radulovic V, Baghaei F, Blixter I, Samuelsson S, Jeppsson A
Comparable effects of recombinant and plasma derived human fibrinogen concentrate on ex vivo clot formation after cardiac surgery
J Thromb Hemost 2012; 10(8): 1696-8

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Abbreviations

A10	Amplitude 10 min after clotting time
ACT	Activated clotting time
Alpha	Alpha-angle
ANOVA	Analysis of variance
APC	Activated protein C
APTT	Activated partial thromboplastin time
ASD	Atrial septum defect
AT	Antithrombin
AUC	Area under the curve
BMI	Body mass index
CABG	Coronary artery bypass grafting
CAT	Calibrated automated thrombography
CFT	Clot formation time
CMV	Cytomegalovirus
CPB	Cardiopulmonary bypass
CT	Clotting time
Da	Dalton
ECAT	External quality control of diagnostic assays and tests
ECC	Extracorporeal circuit
ELISA	Enzyme-linked immunosorbent assay
EPCR	Endothelial protein C receptor
ETP	Endogenous thrombin generation
F1.2	Prothrombin fragment 1 and 2
FFP	Fresh frozen plasma
FGN	Fibrinogen

GAG	Glycosaminoglycans
HCII	Heparin cofactor II
HDR	Heparin dose response
HLM	Heart and lung machine
HMWK	High molecular weight kininogen
HS	Heparan sulfate
ICU	Intensive care unit
IU	International units
LI30	Lysis index 39 min after clotting time
MCF	Maximal clot firmness
ML	Maximum lysis
PBS	Phosphate buffered saline
PC	Protein C
PCI	Protein C inhibitor
pdFGN	Plasma derived fibrinogen
PPP	Platelet poor plasma
PRBC	Packed red blood cells
PS	Protein S
PT INR	Prothrombin time INR
SDS-PAGE	Sodium dodecyl sulphate polyacrylamid gel electrophoresis
TAFI	Thrombin-activatable fibrinolysis inhibitor
TAT	Thrombin-antithrombin complex
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
tPA	Tissue plasminogen activator
TT	Thrombin time
TTP	Time to peak thrombin activity
UFH	Unfractionated heparin
USP	United States Pharmacopeia Units
VTE	Venous thromboembolism
VWF	Von Willebrand factor

Introduction

The first successful surgical intervention of the heart recorded was suturing a knife stab wound by Axel Cappelen at Rikshospitalet, Oslo, in September 1895. Nevertheless, the patient died eventually in mediastinitis. Many years were to pass until the next surgical breakthrough.

Gross closed a patent ductus arteriosus in 1939, Blalock succeeded with the first open heart surgery repairing atrial septum defect (ASD) at Johns Hopkins in Baltimore in 1944, Crafoord and Nylin performed resection of coarctation of the aorta in Stockholm 1945, while Bailey managed to carry out mitral commissurotomy in 1949. Several groups were engaged in intensive pursuit of the means for safe and convenient “surgeon friendly” intra-cardiac surgery. Lillehei at Minnesota Medical School operated using hypothermia and cross circulation from mother/father to a child (having placenta as inspiration), “the only operation with a potential 200% mortality rate” (1). Others focused on constructing a machine that could temporarily replace heart and lungs in the operation theatre. Thus, Mustard experimented with isolated rhesus monkey lungs as oxygenator in Toronto, Dodrill (Wayne State Medical School, Detroit) worked in collaboration with General Motors on a device resembling an old Cadillac V12 engine, Gibbon (Jefferson Medical College, Philadelphia) developed a film oxygenator with help of his wife and Malmros, chief engineer at International Business Machine Corp. (IBM), not so unlike today’s computers, while Dennis (Minnesota Medical School) used rotating oxygenator based on design by Viking Olov Björk and Clarence Crafoord. A couple of years later Gibbon’s device was refined by John Kirklin at Mayo (Mayo-Gibbon machine).

The first successful open heart surgery using heart and lung machine (HLM) was done by John Gibbon and took place in Philadelphia, 5th of May 1953. He closed ASD in an 18-years old patient. Partial bypass time was 45 minutes, complete 26 min; fresh heparinized blood was used for priming, with 10 mg heparin added per 500 ml blood. The clots started to develop in the machine and surgery had to be finished in a rush, but all ended up well eventually. Later that evening, Gibbon went to his office and made two telephone calls – one to Blalock in Baltimore and the other one to Crafoord

in Stockholm. Thanks to Gibbon's modesty as a person and a scientist, this case was first presented on a meeting many months later and was subsequently published in a journal called Minnesota Medicine.

This initial success was unfortunately followed by a series of three patients who died peroperatively, and Gibbon stopped operating and quit further developing of the machine (2).

In 1955-56 there were only two hospitals (surgeons) in the world performing open heart surgery on a daily basis – Lillehei and, some 60 miles away, Kirklin. Åke Senning from Stockholm, who would be the first one to implant a pacemaker a couple of years later, was visiting these two, like many other colleagues from around the world, learning the technique.

Cardiac surgery today

Open heart surgery is performed routinely today and is an established treatment for cardiovascular diseases. There is a huge discrepancy between countries and regions in clinical practice. Some 1222 open heart surgeries per million populations are done in North America, 569 per million in Europe and just 18 per million in Africa (3). Ninety three percent of world's cardiac surgery patients are living outside of USA, Europe and Australia (4). In Sweden, 6178 open heart procedures were performed during 2011 (excluding the pediatric population with congenital heart defects), with overall 30-day mortality 2.9% (5).

Despite advances in surgical technique and anesthesia, cardiac surgery is still accompanied with a risk of serious complications, such as infection, stroke, pulmonary or renal dysfunction or bleeding, especially in an aging population (6).

Cardiac surgery and bleeding

In the US, surgical procedures account for transfusion of almost 15 million units PRBC every year and cardiac operations consume 10-15 % of nation's blood supply (7). Fifty percent of patients undergoing cardiac procedures (entered into The Society of Thoracic Surgeons adult cardiac surgery database) require transfusion, which itself may worsen short and long outcomes (8). One of three undergoing routine cardiac surgery in UK is exposed to allogenic blood products (which is however an improvement comparing with 100 % rate in 1950's) and 10 % of patients utilize 90 % of all blood products issued (9). In a previously mentioned Swedish Registry (5), 308 patients (5.3%) were re-operated because of excessive bleeding during 2011. In another Swedish study on a smaller population, with data from Linköping, Uppsala and Örebro, comprising 4232 patients who underwent primary isolated CABG 2005-2008, 148 patients (3.5%) were re-explored because of bleeding. The mean incremental costs were 6290 Euros per re-exploration per patient, mostly due to prolonged hospital stay, cost of surgery, blood products and hemostatic drugs (10). A surgical cause of such a hemorrhage is found in about half of all cases (in some series 67 % (11)), the rest is due to impaired per- and postoperative hemostasis. In general, bleeding etiology in this setting is complex and multifactorial.

Extracorporeal circulation

The good results of modern open heart surgery could not be possible without stopping the heart temporarily, which requires the use of cardiopulmonary bypass in order to maintain peripheral organ perfusion. The machines have evolved during the last 60 years but are still a major challenge to blood and its homeostasis (12).

In order for blood not to clot, massive doses of anticoagulants are administered. The roller or centrifugal pumps are used to maintain flow of blood, which is exposed to a huge artificial surface. Gas exchange takes place in an oxygenator with a surface of 1.5-2 m² (5% of that in human lungs). This must be compensated for with non-physiological turbulent flow and longer blood dwell time. Furthermore, wound blood from the operation field is often suctioned back to the systemic circulation.

Regarding anticoagulation regime in the circuit, heparin has been used *ab ovo*, mostly due to the fact that there was no other anticoagulant, but even because this drug is old, proven, easily administered, inexpensive and is readily neutralized by protamine. In 1960, thrombin time (TT) was introduced as a measure of complete heparin neutralization by protamine, but no intraoperative monitoring of anticoagulant effect was performed until 1975. Intra-bypass heparin monitoring was introduced first by Bull et al (13), who reported more than 30 different heparin dosing algorithms in US at that time. Today so commonly accepted "safety limit" for providing sufficient anticoagulation while on CPB (activated clotting time (ACT) >400 s) is based mainly on data from Bull and a study measuring soluble fibrin monomer in nine rhesus monkeys (14).

Coagulopathy of surgery with cardiopulmonary bypass

Procedures in cardiac surgery using CPB are among the most extensive iatrogenic trauma human body can encounter. Exposure to artificial flow and surfaces, activation of blood cells and proteins, surgical damage on endothelium and extravascular tissues, massive local and systemic inflammatory response and concomitant medication makes it a very complex and dynamic model to study, impossible to mimic and investigate *in vitro*. There is no research modality today being able to cover all aspects of its complexity. The markers of coagulation are being formed and cleared simultaneously and have different half-lives. Measuring their levels *in vivo* or *in vitro* gives just a cross-sectional picture at the time of blood sampling and cannot capture the dynamics of the whole system (15).

Initiation of CPB causes immense activation of the contact system (16) and formation of FXIIa after contact with anionic surfaces, which in turn converts prekallikrein to kallikrein. Kallikrein exerts positive feedback on FXII and produces bradykinin, which induces tissue-plasminogen activator (tPA) mobilization from endothelium. At the same time, bradykinin clearance in the lungs is decreased due to minimal pulmonary blood flow (17). Fibrinogen is adsorbing on the CPB surface, recruiting and activating the platelets, causing defect in platelet function early during the CPB (18). Consequently, platelets stop reacting towards ECC and this "passivisation" may be due to FXIIa replacement with a high molecular weight kininogen (HMWK)(9). The importance of this contact activation as main coagulation stimulus in vivo has been questioned. Boisclair et al (19) found no correlation between thrombin generation (F1.2) and FXIIa levels. Furthermore, patients with severe FXII deficiency demonstrate coagulation activation while on CPB (20).

The extrinsic pathway with tissue factor-FVIIa interaction is therefore considered as a main trigger for thrombin generation in this setting. Paparella et al (21) found no difference in tissue factor production in "on pump" vs. "off-pump" surgery, underlining the importance of surgical wound as a tissue factor source. Apart from thrombin formed at the site of the wound, there is excessive systemic production of non-wound bound thrombin, a product of dysregulation of the coagulation system (17).

In short, coagulopathy in this setting is attributed to traumatic procedure, hemodilution, platelet and coagulation defects, inflammation and increased fibrinolysis.

Regarding components of plasma coagulation – clotting factors, several studies have shown decrease of factor levels immediately after initiation of CPB (22-25). In earlier studies this finding was thought to be due to per-operative consumption (22, 26), but some authors ascribed it completely to dilution effects (18, 23). Because the concentration of coagulation factors measured in cardiac surgery settings is still much higher than the one seen in single factor deficiency, this per-operative reduction is thought to be of minor importance for bleeding tendency (27). The studies vary however in clotting factors determined, patient populations, sampling timepoints, perioperative procedures and laboratory assessment methods. No trial previously has examined all coagulation factors at the same time.

Heparin

In the beginning of previous century a physiologist William Henry Howell at Johns Hopkins in Baltimore was engaged in studies concerning blood clotting. His theory was based on the existence of balance between clotting inhibitor (termed antiprothrombin) and procoagulant (termed thromboplastin). A second year medical student Jay McLean was assigned of Howell to examine chemical purity of different cephalin preparations (thromboplastin named after the site of primary isolation – canine brain). The preparation from canine liver seemed to demonstrate anticoagulant properties instead and cause bleeding in laboratory animals. Howell continued research on this phenomenon and finally isolated heparin (from Greek *hepar*) and presented his method in 1922.

The real "fathers of heparin" are however Best and Scott in Toronto, who managed to produce it in a larger scale. Erik Jorpes was introduced to their work in Canada and started later himself with heparin research (28). Among the first users of a new drug in a clinical setting was Clarence Crafoord in Stockholm, publishing results from 800 post hysterectomy cases given heparin as thromboprophylaxis in 1947 (29). The first randomized study on heparin for treatment of venous thromboembolism (VTE) was issued in 1960 (30) and the notion of APTT prolongation to 1.5-2.5 times baseline value during heparin treatment was introduced in 1972 (31).

Heparin is a linear polysaccharide, a member of heparan sulfate (HS) family of glycosaminoglycans (GAG). It is extremely complex in its structure and is heterogeneous in both sequence and size. Sulphatation grade is high, but there are even unsulphated domains and more complex structures. Heparin has been localized to secretory granules in mast cells by Hjalmar Holtgren in 1936 (32). It has even been found in hematopoietic and endothelial cells, but then with slightly different structure (33). Strong negative charge of heparin molecule secondary to sulphatation is important for interaction with other constituents in granules. Its primary function seems not to be anticoagulation but storage and retention of structures mentioned. Heparan sulfate (HS) shares the same basic structure but is less heavily sulphated and is produced by virtually all the cells of the human body.

The complete chemical characterization of any naturally occurring heparin is not possible with today's physicochemical techniques. Its molecular weight is an experimental estimation, since there are no calibrants for determination (34). The estimated molecular weight of unfractionated heparin (UFH) is thus 10000 – 20000 Da, but contains even molecules under 3000 and over 100 000 Da. Purity of preparations in clinical use is a matter of convention because of mixture of mucosal glycosaminoglycans that is used for industrial production.

In a process of establishing the 5th International Standard Unfractionated Heparin, all previous standards have been compared with the current ones (European Pharmacopoeia, United States Pharmacopoeia and Chinese) by Mulloy et al (35). During the 50 years there has been a shift from bovine to porcine mucosa sources and increase in heparin specific activity, from 140 IU/mg in 40s and 50s to today's 180 IU/mg or more. Current heparins have also less material of low molecular weight. United States Pharmacopoeia calibrated its heparin potency reference standard with the international standard issued by World Health Organization by decreasing the potency 10 %, as announced on August 21, 2009 (36).

In 1939, it was speculated by two independent authors that heparin needed yet unknown substance for its effect (37, 38). Antithrombin was discovered by Abildgaard first in 1968. Heparin's most significant anticoagulant function *in vivo* is potentiating the endogenous antithrombin action. The heparin-binding site of antithrombin (AT) binds to a specific pentasaccharide sequence in the heparin molecule, inducing conformational changes in AT, and exposing its reactive centre loop (39). This makes the interaction between AT and a certain active protease (thrombin (FIIa) for example) easier, the loop is cleaved and thrombin trapped in a covalent bound. It is essential that thrombin connect with the AT-bound heparin as well, and that is why the heparin molecule must be at least 13 polysaccharides long (which makes it 18 with a pentasaccharide sequence added) to bridge the gap between AT and FIIa molecules. AT mediated inhibition of other proteases (FXa, IXa and XIa) can occur even in presence of shorter heparin molecules, with a molecular mass < 5400. This explanation model is probably oversimplified regarding the fact that there is actually heparin octasaccharide needed for AT connection (40), antithrombin occurs in two variants, with different heparin affinity (41) and only one third of heparin molecules bind to AT (42). In that sense, Jaques (43) was right talking about

“...misgivings of the pioneers in accepting heparin as a simple anticoagulant substance”.

Moreover, heparin presence potentiates activity of heparin cofactor II (HCII), protein C inhibitor (PCI) and tissue factor pathway inhibitor (TFPI).

Protamine

Protamines are highly basic, polycationic, arginine-rich molecules, that act in a nucleus as DNA stabilizers during spermatogenesis (44). The protamine sulphate is a purified mixture of proteins obtained from the sperm of salmon species fished off the coast of Japan (previously Honshu but nowadays Hokkaido, after tsunami 2011)(45).

Its initial use was in delaying insulin absorption (46). Experiments were then made in attempt to prolong the heparin effect, but showed surprisingly heparin neutralization instead (47). Heparin and protamine react electrostatically, building an ion complex with no anticoagulant activity. The higher sulphate content in a heparin preparation the better neutralization by protamine. According to Schulman et al (48), 1 mg protamine sulphate neutralizes 90 United States Pharmacopoeia units (USP) of bovine UFH and 115 USP units of porcine origin UFH. Exact ratio is impossible to determine however, and depends on protamine and heparin preparations and the coagulation system used for testing (43). The substances have also different half-lives ($T_{1/2}$) - protamine 7 minutes and UFH 1-2 hours. Protamine is able to activate platelets and complement system, to cause histamine release from mast cells and is potentially antigenic (44).

Thrombin

Blood flow in vivo and preventing exsanguination in case of eventual endothelial damage are tightly regulated by a fine-tuned hemostatic system. One of its components is a complex network of (mostly) serine proteases,

acting in an amplification cascade, sometimes called "secondary" or "plasma" coagulation. The final product is factor IIa (thrombin), which cleaves fibrinogen to its active form fibrin, which together with a platelet plug forms a clot.

Thrombin is a serine protease with structural similarity to trypsin and chymotrypsin and is by many considered the central player in the coagulation cascade. Its precursor is prothrombin (FII), synthesized in the liver. The half-life of thrombin in plasma is physiologically 10-15 s because of a rapid inhibition by antithrombin (49). It has one active- and two exosites (I and II), and is Na⁺-dependant. Thrombin's role in the system of blood coagulation is dual because it can act both as pro- and anticoagulant. In procoagulant regard, its primary role is in conversion of fibrinogen to fibrin, but it also activates FV, FVIII, FXI and FXIII; stimulates platelets via protease-activated receptors (PARs) and glycoprotein V (GPV), binds even to GPIb α , activates (in complex with thrombomodulin (TM)) thrombin-activatable fibrinolysis inhibitor (TAFI) and finally, protects von Willebrand factor (VWF) from ADAMTS-13 action by proteolysis. Thrombin's anticoagulant function is primarily activation of protein C (PC), reaction that is greatly enhanced by thrombin binding to thrombomodulin (TM) on the intact endothelium and PC being linked to its endothelial receptor (EPCR). Activated protein C (APC) can then, in complex with its cofactor protein S (PS), inhibit FVa and FVIIIa by proteolytic cleavage. Thrombin propagation is being controlled and limited to the endothelial injury site by antithrombin action, potentiated by heparin or heparan sulfate presence. Apart from blood coagulation, thrombin seems to be involved in inflammation, angiogenesis and tissue repair, but this has been only investigated in vitro (in cell culture) or in animal models so far (50). There is certain experimental and clinical evidence suggesting that thrombin also is a mediator of myocardial ischemia-reperfusion injury (for review see (51)).

Giving the central role of thrombin in hemostasis, the ability of blood to generate adequate thrombin burst is crucial for timely forming of sufficient blood clot, especially in surgical setting. Early methods for determining thrombin generation (52) were laboursome, involving repeated manual subsamplings. As soon as small amounts of thrombin are formed (upon sample activation in a test tube) blood clotting occurs and the time needed therein comprises the result of most of routine coagulation tests (53). Further thrombin production remains thus undetected. Recently, a new

method has been described (54), allowing continuous automated detection of thrombin generation in vitro. It has been tested in assessing venous thromboembolism and bleeding risk in clotting factors deficiencies, but is still relatively unproven in surgical settings (55).

Fibrinogen

Denis de Commercy suggested existence of a fibrin precursor he termed fibrinogen in 1859. It was purified from horse plasma by Olof Hammarsten in Uppsala twenty years later (56). Fibrinogen is a glycoprotein synthesized by hepatocytes and secreted as assembled hexamer $(A\alpha B\beta\gamma)_2$, with a half-life in plasma of approximately 120 hours (57). It is the only clotting factor with concentration measured in grams and next to immunoglobulins and albumin the most abundant protein in plasma.

Apart from its role in coagulation, fibrinogen seems to be linked to inflammation, cancer, host defense and neuropathology (58). Under controlled conditions in vitro, increasing concentrations of fibrinogen, thrombin or both makes blood clot more densely-woven and homogeneous in architecture.

Lately, several experimental, animal and human studies on fibrinogen concentrate have been published (59). Current European trauma treatment guidelines recommend treatment with fibrinogen concentrate in case of bleeding accompanied with functional fibrinogen defect or low levels (60). In small randomized clinical trials it was demonstrated that prophylactic use of fibrinogen decreased transfusion rate and postoperative bleeding in urologic (61) and cardiac surgery population (62). In some countries fibrinogen products are already granted not only for hereditary a- and hypofibrinogenemia but even for acquired deficiency (63). However, all fibrinogen concentrates available today are plasma derived and, in spite of several viral elimination steps during production, cannot be considered completely safe regarding eventual infectious risk, especially concerning non-lipid-enveloped viruses (entero-, circo-, parvo- or polyomavirus) and prions (64, 65). In a hypothetical model of occurrence of a new pathogen resistant to inactivation, there is 1000-fold greater risk for exposure using

fibrinogen concentrate in comparison to cryoprecipitate (66). That is why manufacturers, physicians, regulatory agencies and public are showing a great interest in developing products completely free from human or animal proteins, in any part of manufacturing process (67).

Aims of the study

- (1) To investigate activity of individual coagulation factors in cardiac surgery with cardiopulmonary bypass (CPB) in relation to hemodilution and bleeding volume after surgery (**Paper I**)
- (2) To investigate the thrombin generation capacity after cardiac surgery with CPB in correlation to reduction in activity of individual coagulation factors (**Paper II**)
- (3) To investigate standard weight-based heparin dosing compared to dosing using heparin protamine titration regarding thrombin generation capacity in cardiac surgery with CPB (**Paper III**)
- (4) To investigate ex vivo functional properties of fibrinogen derived from human plasma and recombinant fibrinogen derived from a human cell line, in blood samples from cardiac surgery patients (**Paper IV**)

Material and methods

Ethics

All studies were approved by the human ethics committee at the Sahlgrenska Academy at University of Gothenburg. Patients were included after obtained written informed consent. The investigations were undertaken at the Department of Cardiothoracic Surgery at Sahlgrenska University Hospital, Gothenburg, Sweden.

Patients

Paper I and II

Originally, 59 adult patients (age > 18 years) undergoing first time elective CABG with cardiopulmonary bypass were enrolled in a prospective observational study between September 2007 and February 2008. Two were excluded eventually (one because of change in surgical approach and the other one because of ongoing clopidogrel treatment). In Paper II further nine patients were omitted in analysis due to missing thrombin generation measurements. Predefined exclusion criteria were acute operation, known bleeding disorder and ongoing treatment with clopidogrel, warfarin or low molecular weight heparin (LMWH). All patients had ongoing therapy with acetylsalicylic acid.

Paper III

Initially two hundred thirty four (234) adult patients were assessed for eligibility. One hundred seventy four did not meet the inclusion criteria or were excluded for other reasons. Finally, sixty (60) elective, first time CABG or valve replacement surgery patients were included in the trial. The study participants were randomized (using opaque envelopes) to either heparin and protamine titration using the heparin dose response

(HDR) curve (Intervention) or weight based heparin and protamine dosing treatment, guided by activated clotting time (ACT) (Control). Predefined exclusion criteria were acute operation, known bleeding disorder, liver or kidney disease, previous stroke and on-going treatment with P2Y₁₂ receptor antagonist. Surgeons and intensive care unit (ICU) personnel were blinded to the randomization.

Paper IV

Ten elective first time CABG patients, with ongoing acetylsalicylic acid therapy were included. All had preoperative fibrinogen concentration < 3.5 g/L and APTT, PT INR and platelet count within the reference range.

Clinical management

During surgery, anesthesia was induced with 200-300 µg of fentanyl and 3-5 mg/kg of thiopentone (or 1-2 mg/kg propofol), followed by 0.1 mg/kg procuronium and maintained with sevoflurane. During CPB, anesthesia was maintained with propofol. The CPB circuit included a phosphorylcholine coated membrane oxygenator and roller pumps. Standard non-pulsatile CPB technique with moderate hypothermia (bladder temperature 34-36°C) or normothermia (bladder temperature 36-37°C), and hemodilution was used. The CPB circuit was primed with 1500 ml of Ringer-Acetate (Fresenius Kabi AB, Uppsala, Sweden) and 200 ml of Mannitol (150 mg/ml)(Fresenius Kabi AB). Cardioprotection was achieved with intermittent antegrade cold blood cardioplegia. Weaning off CPB was performed after rewarming to a bladder temperature of 36°C. The patients received 350 units UFH per kg body weight. In addition, 10 000 IU UFH was added to the priming solution. Heparin monitoring intraoperatively was performed by standard ACT (HEMOCHRON Jr. ACT+ [ITC, Edison, NJ]). After CPB, the heparin was reversed by administration of protamine sulfate (1mg protamine/100 units of the initial heparin dose).

In the Intervention group in Paper III, HEPCON Hemostasis Management System Plus device (Medtronic Inc, Minneapolis, Minnesota) was used,

according to manufacturer's recommendations. After estimating the patients' blood volume and individualized heparin sensitivity (HDR), the initial bolus heparin dose, heparin concentration and ACT were determined using a six channel cartridge (two channels with heparin concentration 2.5 U/ml, two with heparin concentration 1.5 U/ml and two without added heparin). Eventual bolus doses of heparin were estimated (and given) every 30 min throughout the surgery, in order to maintain target ACT above 480 s. At the end of the CPB, the protamine dose required for heparin neutralization was also established using the device and the effect controlled by additional ACT check 10 min after weaning.

Two grams of tranexamic acid was administered at anesthesia induction and at the end of the surgery in all patients. Aprotinin was not used.

Study design

The following perioperative variables were registered: age, gender, weight, body mass index (BMI), Euroscore, systolic ejection fraction, preoperative medication, number of grafts, CPB time and aortic clamp time (Papers I-III). Intraoperative blood loss was calculated by the operation nurse based on waste suction volume and number of sponges used (Paper III). Postoperative bleeding was assessed as total amount of chest tube drainage during the first 12 postoperative hours. Blood samples were collected at three time points: the day before surgery and 2 and 24 hours after (Paper I and II) or at four time points (Paper III): preoperatively, 10 minutes after weaning from CPB and 2 and 4 hours after surgery. The samples were drawn from an antecubital peripheral vein (Paper I and II - before surgery) and nonheparinized arterial line (Paper I and II - after surgery), while in Paper III, preoperatively placed central venous line was used at all time points. For all sampling the first 10 ml of blood was discarded. Specimens were collected in sodium citrate tubes (0.13M, 9 parts blood, 1 parts sodium citrate), and centrifuged at 2000 g for 20 min. The supernatant was filled in separate tubes and frozen at -70°C until further analysis. The samples for thrombin generation capacity studies were additionally centrifuged at

10000g for 10 min in order to obtain platelet poor plasma (PPP). ROTEM analyses were done on citrated fresh whole blood specimens.

In Paper IV arterial whole blood samples (10 ml) were collected before anesthesia and 30 min after heparin reversal. Increasing doses of recombinant or plasma-derived fibrinogen were added to the postoperative samples. Each study sample consisted of 1.2 mL of whole blood. Phosphate buffered saline (PBS) in various doses was used to maintain the same hemodilution in all study samples. Measurements were performed pre-operatively (with and without the addition of PBS) and postoperatively without any additives and with the addition of: 1.) 180 μ L PBS only (baseline for effect studies of fibrinogen), 2.) 60 μ L fibrinogen (either plasma derived or recombinant) + 120 μ L PBS, 3.) 120 μ L fibrinogen + 60 μ L PBS and 4.) 180 μ L fibrinogen + 0 PBS. All analyzes were performed in duplicate. The chosen doses increase the fibrinogen plasma concentration with 0.5, 1.0 and 1.5 g/L, respectively.

Analyses

All analyses, except CAT in study II, ROTEM and activated clotting time (ACT) were performed at coagulation laboratory at Sahlgrenska University Hospital. Hemoglobin, hematocrit and platelet count were measured using routine laboratory techniques. Activated partial thromboplastin time (APTT) was measured with STA APTT reagent containing cephalin as a source of phospholipids and silica as activator (reference range 30-42 seconds).

Fibrinogen (reference range 2.0-4.5 g/L) was measured by the modified method of Clauss. Factor II (FII) (reference range 70-130%), FV (reference range 60-140%), FVII (reference range 50-160%), FVIII (reference range 50-200%), FIX (reference range 45-190%), FX (reference range 70-130%) and FXI (reference range 60-140%) were determined using one stage clotting assays with specific factor deficient plasma samples on the instrument STA-R (Diagnostica Stago, Asnieres, France). Activity of FXIII (reference range 70-140%) was measured by a photometric method on the instrument Cobas Mira (Roche, Basel, Switzerland). Coagulation

factor activity is reported both as absolute value and value adjusted for hemodilution according to the formula: adjusted activity = absolute activity \times (preoperative hematocrit/actual hematocrit) (68).

F1.2 and TAT were measured using an enzyme-linked immunosorbent assay technique with commercially available tests (Enzygnost[®] F1+2 micro and Enzygnost[®] TAT, Dade Behring, Marburg Germany). The reference ranges are 70 – 230 pmol/L for F1.2 and 1.0 – 4.1 μ g/L for TAT. Anti-Xa activity was assessed by quantifying residual factor Xa activity by cleavage of a chromogenic substrate (COATEST[®] Anti Xa Heparin, Chromogenix/IL, Italy, Milano)(Paper I and II) and (STA[®]-Liquid Anti Xa, Diagnostica Stago) (Paper III), both reference value < 0.05 kIU/L. Determination of thrombin time (TT) was done by standard commercial STA Thrombin test (Diagnostica Stago), adding thrombin to patient plasma and measuring the clotting time (reference range 14–21 seconds).

Antithrombin was measured by chromogenic substrate method (STA[®]-STACHROM[®] AT III, Diagnostica Stago) (reference range 0.8 – 1.2 kIU/L). All analyses were performed on STA-R coagulometer (Diagnostica Stago).

Thrombin generation capacity

The Calibrated Automated Thrombogram (CAT) assay was performed in round bottom 96-well plates (Greiner microlon, U-shaped, high binding, USA or Immulon 2HB transparent U-bottom 96-well, ThermoFisher). Citrated plasma samples (80 μ l) and trigger solution (20 μ l) (PPP reagent Cat# TS30.00, Thrombinoscope, Maastricht, The Netherlands) containing 1 (Cat# TS30.00) (Paper II) or 5 pM tissue factor (Cat# TS31.00) (Paper III) were mixed in sample wells. In parallel, a calibrator, (Cat# TS20.00 Thrombinoscope), was analyzed by mixing 20 μ l of thrombin calibrator and 80 μ l test plasma in wells coupled to the sample wells. In addition, two controls were used on each plate, one with normal pooled plasma and the other with warfarin treated plasma with PT INR 2.0. Then the plate was moved to a fluorometer (Ascent reader and Fluoroskan Ascent FL, both Thermolabsystems OY, Helsinki, Finland) and 20 μ l of FluCa solution (Cat# TS50.00 Thrombinoscope) containing fluorogenic

substrate and CaCl₂ was dispensed by the instrument. The fluorogenic signal was measured at λ_{ex} 390 nM, λ_{em} 460 nM during 60 min. Start of thrombin activity (lag time), time to peak thrombin activity (TTP), peak thrombin activity (peak), and total endogenous thrombin potential (ETP) were calculated using the software Thrombinoscope (version 3.0.0.29) from Thrombinoscope BV (Maastricht, Netherlands).

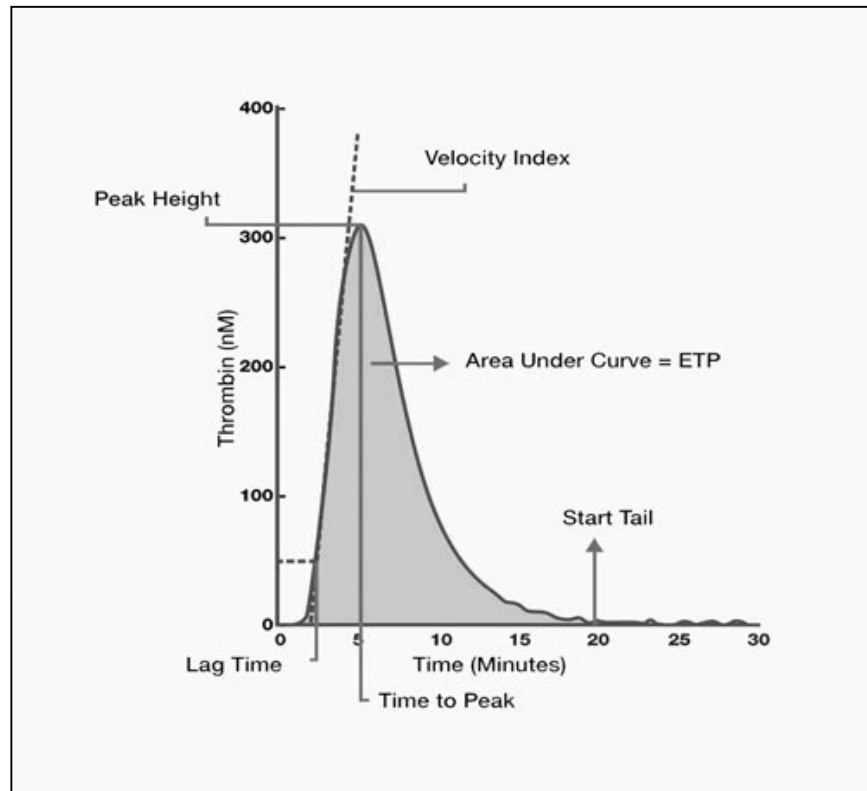


Figure 1. Calibrated automated thrombogram (with permission from the manufacturer).

Rotational thromboelastometry

Rotational thromboelastometry was performed on ROTEM[®] delta instrument (TEM International, Munich, Germany) using 300 μ l citrated whole blood, with previously described technique (69). Assays using contact activation (INTEM), contact activation with addition of heparinase in order to neutralize the heparin effect (HEPTEM), tissue factor activation (EXTEM) and tissue factor activation with platelet inhibition to assess the fibrinogen status (FIBTEM) were run on all

samples. The results are presented in graphical form, showing ROTEM parameters: clotting time (CT), clot formation time (CFT), alpha-angle (alpha), amplitude 10 min after CT (A10), maximal clot firmness (MCF), lysis index 30 min after CT (LI30) and maximal lysis (ML).

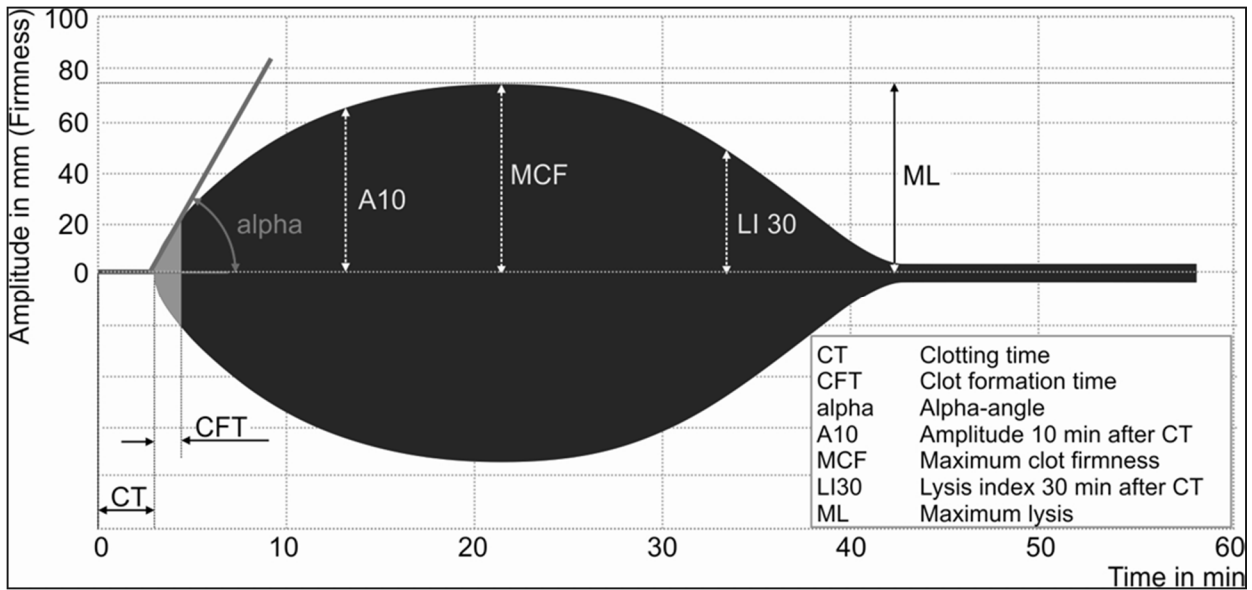


Figure 2. Rotational thromboelastometry (with permission from manufacturer).

Fibrinogen

Human plasma-derived fibrinogen (Haemocomplettan®; CSL Behring, Marburg, Germany) and wild-type recombinant human fibrinogen (ProFibrin, Leiden, The Netherlands) were dissolved to 10 mg/ml in PBS and frozen until analysis. Wild-type recombinant fibrinogen was produced using the human PER.C6® cell line (Paper IV).

Statistics

Results are expressed as mean and standard deviation (SD) or number and percent (%). Statistical significance was defined as a p value <0.05. Since bleeding was not normally distributed, all statistical analyses involving bleeding were performed with non-parametric tests. Intergroup comparisons were performed with Mann-Whitney test, Kruskal–Wallis test or Chi-square-test, when appropriate. Correlation testing was performed with Pearson's test (normally distributed data) or Spearman rank sum test. Correlation between coagulation factor activity and postoperative bleeding volume was performed on absolute activities, without correction for hemodilution (Paper I). Coagulation factor activity after surgery (normally distributed) was compared to baseline with paired T-test.

In Paper III, intra- and inter-group differences between control and intervention group, with respect to CAT, ROTEM parameters, antithrombin, anti-FXa, ACT, hemoglobin, platelet count, PT INR, APTT and TT were compared using analysis of variance (ANOVA) for repeated measurements. If the ANOVA analysis group or interaction between group and time indicated a significant difference ($p < 0.05$), student-T-test was used to test the different time points. STATISTICA 10 software was used for statistical analyses (StatSoft, Tulsa, OK, USA).

In Paper IV, the effects of recombinant and plasma-derived fibrinogen were compared with a general linear model using dose, treatment and dose x treatment as fixed effects.

Results

Paper I

Patient characteristics (n=57) are given in Table 1.

Age	65±7
Male gender	44 (77%)
BMI	27±3,4
Euroscore	2.9±3.1
ECC (min)	72±27 (40-187)
Clamptime (min)	44±17 (23-112)
Number of grafts	3.3±0.9
Preop ASA	57 (100%)
Hemoglobin (g/L)	148±14
Platelet count (per L)	279±64 x 10 ⁹
Hematocrit	0.44±0.04

Table 1. Patient characteristics, study I.

All patients could be discharged from hospital without serious complications. Median postoperative bleeding was 380 ml (range 150-1560 ml).

Apart from one patient with subnormal FX concentration (68%, lower normal limit 70%), all others had coagulation factor activity within normal reference range preoperatively.

At two and twenty four hours after surgery there was pronounced variation in coagulation factor levels (Table 2).

	Before surgery	2 h Postop	24h postop
Fibrinogen (g/L)			
Absolute	3.7±0.9	2.5±0.7***	3.9±0.7*
Adjusted		3.1±0.7***	5.2± 1.0***
FII (%)			
Absolute	108±12	78±13***	79±11***
Adjusted		99±13***	106±20
FV (%)			
Absolute	111±19	76±18***	81±16***
Adjusted		96±20***	106±19*
FVII (%)			
Absolute	121±31	98±28***	57±20***
Adjusted		124±32	76±27***
FVIII (%)			
Absolute	194±66	175±59*	263±76***
Adjusted		221±70**	347±95***
FIX (%)			
Absolute	149±24	143±30	146±26
Adjusted		180±29***	194±38***
FX (%)			
Absolute	104±18	72±17***	70±14***
Adjusted		91±18***	93±20***
FXI (%)			
Absolute	117±25	91±19***	83±18***
Adjusted		115±18	110±24*
FXIII (%)			
Absolute	135±30	97±26***	94±19***
Adjusted		122±31***	125±26**

Table 2. Coagulation factor concentrations before surgery and at 2 and 24 hours after surgery (absolute and adjusted), *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$ vs before surgery.

Mean hematocrit fell from 44.1 ± 4.0 % preoperatively to 34.7 ± 3.9 % two hours after surgery and 33.7 ± 3.6 % after 24 hours (both $p < 0.001$ vs preoperative).

After adjustment for hemodilution, measurements at two hours after surgery showed increase in FVIII and FIX, unaltered FVII and FXI and decrease in fibrinogen, FII, FV, FX and FXIII, Figure 3.

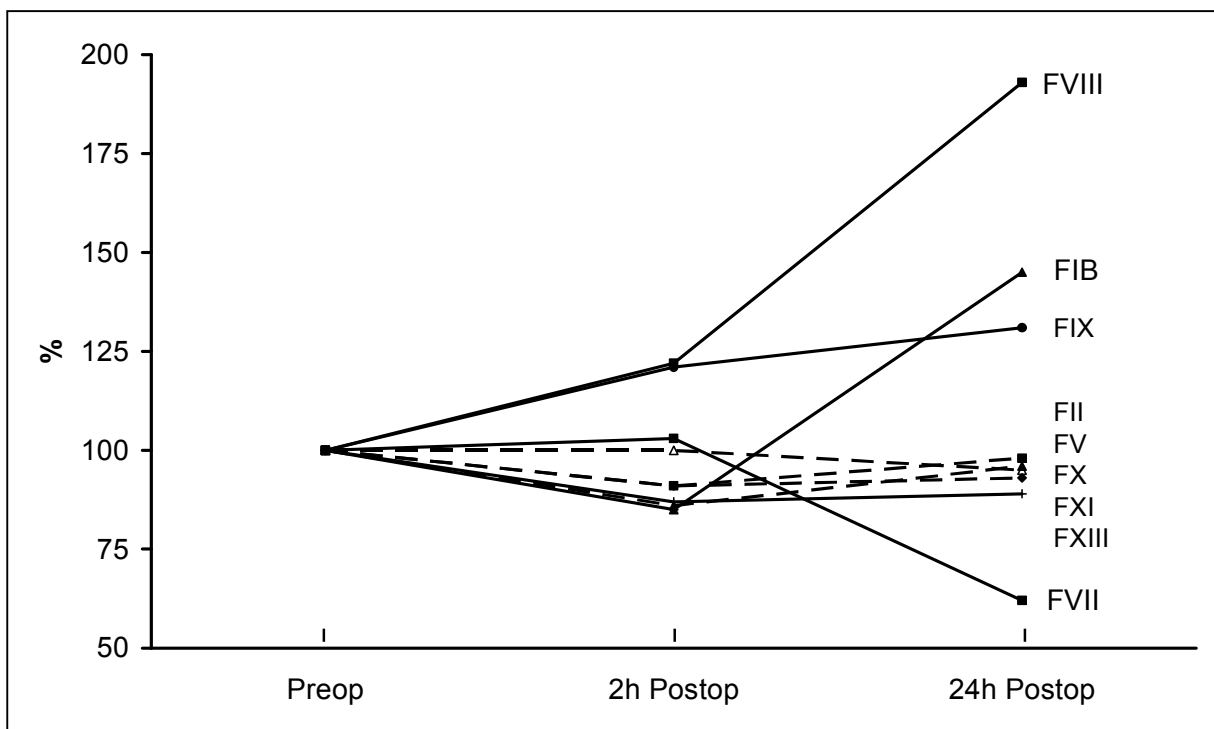


Figure 3. Plasma concentration of coagulation factors before, and 2 and 24 hours after surgery (adjusted for hemodilution), in percents of preoperative value.

At 24 hours, factor levels adjusted for hemodilution demonstrated increased fibrinogen, FVIII and FIX, unchanged FII and decreased FV, FVII, FX, FXI and FXIII, all compared to baseline.

The only correlations between absolute factor level and volume of postoperative blood loss were the weak ones with fibrinogen at 2 hours

postoperatively ($r = -0.33$, $p = 0.019$) and FXIII, both pre- ($r = -0.34$, $p = 0.009$) and postoperatively ($r = -0.41$, $p = 0.003$).

Postoperative bleeding volume did not correlate to any other clinical or laboratory preoperative parameters.

Paper II

Patients were the same cohort as in Paper I, with seven patients excluded.

Calibrated automated thrombography variables were markedly altered 2 h after surgery compared to the preoperative values, Figure 4. Median lag time and time to peak increased with 162% (102–261) (interquartile range) and 98% (61–138), respectively (both $p < 0.001$), whilst peak levels and ETP decreased with 74% (54–91) and 65% (43–86), respectively (both $p < 0.001$). Twenty-four hours after surgery there were statistically significant shorter lag time and time to peak and higher peak level than at baseline (all $p = 0.001$), while ETP did not differ significantly.

Ten patients had >90% reduction in ETP 2 h after surgery. These patients showed higher median anti-Xa activity (0.18, range 0.13–0.37 kIU/L, versus 0.06, range 0.04–0.08 kIU/L, $p = 0.038$), longer thrombin time (24, range 19–34 s, versus 17, range 16–18 s, $p = 0.021$) and longer APTT (44, range 38–47 s, versus 34, range 32–38 s, $p = 0.011$) in comparison to patients with <90% reduction in ETP.

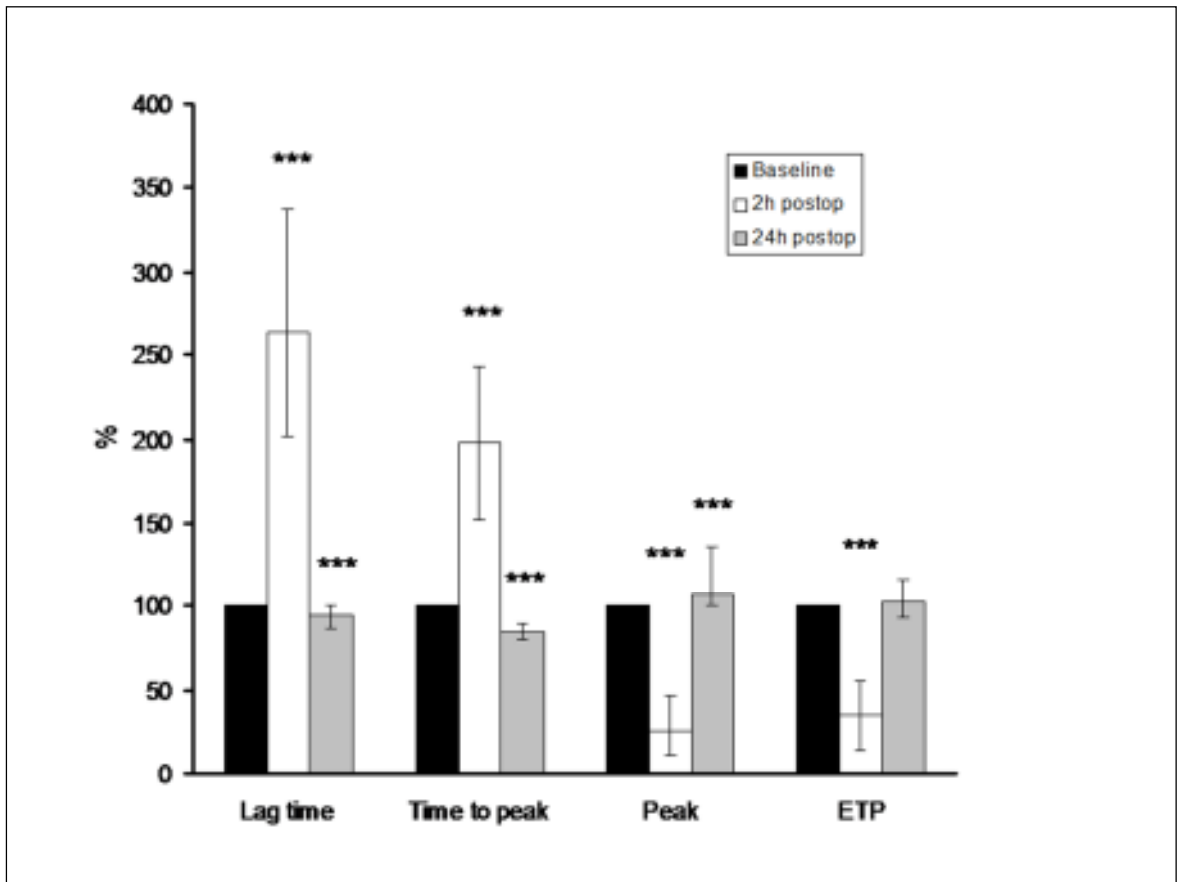


Figure 4. Changes in percent from baseline in CAT parameters. Median values and interquartile range. ***= $p < 0.001$.

There were moderate correlations between ETP and anti-Xa ($r = -0.50$, $p = 0.01$), ETP and thrombin time ($r = -0.42$, $p = 0.037$) and ETP and APTT ($r = -0.44$, $p = 0.027$), all at two hours after operation.

Median postoperative bleeding (380 ml, range 160-1520 ml) did not correlate with CAT variables at any point. In contrast, postoperative bleeding volume correlated to TAT ($r = 0.57$, $p < 0.001$) and F1.2 ($r = 0.56$, $p < 0.001$) two hours postoperatively.

Paper III

All of the study participants completed the study. There was no difference between Intervention group and control group in any of the baseline variables, inclusive platelet count, PT INR, APTT, hematocrit and antithrombin.

	Control group (n=30)	Intervention group (n=30)	p
Valve surgery (n)	5 (17%)	2 (7%)	0.23
Total protamine dose (mg)	314 ± 58	319 ± 96	0.78
Total heparin dose (IU)	37167 ± 11573	37150 ± 8734	0.99
Clamp time (min)	57 ± 4	44 ± 3	0.009*
ECC time (min).	87 ± 6	69 ± 4	0.023*

Table 5. Intra- and postoperative characteristics.

Despite randomization, aortic clamp time and ECC time were significantly longer in control group than in the intervention group, reflecting more valve surgery patients in that group, Table 5.

The intervention group received higher bolus dose heparin ($p=0.009$), but the total heparin dose did not differ between the groups ($p=0.99$). Fourteen patients in control- and twelve patients in intervention group received extra heparin during surgery. One patient in each group did not reach ACT 480 s after the initial heparin bolus dose. The total protamine dose did not differ significantly ($p=0.78$).

There was a significant reduction in thrombin generation capacity in both groups postoperatively compared to the preoperative measurements but to a similar degree in both groups (Figure 5). This change was significant in all CAT variables (lag time, time to peak, peak value and ETP). The lowest ETP was registered 4 hours after CPB in both groups. Time to peak (TTP) was significantly longer in the intervention group at 2 and 4 hours after surgery but no other statistically significant difference between the groups was observed.

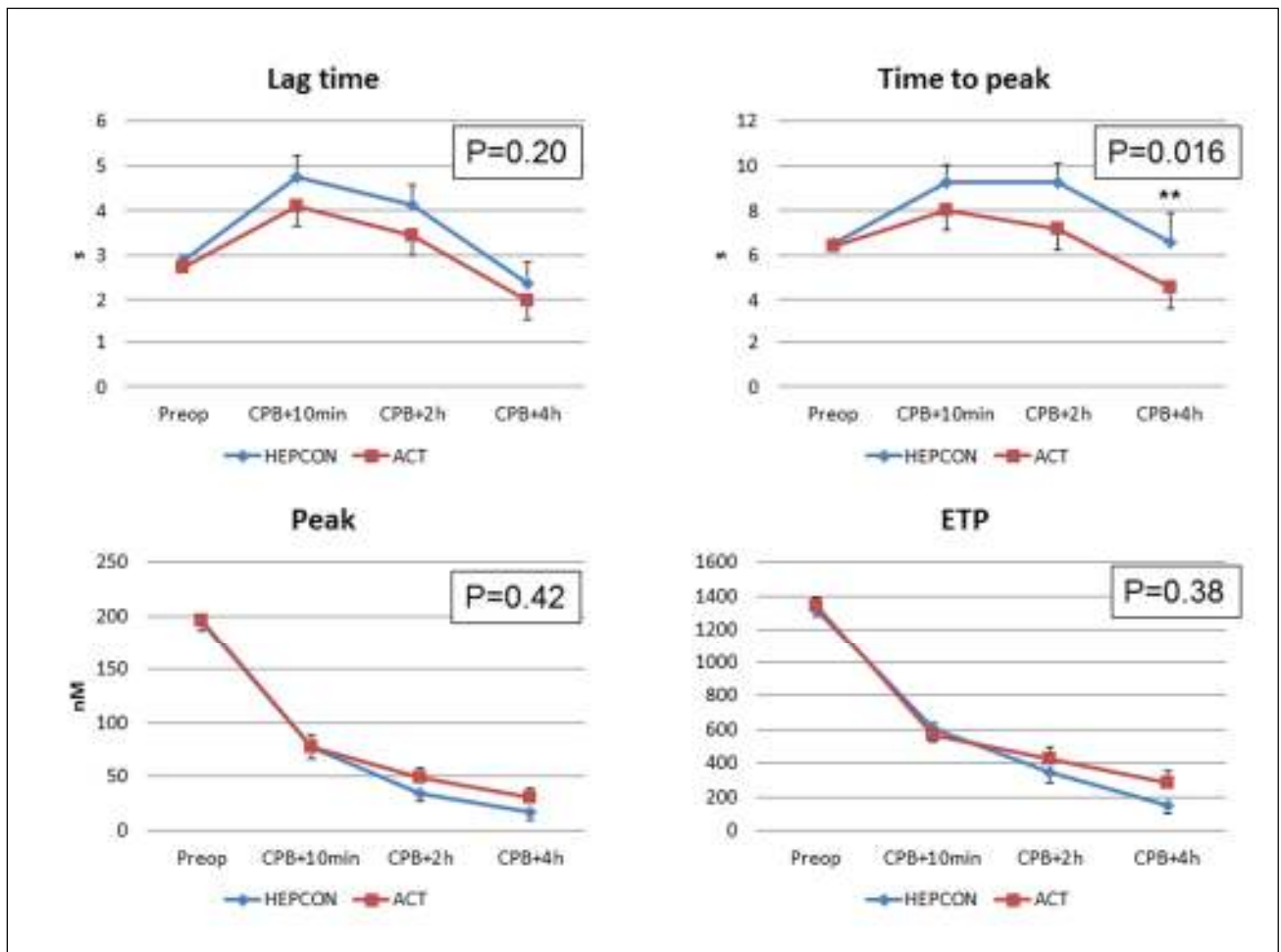


Figure 5. CAT variables between the groups at four time points. Mean and standard error of mean. P value for group comparisons with repeated measures ANOVA.

The heparin effect measured as anti-Xa was present in both groups, with no significant difference (Figure 6). There were significant inverse correlations between anti Xa levels and ETP 10 min ($r = -0.43$, $p = 0.001$), 2 hours ($r = -0.66$, $p < 0.001$) and 4 hours after surgery ($r = -0.58$, $p < 0.001$). Postoperative INTEM/HEPTM ratio correlated also with ETP levels, two ($r = 0.44$, $p = 0.001$) and four hours ($r = 0.44$, $p = 0.001$) after surgery.

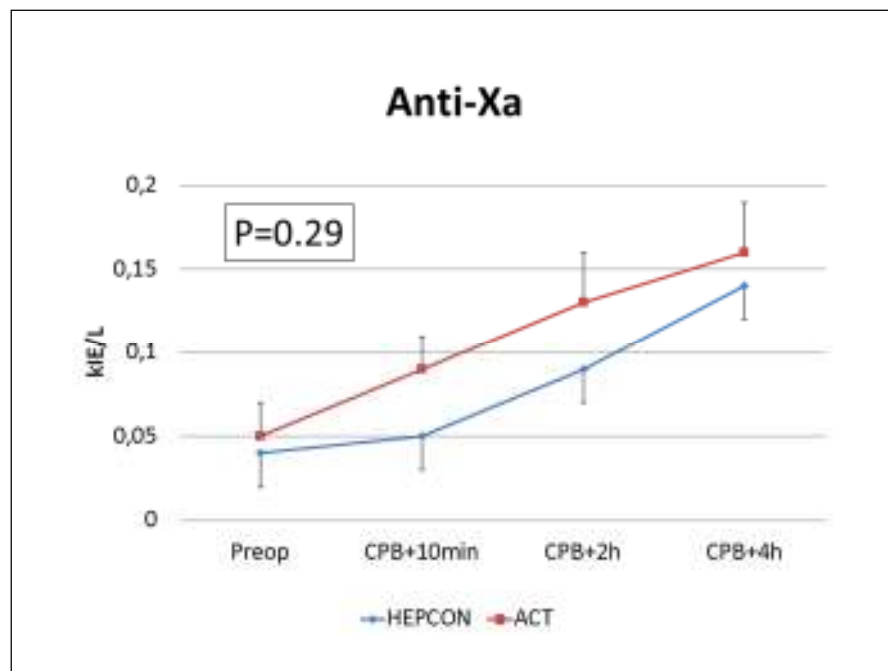


Figure 6. Anti-Xa levels in intervention and control group at four time points. Mean and standard error of mean. P value for group comparison with repeated measures ANOVA.

The groups did not differ in per- or postoperative bleeding, transfusion rate, ICU stay or time spent on mechanical respiratory support.

Paper IV

The median preoperative and postoperative fibrinogen plasma concentration was 2.9 g/L (range 1.6–3.4) and 2.0 g/L (range 1.3–2.8) respectively, $p=0.005$. There were no significant differences between recombinant and plasma-derived fibrinogen in any of the thromboelastometric analyzes at any concentration, Figure 7.

EXTEM-CFT decreased dose dependently after the addition of both plasma-derived and recombinant fibrinogen ($p=0.031$ and $p=0.007$ for trend, respectively), whereas EXTEM-MCF ($p<0.001$ both), EXTEM- α ($p=0.002$ and $p<0.001$, respectively), FIBTEM-A10 and FIBTEM-MCF ($p<0.001$ all) increased dose dependently. EXTEM-CT, FIBTEM-CT, FIBTEM- α and FIBTEM LI30 did not change significantly with any of the fibrinogens.

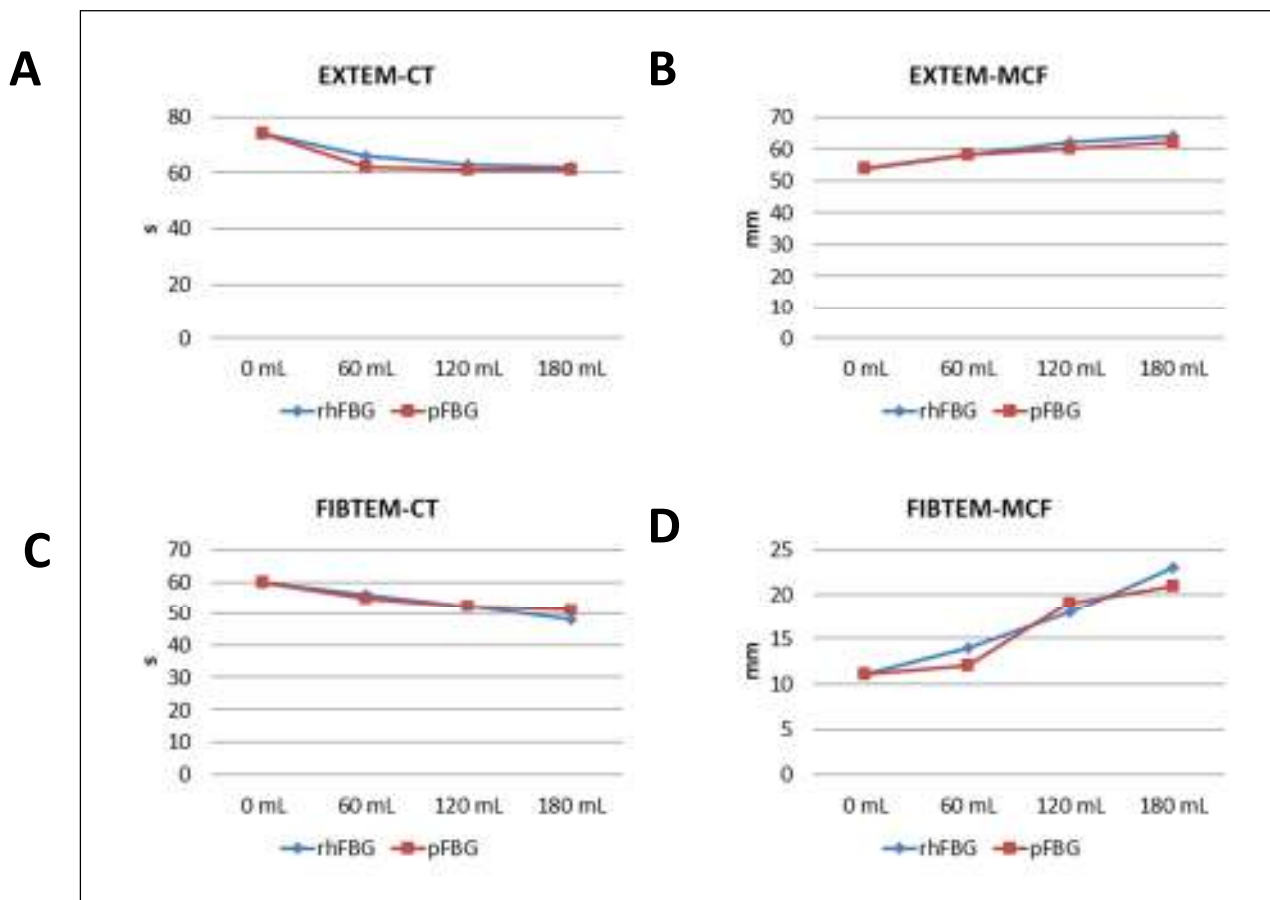


Figure 3. EXTEM-CT (A), EXTEM-MCF (B), FIBTEM-CT (C), FIBTEM-MCF (D)

Discussion

Cardiac surgery with cardiopulmonary bypass (CPB) induces profound alteration of the whole hemostatic system. Plasma coagulation may contribute to perioperative coagulopathy but also hemodilution, and platelet dysfunction may be equally or more important (17, 18). However, most of the studies measuring coagulation proteins during cardiac surgery are of older date (18, 22-24, 26), reflecting, in some ways not actual clinical and perfusion management and laboratory methods. That is why we conducted study I, which aimed to prospectively determine levels of all coagulation factors pre- and postoperatively in a modern surgery setting.

The absolute levels of all factors measured (apart from FIX) decreased significantly at two hours after surgery (-4 to -32% of preoperative values), to an extent greater than the change in hematocrit level solely, implying a certain consumption mechanism perioperatively. After all, CPB is known to induce massive thrombin generation (70). During the postoperative course, the hemostatic balance shifts to a procoagulant state, with increases in fibrinogen, FVIII and FIX levels. Although the other factors are still statistically lower than baseline, this difference is small, apart from more pronounced decrease in FVII. This decrease could theoretically be due to a FVII's short half-life and its rapid recruitment by huge amounts of tissue factor, both wound bound and circulating. The observed variation in factor concentrations does not seem to be directly associated with postoperative blood loss. The only two factors that correlated to chest drainage volume at 12 hours were postoperative fibrinogen and pre- and postoperative FXIII.

The correlation between fibrinogen concentration and postoperative bleeding has previously been demonstrated by our group (71) and others (72). Furthermore, prophylactic substitution with fibrinogen concentrate reduces the blood loss (62). In a recent meta-analysis on use of fresh frozen plasma (FFP) or fibrinogen in perioperative and massive trauma settings, the latter seemed to have superior effects regarding blood loss, transfusion rate and duration of ICU- and hospital stay (73). Fibrinogen substitution is also known to compensate for hemodilution (74) and thrombocytopenia (75) in animal

models. There are several ongoing trials investigating fibrinogen concentrate use in heart surgery (76).

Findings for FXIII are more contradictory in regard to bleeding, with some authors reporting relation to bleeding volume (77, 78), while others not (79). A recent study on substitution with recombinant FXIII before surgery failed to show any benefit concerning transfusion rate (80). This endpoint is however elusive and in spite of predefined criteria, transfusion practice in the trial varied between the study centers.

There is massive thrombin production during the course of cardiac surgery with CPB (17, 70) with typical bursts immediately after going on CPB and after reperfusion of the ischemic heart (51, 81). Indirect markers of already formed thrombin (such as F1.2, D dimer, TAT complex) have predominately been used in this research. New automated technique for assessing plasma's potential to form thrombin has made it potentially easier to study thrombin generation in surgical setting in real time. By this method, both procoagulants and anticoagulants in plasma are taken into account and plasma's ability to trigger thrombin formation can be quantified (54). However, there are still problems with intra- and inter-individual variation (82) and lack of standardization (83), just to name some.

In conducting the study II we speculated that decreased thrombin generation potential (measured by CAT), would be found postoperatively and that it would be associated with decline in concentration of individual coagulation factors. Indeed, the results demonstrated a substantial drop in plasma ability to generate thrombin two hours after surgery. This finding is in accordance with some other studies (25, 84-86), but in contrast with others (87, 88), perhaps due to different sampling time points, overall study design and modifications in laboratory technique. The concentration of tissue factor (TF) is of importance for the test result. We deliberately used low TF concentration (1 pM) in order to improve sensibility of the assay. According to the recommendation of the manufacturer, the reagent containing 20 pM TF should be used in plasma containing UFH, but various concentrations have been utilized in research. Hemker's group reported recently applying 30 pM TF while testing the cardiac surgery population (89). Furthermore, heparin presence is actually not expected, after the protamine neutralization postoperatively.

In our study, there was no correlation between CAT variables and changes in individual coagulation factor activity. In search for plausible explanation,

samples were then tested in respect to heparin effect, showing association between anti Xa, APTT and thrombin time assays and CAT parameters, thus implicating inadequate neutralization by protamine and/or heparin rebound. Increased anti-Xa levels in spite of heparin neutralization have been described previously (90-92). CAT results in study II did not correlate to postoperative bleeding volume at any time point.

In light of these results, the study III was designed (in prospective, randomized manner), in order to optimize per-operative heparin dosing and heparin-protamine ratio and in that sense improve plasma capacity to generate thrombin. As the counterpart to the widespread weight based heparin dosing in the control group, Hepcon HMS device was used in the intervention group, assessing individual sensitivity to heparin.

In spite of this intervention however, the findings were similar to those in our previous study, with clear postoperative deterioration of CAT variables, caused probably by heparin effect, as determined with anti Xa, TT and INTEM/HEPTEM ratio. There was no significant difference between the study groups in this regard. Furthermore, similar amounts of heparin and protamine were used during and after surgery. The groups did not differ in postoperative bleeding, nor did they in any other clinical parameter. Our population was however undersized concerning the clinical endpoints.

These results are in contrast with some of the other investigations, which demonstrated increased heparin and lower protamine doses when guided by heparin dose response curve (93, 94). That change in given heparin and protamine dose should imply a hypothetical advantage in terms of more potent anticoagulation during surgery and at the same time reduction of the negative effects of protamine overdose on platelet function. Trials in the matter show great variability, making it very difficult to make direct comparisons.

Regarding the decrease in blood loss after surgery by using protamine titration, the results are conflicting (95). The most recent meta-analysis found beneficiary effects of this technique (96). Data in that study were obtained from four studies, comprising 20 to 247 patients per trial. Each of the trials described used different protamine dosing regimen in the control group.

In summary, the more expensive and labor intensive titration technique has in our hands shown no benefit compared to a standard weight-based approach.

There has been a revival of fibrinogen as a crucial coagulation factor during major trauma and surgery. Several guidelines for treatment of trauma have revised the previously recommended fibrinogen threshold of 1 g/L. The predominant means for fibrinogen substitution today are cryoprecipitate and plasma derived fibrinogen concentrate (pdFGN). While cryoprecipitate is still the treatment of choice for acquired hypofibrinogenemia associated with major trauma, surgery or postpartum hemorrhage in USA and United Kingdom, in many European countries purified pdFGN is used instead (97). This product has recently been licensed for patients with congenital a- and hypofibrinogenemia also in USA.

Large pools of donor plasma are used in production and it takes up to nine months from plasma donation to the final product. According to a recent WHO report (98), there are 92 million blood donations worldwide annually. In 39 countries (out of 159 reporting) blood donations are still not routinely tested for transfusion transmissible infections. On the other hand, plasma derived fibrinogen concentrates in the western world have proven extremely safe, and some 3 million grams of fibrinogen have been administered in Europe since 1985 without viral transmission (97). However, theoretical potential for transmitting the new infectious agents remains possible.

Regarding plasma, majority of donations comes from 400 centers across US (99). Giving the fact that manufacturers produce plasma products for the worldwide market, a possible shortage in any product causes domino effect on the worldwide supply. Just four months after licensing the pdFGN in Canada, the demands increased so dramatically that Canada Blood Service was forced to issue an inventory alert on June 10, 2013 (100), due to the fact that the current inventory would last only for another three weeks. The use of fibrinogen concentrate is increasing in US as well. According to (101), the amount of fibrinogen needed to satisfy 75% of US annual demand (just for topical administration, as a tissue sealant) is 2650 kg.

Taken all together, the study IV is of interest, investigating for the first time functional effects of recombinant human fibrinogen. Three different concentrations of plasma derived and recombinant fibrinogen, targeted to increase plasma fibrinogen by 0.5; 1 and 1.5 g/L, were tested with thromboelastometry (TEM) on blood samples collected from cardiac surgery patients. This method has previously been proven for monitoring fibrinogen substitution (63, 102, 103). Comparable ex vivo results on fibrin specific and

tissue factor activated whole blood clot formation were demonstrated. Similar findings regarding the viscoelastic properties of a blood clot were subsequently reported by another group, which used different form of recombinant fibrinogen, the one obtained from the milk of transgenic cows (101). Recombinant fibrinogen concentrates remain to be tested in a future research, in larger scale studies. At present, it is hard to predict which manufacturing method will prevail (if any).

Summary

- (1) Concentration of plasma coagulation factors varies after cardiac surgery with CPB. Apart from FVIII and FIX, all others decrease, secondary to hemodilution and probably consumption. Factors engaged in blood clot stability (fibrinogen and FXIII) are the only ones demonstrating association with postoperative bleeding volume.
- (2) Thrombin generation capacity as measured with calibrated automated thrombography (CAT) decreases profoundly early after cardiac surgery due to sustained heparin effect.
- (3) Heparin/protamine titration technique does not improve thrombin generation capacity in plasma at two and four hours postoperatively.
- (4) Similar effects on clot stability ex vivo were shown after addition of recombinant human fibrinogen concentrate and plasma derived fibrinogen concentrate.

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