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RĪGAS STRADIŅA UNIVERSITĀTE

Agnese Ozolina

INFLUENCE OF COAGULATION SYSTEM CHANGES AND GENETIC POLYMORPHISMS ON POSTOPERATIVE BLEEDING AFTER ON PUMP CARDIAC SURGERY

For Obtaining the Degree of Doctor of Medicine
Speciality – Surgery

Riga, 2013

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LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
ACE 16 intron I/D	Angiotensin converting enzyme gene insertion/deletion polymorphism at 16-intron position (rs4646994)
ACT	Activated coagulation time
ADP	Adenosine dipshosphate
Angle A	Angle Alpha
APC	Activated protein C
APTT	Activated partial thromboplastin time
ATIII	Antithrombin III
ATP	Adenosine triphosphate
BMI	Body mass index
bp	Base pairs
BSA	Body surface area
C^0	Celsius degrees
COPD	Chronic obstructive pulmonary disease
CPB	Cardiopulmonary bypass
CRIO	Cryoprecipitate
CTD	Chest tube drainage
DIC	Disseminated intravascular coagulopathy
DNA	Deoxyribonucleic acid
DTIs	Direct thrombin inhibitors
EF	Ejection fraction
EPI	Extrinsic pathway inhibitor
ESA	European Society of Anaesthesiology
ETP	Endogenous thrombin potential
F	Coagulation factor
FF	Functional fibrinogen
FFP	Fresh frozen plasma

F/R	Forward/reverse primers
GPIIb/IIIa	Glycoprotein IIb/IIIa receptors
Hb	Hemoglobin
hep.kTEG	Heparinase-modified kaolin activated thromboelastography thromboelastography
HIT	Heparin induced thrombocytopenia
HITT	Heparin induced thrombocytopenia with thrombosis
Ht	Hematocrit
ICU	Intensive care unit
INR	International normalized ratio
IV	Intravenously
K	Clot formation time
kDA	Kilodalton
kTEG	Kaolin activated thromboelastography
LMWH	Low molecular weight heparin
LV	Left ventricle
mA	Miliampers
MA	Maximum Amplitude
NO	Nitrous oxide
NSAID	Non-steroidal anti-inflammatory drugs
PAI-1	Plasminogen activator inhibitor type-1
PAI-1 gene -675 4G/5G	Plasminogen activator inhibitor type-1 gene 4 Guanine/5 Guanine polymorphism at 675 position (rs1799768)
PAI-1 gene -844 A/G	Plasminogen activator inhibitor type-1 gene Adenosine/ Guanine polymorphism at 844 position (rs2227631)
PCC	Prothrombin complex concentrate
PCCoef.	*Pearson's* correlation coefficient
PCR	Polymerase chain reaction
PLT	Platelets

PI	Prothrombin index
PO	Per Oral
POC	Point-of-care
R	Reaction time
RAAS	Renin angiotensin aldosteron system
ROTEM®	Rotation thromboelastometry
SD	Standard deviation
s	Seconds
SQ	Subcutaneous
t-PA	Tissue plasminogen activator
t-PA/PAI-1	Complex of tissue plasminogen activator and plasminogen activator inhibitor type-1
T0	Time point before surgery (preoperatively)
T1	Time point immediately after surgery on admission in ICU
T4	Time point four hours after surgery
T6	Time point six hours after surgery
T24	Time point twenty-four hours after surgery
TEG®	Thromboelastography
TF	Tissue factor
TRALI	Transfusion related acute lung injury
TxA2	Thromboxane A2
u-PA	Urinary type plasminogen activator
VLHL PAI-1	PAI-1 protein with a very long half-life > 700 hours
VWD	Von Willebrand disease
vWF	Von Willebrand factor

INTRODUCTION

Perioperative bleeding is a concern for all patients undergoing cardiac surgery. The incidence of severe bleeding after cardiac surgery exceeds 10% [1]. Bleeding continues to be the most frequent complication requiring early mediastinal re-exploration after open heart surgery and is associated with worse outcomes increasing the incidence of respiratory insufficiency, delayed extubation, transfusion reactions and transmission of viral diseases due to massive blood transfusions as well as increasing risk for developing myocardial infarction, low cardiac output syndrome, cardiac tamponade, severe arrhythmias, deep sternal wound infections, hepatic and renal insufficiency and need for hemofiltration, finally resulting in increased mortality [2, 3]. Surgical re-exploration due to postoperative bleeding has an incidence in the range of 2% - 6% [4-7], and in less than 50% of the cases a specific site of bleeding is identified during re-exploration.

Cardiac surgery with concomitant cardiopulmonary bypass (CPB) can profoundly alter hemostasis, predisposing patients to major hemorrhagic complications, and possibly early bypass conduit-related thrombotic events as well [8, 9]. Alterations in hemostasis may have a diversity of etiologies. These include the cardiac surgery per se as well as effects of the CPB on the coagulation and the inflammation cascades, and their cross-reactions with the fibrinolytic and the kinin-kallikrein systems [6, 9, 10]. Pathophysiologically, the balance between bleeding, normal hemostasis and thrombosis is markedly influenced by the rate of thrombin formation, platelet aggregation and activation of the fibrinolytic system. Recent evidence suggests that genetic variability modulates the activation each of these pathways [11-14]. The fibrinolytic system plays a pivotal role in the prevention of intravascular thrombosis but increasing evidence reports its importance in bleeding especially in patients undergoing cardiac surgery with the use of CPB [15, 16].

In the most recent literature, information about genetic polymorphism and its association with bleeding after cardiac surgery appears more frequently [11-14, 17, 18]. Previous studies have identified clinical complications associated with excessive bleeding and massive blood transfusions after cardiac surgery [7, 19]. Genetic factors modulate the variability in blood loss after cardiac surgery, and increasing knowledge

shows that combining genetic and clinical factors doubles our ability to predict bleeding [12, 13].

At the Cardiac Surgery Center of Pauls Stradins Clinical University Hospital are carried out more than 1000 cardiac operations with CPB every year. Most of them are coronary artery bypass grafting (CABG), valve replacement and mixed (CABG + valve) operations. Figure 1 shows data from the Center of Cardiac Surgery including types of heart surgery and the number of operations between 2008 and 2012.

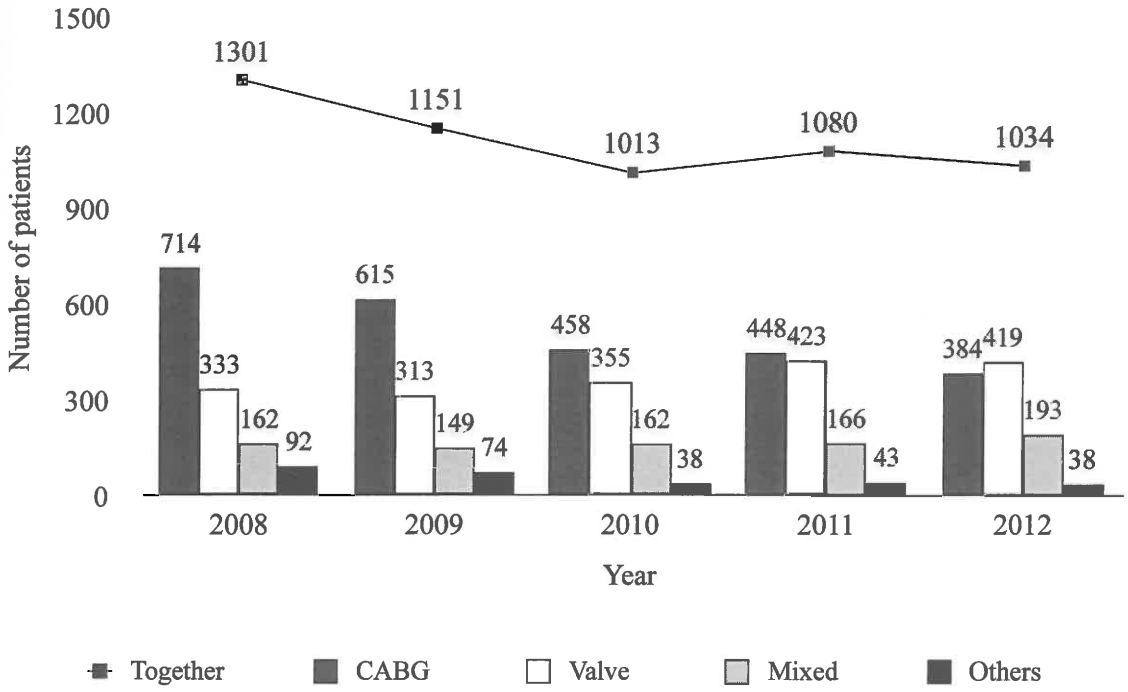

Figure 1. Number of operations performed between 2008 and 2012 at the Center of Cardiac Surgery

Together, total number of the subsequent types of cardiac surgery; CABG, coronary artery bypass grafting; Valve, valve replacement; Mixed, mixed CABG+ valve surgery; others - myxoma, atrial septal defect closure, congenital heart disease, asc aorta replacement etc.

The local importance of the problem is emphasized by the fact that our incidence of re-exploration due to increased bleeding are similar with reported data in literature [4, 7]. In the last 5 years the incidence of re-operations because of bleeding is almost 5.9%. Moreover, after CPB it is often hard to distinguish a surgical from a hemostatic bleeding at the onset. Later on blood loss due to surgical reasons also may cause alterations in hemostasis.

Additinoally, aproximately 16 % of patients have to underwent urgent - and emergency heart operations. These patients have a higher risk for perioperative bleeding

complications because many of them use antiaggregants, anticoagulants without adequate cancelation before surgery, they also undergo longer and more difficult surgical procedures. Their condition might also be more severe due to lacking of inadequate treatment of heart failure.

The goal of the present thesis is to identify and evaluate predictive potential markers of bleeding in patients undergoing elective cardiac surgery employing CPB.

To achieve this goal, the following objectives were analyzed:

1. By evaluating changes in the standard coagulation tests (activated partial thromboplastin time - APTT, prothrombin index - PI, fibrinogen, platelet count - PLT) during CPB and their correlation with 24-hour postoperative bleeding volume.
2. By analyzing quantitatively the plasma concentrations of markers of fibrinolysis: plasminogen activator inhibitor type-1 (PAI-1) and complex of tissue plasminogen activator/PAI-1 (t-PA/PAI-1) and their association with 24-hour postoperative bleeding volume.
3. By identifying genetic polymorphisms in PAI-1 and Angiotensin converting enzyme (ACE) genes and determining their associations with individual fibrinolytic activities and bleeding.
4. By estimating changes in the coagulation state after CPB, as determined by thromboelastography (TEG) and the standard coagulation tests.

Working hypothesis

1. As the standard coagulation tests are regarded, the plasma concentration of fibrinogen possibly could be one of the most precise predictor of greater postoperative bleeding.
2. Decreased plasma concentrations of PAI-1 preoperatively and t-PA/PAI-1 postoperatively may lower inhibitory potential, and consequently, cause greater bleeding tendency.
3. Genetic polymorphism can influence postoperative bleeding volume due to different individual fibrinolytic activity.
4. Parameters of TEG more precisely reflect changes of coagulation state in comparison with standard coagulation tests after CPB.

Scientific and practical diagnostic novelty

Scientific novelty mostly is based on the understanding of the individual kinetic of the markers of fibrinolytic system at different time points, as well as to evaluate the genetic predisposition to bleeding due to changed fibrinolytic activity in our population after cardiac surgery with CPB. Current knowledge of the effect of genetic variability on fibrinolysis and bleeding is sparse but our knowledge about gene polymorphisms and their influence on individual plasma concentrations of PAI-1 and t-PA/PAI-1 may help us in the preoperative patient risk stratification before cardiac surgery employing CPB.

Determination of fibrinolytic markers might become a part of an obligatory test regiment with the aim to identify patients who are at increased risk of postoperative bleeding due to excessive fibrinolysis, and who could benefit the most from antifibrinolytic prophylaxis.

Evaluation of coagulation state after CPB by means of TEG parameters and standard coagulation tests could help to estimate the effect of hemodilution on hemostasis with a potential to improve hemostatic management.

Organization and laboratory basis

The study was carried out in Pauls Stradins Clinical University hospital, Center of Cardiac surgery, Department of Anesthesiology and Cardiac surgery. As well as in 21 and 16 Cardiac Care wards where patients were selected, enrolled and informed for participation in the study. A collaboration was established between Pauls Stradins Clinical University hospital and the Clinical Immunology Center represented by dr. Inta Jaunalksne and the laboratory assistants Jelena Serova and Tatjana Romanova, and with dr. med. Liene Nikitina-Zake, the Latvian Biomedical Research and Study Center.

Personal contribution

The author was involved in all stages of the study. The author participated in the design and administration of the study, informed the patients and obtained their written consent, selected genetic polymorphism and fibrinolytic markers under investigation, collected the clinical and laboratory data for analysis. Moreover, the author reviewed the literature, collected the data, performed the statistical analysis and interpreted the results.

Ethical concerns

The study protocol and the informed consent form were approved by the Ethics Committee of Development Society (approval Nr. 151209-4L) of Pauls Stradins Clinical University hospital. All patients gave their informed consent for participation in the study. A separate consent form was approved by Latvian Biomedical Research and Study Center and the patients gave in writing their informed consent that their genome could be included in the Latvian genome database.

1. LITERATURE REVIEW

1.1. Normal hemostasis

The concept of blood coagulation dates back to the 1960s, when *Davie*, *Ratnhoff* and *Macfarlane* published articles in Nature and Science outlining the fundamental principle of a cascade of proenzymes activated through proteolytic cleavage of proteins that in turn activate "downstream" enzymes [20, 21].

The hemostatic system is an interaction with the endothelial cells keeping the blood flowing, but making it coagulate in order to prevent excessive blood loss when a blood vessel is injured. Any disequilibrium to the system may result in bleeding or thrombosis [22, 23]. Blood coagulation can be divided into three parts [24]:

1. Primary hemostasis, consisting of the formation of a platelet plug and occurrence of vasoconstriction;
2. Fibrin formation, as a result of the activation of various coagulation proteins, that ultimately results in the generation of thrombin and subsequent fibrinogen-to-fibrin conversion;
3. Removal of fibrin, which is a function of the fibrinolytic system.

More precisely normal hemostasis depends and consist of five stages including local reaction in blood vessels, primary hemostasis, coagulation, anticoagulation and fibrinolysis. Stages of blood coagulation, with the main function for each stage, is presented in Table 1.1.

Table 1.1.

Stages of normal hemostasis

Normal hemostasis depends on:	The main function
Blood vessels	Normal endothelial cells and vascular contractile function
Primary hemostasis	Leads to creation of a platelet plug
Coagulation	Creates a fibrin network which stabilizes the platelet plug

Normal hemostasis depends on:	The main function
Anticoagulation	Coagulation inhibitors prevent the clot from growing too large
Fibrinolysis	Dissolves the fibrin clot and restores the blood vessel and blood flow

1.1.1. Blood vessel contraction

A fast vascular constriction limits blood loss after injury to a blood vessel. Several mechanisms - serotonin and thromboxane A2 (TxA2) from platelets contribute to the vasoconstriction.

Endothelial cells

Blood vessels are lined with endothelial cells. The latter cells stay in direct contact with blood and modulate both anticoagulation and coagulation *[25]*. Normal endothelial cells have several properties that prevent coagulation. On the endothelial surface antithrombin III (ATIII) prevents coagulation by binding to factors II and X. These reactions are facilitated by heparin sulphate. Furthermore, the thrombomodulin-thrombin complex turns into a protein C activator, which catalyzes the proteolytic conversion of protein C into activated protein C (APC). The latter acts against coagulation by inactivating coagulation factors Va and VIIIa. Endothelial cells release tissue factor - FIII (TF), tissue plasminogen activator (t-PA), prostacyclin and nitric oxide that inhibits platelet activity and relax blood vessels, and von Willebrand factor (vWF) that promotes hemostasis *[22, 23]*.

1.1.2. Primary hemostasis

When a blood vessel is damaged, a platelet plug is formed within minutes, which temporarily stops the leak. The process leading to the formation of a platelet plug is designated primary hemostasis. This platelet plug is fragile and might easily dissolve if

not stabilized by a fibrin network. The main steps are platelet adhesion, platelet secretion and aggregation.

The main steps of primary hemostasis:

The first step is ***platelet adhesion***. Platelets become activated and adhere to endothelial cell surface with membrane glycoproteins receptor (GP) Ib if vessel wall is disrupted [26]. Platelets with GP Ib bind to collagen and to activated vWF in the subendothelium. Adhered platelets form pseudopodia and undergo a metamorphosis [22].

Normally, platelets circulate in an inactive state, becoming activated in response to vessel wall injury. Main platelet physiological activators are vWF, thrombin, collagen, adrenalin, adenosine diphosphate (ADP), platelet activating factor, arachidonic acid and TxA2 [22].

There are 2 independent pathways of platelet activation. The first is a TF-dependent pathway. TF which is expressed on the vessel wall is activated by disulfide isomerases leading to thrombin generation. Thrombin than activates platelets by cleaving the platelet thrombin receptor Par4. This pathway does not require disruption of the endothelium. The second pathway is dependent on the exposure of subendothelial matrix proteins after endothelium disruption. The exposed collagen interacts with platelet GP VI receptors, vWF and GP Ib-V-IX leading to platelet activation [27]. Moreover, ADP activates platelets through binding to the platelet surface receptors P2Y1 and P2Y12 [25, 28].

The second step is ***platelet secretion*** when activated platelets release their granule contents. Platelets have alpha, dense and lysosomal granules. Platelet alpha granules contain fibrinogen, vWF, plasminogen, alpha-2-antiplasmin, plasminogen activator inhibitor type-1 (PAI-1), growth factors and membrane-bound adhesion factors. Platelet dense granules contain several platelet agonists such as ADP, adenosine triphosphate (ATP), serotonin, and Ca^{2+}. Platelet surface becomes procoagulant and the platelet receptors GPIIb/IIIa are exposed. GPIIb/IIIa receptors further strengthan the adhesion to the subendothelium and are essential for normal platelet aggregation [22]. In addition, the phospholipid membrane of the activated platelets provides an excellent surface on which the generation of thrombin and subsequently the fibrin formation may take place [26].

The third step is ***platelet aggregation***. During aggregation platelets stick to each other and form a platelet plug. The platelets are bound to each other by fibrinogen or vWF that forms bridges between GPIIb/IIIa receptors on different platelets. ADP and TxA2 released from activated platelets contribute to the growth of the platelet aggregate by activating other platelets. Platelet aggregation is inhibited by prostacyclin PGI2. The platelet plug may temporarily stop the bleeding but the plug is not firm and needs formation of a fibrin network to become permanent.

Red blood cells appear to play an important role in platelet adhesion and aggregation, potentially because of their physical capability to facilitate platelet transport to the surface. Therefore, adequate function of primary hemostasis is dependent on a sufficiently high hematocrit (Ht) concentration [24, 29].

1.1.3. Coagulation

The coagulation is the process that produces the fibrin network, which stabilizes the platelet plug and transforms it into a firm and durable clot [22].

The coagulation system consists of coagulation factors. Activated platelets and damaged endothelial cells establish a platform of negatively charged phospholipids, that bind coagulation factors and convert inactive zymogens to their active forms as serine proteases [25]. The coagulation system has traditionally been divided into intrinsic, extrinsic and common pathways, but *in vivo* such a division does not exist [30].

The principal route of activation of blood coagulation is via the TF, a glycoprotein which is present in all tissues, especially in brain, lungs and placenta. TF is not in contact with the blood under physiological circumstances [26]. TF is released from sub-endothelial cells when the blood vessel is damaged and as response to inflammation process it is released form endothelial cells and from monocytes.

Coagulation factor (F) VII binds to TF and becomes activated. This is a trigger that starts the coagulation process. TF - FVII pathway is called the ***extrinsic pathway*** of coagulation. The alternative route for FXa activation is by activation of FIX. Traditionally, this is called the ***intrinsic pathway*** of coagulation. The role of the intrinsic pathway is less clear *in vivo,* but becomes important when the blood is in contact with

foreign surfaces, for example, glass, collagen, a CPB circuit or mechanical circulatory assist device [31]. Both coagulation pathways are presented in Figure 1.1.

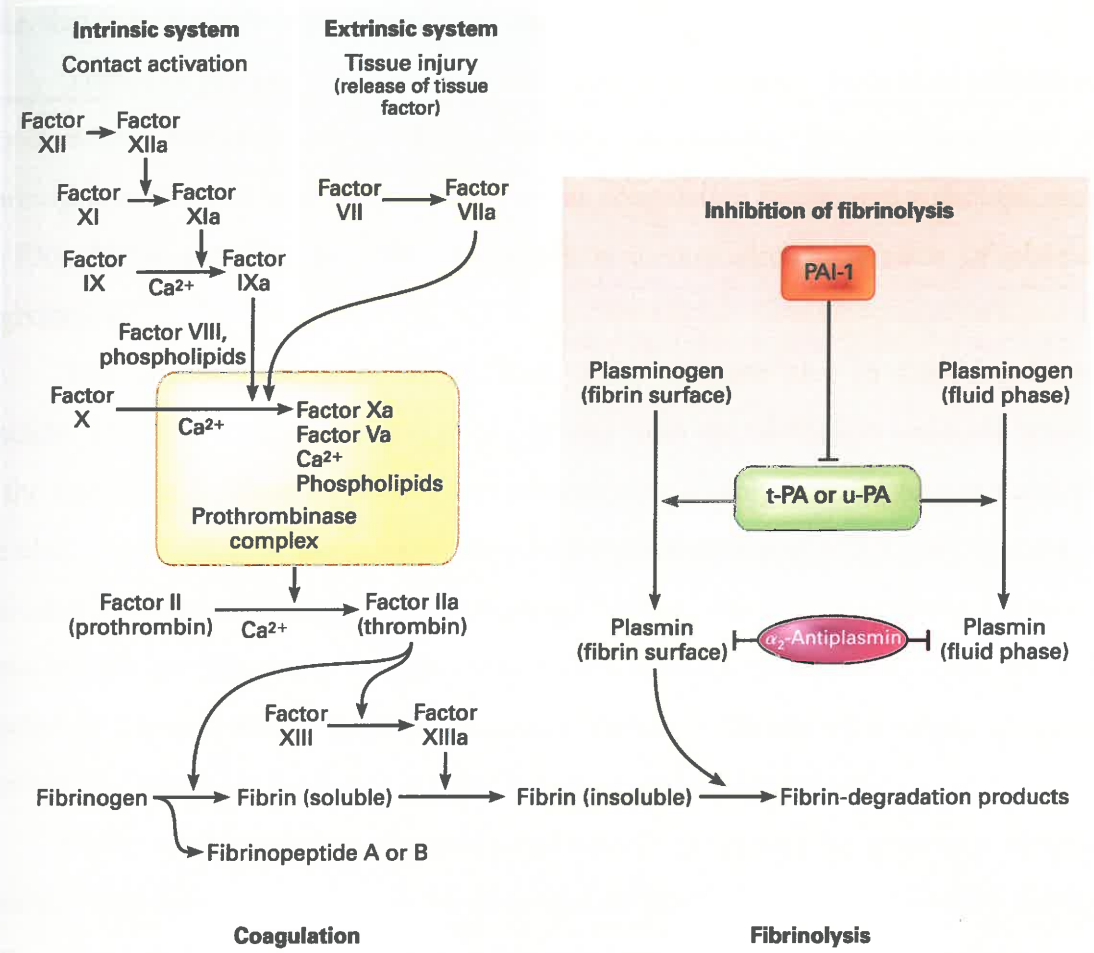

Figure 1.1. Extrinsic and intrinsic coagulation pathway and fibrinolysis
Reprinted from Kohler HP et al. *Engl J Med.* 2000; 342: 1792–1801
Abbreviations: PAI-1, plasminogen activator inhibitor type-1; t-PA, tissue plasminogen activator; u-PA, urinary type plasminogen activator

These pathways are interconnected on many levels and converge at the prothrombinase complex, which consist of FXa and FVa bound together by calcium ions on a phospholipid membrane. These events start the ***common pathway*** of coagulation. The prothrombinase complex converts prothrombin (FII) to thrombin (FIIa). Thrombin activates FXIII to FXIIIa, which stabilizes the fibrin clot by covalently cross-linking fibrin [25].

The coagulation FII (prothrombin), FVII, FIX, FX and the coagulation inhibitors protein C and protein S are vitamin K-dependent. Vitamin K is necessary cofactor for the synthesis of a gamma-glutamyl region.

Thrombin

Activation of FVIIa binds TF from the wound leading to activation of FIXa and FXa, that in turn converts prothrombin to thrombin [32].

Thrombin is a key enzyme in the activation of coagulation. Its normal half-life in blood is approximately 30 sec [15]. Thrombin is essential for the conversion of fibrinogen to fibrin. It is able to activate various coagulation factors and cofactors, such as FXI, FVIII and FV, the vWF. Thrombin is a very strong activator of platelet aggregation.

The formation of cross-linked fibrin is the ultimate step in the coagulation cascade. Thrombin mediated cleavage of peptides from the fibrinogen molecule results in the formation of fibrin monomers and subsequently in polymers. To further stabilize the clot, cross-linking of fibrin takes place by thrombin activated FXIII [26]. Thrombin activated FXIII creates strong covalent bindings between the threads of fibrin. Up to six cross bridges per fibrinogen subunit create a firm network of insoluble fibrin, the end product of the coagulation process. Thrombin formed at the site of a wound is called "hemostatic" thrombin because it is participating in normal hemostasis.

Under some conditions, thrombin and soluble fibrin may be generated without wounds. This can occur locally as in deep venous thrombosis or systemically during CPB, during wide spread organ damage (sepsis) or in cases of systematic release of procoagulants such as TF. Excessive local or systemic thrombin and fibrin formation that are not being made in response to wound reflects dysregulation of the coagulation system and may result in consumptive coagulopathy and in some cases bleeding and thrombosis. This is called "nonhemostatic" thrombin and fibrin [9].

Thrombin also acts as an antithrombin. On the surface of endothelial cells thrombin binds to thrombomodulin and is transformed into an anticoagulant factor that activates the coagulation inhibitor protein C [22].

1.1.4. Anticoagulation

The coagulation inhibitors are activated on the surface of endothelial cells. The main anticoagulants are:

Antithrombin III (ATIII)

ATIII is serine protease inhibitor that is synthesized by the liver and circulating in plasma. It is the most important inhibitor of FXa and thrombin. It also inhibits the active forms of the other coagulation factors in the intrinsic pathway like FXIIa, FXIa and FIXa by forming a complex with the active factor. This complex formation is accelerated by heparin sulphate on the surface of endothelial cells or in the presence of heparin.

Protein C and protein S

They are vitamin K-dependents plasma proteins synthesized by the liver. Protein C circulates in plasma as an inactive proenzyme of the serine protease APC. It becomes activated by the thrombin/thrombomodulin complex when protein C is bound to it's endothelial receptor (endothelial cell protein C receptor). By acting together with protein S, APC prevents generation of thrombin in a negative feedback loop by proteolytic cleavage of activated factors V and VIII [22].

Extrinsic pathway inhibitor

It is synthesized by endothelial cells and megakaryocytes and is present in plasma and platelets. It forms a complex with FXa which effectively inhibits the TF-FVIIa complex.

1.1.5. Fibrinolysis

The fibrinolytic system cleaves fibrin into fibrin degradation products and clear blood vessels from thrombi. Fibrinolytic activators such as t-PA and urinary type plasminogen activator (u-PA) convert the plasminogen into the fibrin degrading enzyme plasmin. Different inhibitors like PAI-1, alpha 2 antiplasmin effectively inhibits this activation but in the presence of a fibrin surface the inhibitors are inefficient and the fibrinolytic process is allowed to proceed. Plasmin also can be activated by tumor cells and bacteria [22]. Figure 1.1. represents inhibition of fibrinolysis.

1.2. Fibrinolytic system

The main fibrinolytic components of plasma are plasminogen, PAI-1, alpha-2 antiplasmin, t-PA, u-PA. Fibrinolytic activity depends of balance between plasminogen activators and plasminogen activator inhibitors. Known plasminogen activators in the vascular system include t-PA and u-PA. The fibrinolytic system, resembling the cascade mechanism of blood coagulation, comprises zymogen-to-enzyme conversions, feedback amplification and inhibition, and a finely regulate balance with various inhibitors [33]. The most important fibrinolytic system inhibitors in circulation are PAI-1 a fast actin inhibitor of both t-PA and u-PA, and alpha-2 antiplasmin, a specific plasmin inhibitor that is covalently bound to polymerizing fibrin by FXIIIa and thrombin activatable fibrinolysis inhibitor [26].

t-PA circulates in plasma as a complex with PAI-1. The fibrin clot provides the surface on which the reactions occur. Plasminogen, t-PA and fibrin form a complex that promotes the formation of plasmin and the subsequent lysis of cross-linked fibrin [34]. The process of fibrinolysis activation and inhibition is shown in Figure 1.2.

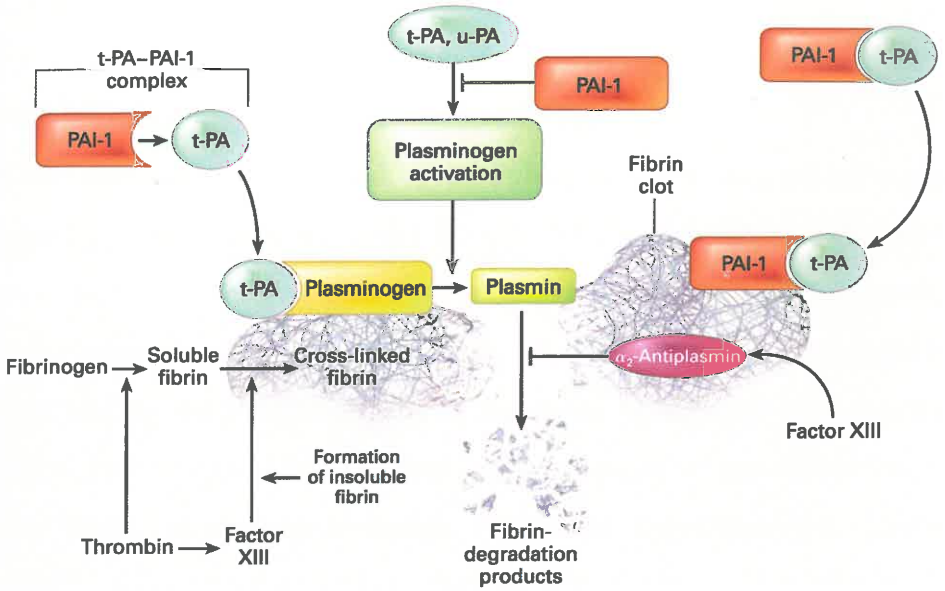

Figure 1.2. Activation and inhibition of the fibrinolytic pathway
Reprinted from Kohler HP et al. *N Engl J Med.* 2000; 342: 1792–1801
Abbreviations: PAI-1, plasminogen activator inhibitor type-1; t-PA, tissue plasminogen activator; u-PA, urinary type plasminogen activator; t-PA-PAI-1, complex of tissue plasminogen activator and plasminogen activator inhibitor type-1

Numerous studies have demonstrated a role for genetic factors in individual fibrinolytic system activity [10-12, 14, 17, 18, 35-37]. Associations have been shown between genetic polymorphisms, fibrinolytic system activity and thrombotic events. Only a few reports have focused on increased bleeding in relation to the fibrinolytic system and genetic factors in cardiac surgery.

Recent evidence suggest the importance of PAI-1 gene -675 (4G/5G) and PAI-1 gene -844 A/G promoter polymorphisms as well as Angiotensin-converting enzyme (ACE) gene intron 16 I/D polymorphism association with increased fibrinolytic activity and bleeding [17, 36, 37].

1.2.1. Plasminogen activator inhibitor type 1

PAI-1 was first identified in cultured human endothelial cells and subsequently in plasma, platelets, placenta and hepatocytes. The serine protease inhibitor PAI-1 is a linear glycoprotein that is composed of 379 amino acids and has a molecular weight of 48 kilodalton (kDa) [38]. The active form of PAI-1 is synthesized in platelets as well as in endothelium and adipose tissues. It binds rapidly to t-PA and u-PA forming a stable

complex in the ration 1:1 that is cleared from circulation in hepatic cells. The active form of PAI-1 is unstable with a half life of 30 minutes.

When platelets are stimulated by thrombin, PAI-1 is released on the platelet surface, thereby protecting the blood clot from premature lysis. This mechanism causes a rapid local increase in the PAI-1 concentration in the circulation [39]. Thrombin also stimulates the synthesis of PAI-1 in endothelial cells. PAI-1 plasma concentration plays a significant role for the vascular effects of the Renin Angiotensin Aldosteron system (RAAS), since PAI-1 expression is regulated by Angiotensin IV and Aldosteron.

Low PAI-1 plasma concentration results as hyperfibrinolytic hemorrhage characterized by a normal primary hemostasis: normal clot is readily formed by lysis as there is no inhibitor to abate plasmin activity [40].

Increased PAI-1 level in blood appears to be associated with thromboembolic diseases such as myocardial infarction, deep vein thrombosis and pulmonary artery thromboembolism. Moreover, elevated PAI-1 levels have been associated with an elevated body mass index (BMI), hypertension, hyperinsulinemia, lipid disorders and increased cytokine levels, a constellation of pathologies referred to as the metabolic syndrome [41, 42].

PAI-1 antigen concentration can be measured in plasma using double-antibody enzyme-linked immunosorbent assay (ELISA test) and PAI-1 activity can be detected by spectrophotometry. PAI-1 antigen normal range in human plasma is 2-48 ng/mL. PAI-1 activity usually is less than 15 IU/mL as most of the PAI-1 exist in the latent or inactive forms.

Following cardiac surgery with the use of CPB, plasma levels of PAI-1 are known to increase as a part of the „fibrinolytic shut-down" [43]. The PAI-1 level starts to rise immediately peaking on the first or second postoperative day and decreases slowly over the following days or weeks [44]. There are clinical data associating deficiencies in PAI-1 production in cases of increased perioperative bleeding [10, 40, 45].

1.2.2. Tissue-plasminogen activator

First identified in tissues and tissue extracts, t-PA was purified from human plasma in the 1970s and t-PA cDNA was cloned and expressed in 1983. *In vivo* t-PA is synthesized and secreted by vascular endothelial cells. Plasma concentration of t-PA antigen is usually 5 - 10 ng/mL, whereas the concentration of free t-PA is probably less than 1 ng/mL. Human t-PA - is a serine protease, consisting of 527 amino acids and in plasma circulates in a single-chain form [46]. This form is cleaved by plasmin, by tissue kallikrein and FXa into a double-chain form, consisting of an amino-terminal H chain and a carboxyl-terminal L chain held together by a disulphide bond [47]. FXIIa, FXIa, FIXa and FVIIa and protein C are reported not to catalyze the conversation of t-PA [48].

Fibrinolysis is dependent on the plasminogen and t-PA binding via specific domain interaction. There are 5 distinct structural domains in t-PA (F-E-K1-K2-P). The most important sites for fibrinolysis appear to be in the F and K2 domains [49].

As an enzyme t-PA initiates fibrinolysis by catalyzing the conversation of plasminogen to plasmin into the fibrin clot surface. It does so by cleaving the single-chain plasminogen into two chains. In blood t-PA is rapidly inactivated by PAI-1. Circulating t-PA is than present predominantly in an inactive stable complex of t-PA/PAI-1. Clearance of t-PA is biphasic, phase 1 having a half-life of about 5 minutes and phase 2 having a half-life of about 45 minutes. It binds to receptors on liver. Regulation of t-PA depends on fibrin binding and fibrin structure [50].

t-PA antigen concentration can be measured in plasma using ELISA test and t-PA activity can be detected by spectrophotometry. Antigen normal range in human plasma is < 5 ng/mL and activity usually is about 0.2-2 IU/mL.

Elevated t-PA plasma concentrations and activity are observed in myocardial infarction, septicemia, stroke, liver diseases etc. t-PA is also involved in metastasis and tumor development. Increased t-PA activity and plasma concentration causes hyperfibrinolysis, which may manifest as excessive bleeding.

1.2.3. Complex of tissue-plasminogen activator/plasminogen activator inhibitor type-1

The complex of t-PA/PAI-1 has a molecular weight of 120 kDa (70 kDa for t-PA and 50 kDa for PAI-1).

PAI-1 binds rapidly to t-PA with at a ratio of 1:1 forming a stable complex, which is cleared from circulation by macrophages lining the walls of the liver sinusoids. The formation of complexes depends on the function and plasma concentrations of the two proteins: the more t-PA and PAI-1, the more complex will be formed in the circulation [51]. The complex is considered to be an indicator of the concentration and function of active PAI-1 and t-PA in the blood [52].

The concentration of t-PA/PAI-1 complexes can be measured in plasma using ELISA test and in normal human plasma is usually low < 5 ng/mL.

Increased concentrations of t-PA/PAI-1 are observed together with elevated PAI-1 levels during pregnancy, sepsis and also might be associated with occurrence of myocardial infraction. Decreased levels of t-PA/PAI-1 suggest increased fibrinolytic activity and enhanced risk of bleeding.

1.2.4. The relation between the renin angiotensin aldosteron system and fibrinolytic system

Many links have been established between the renin angiotensin aldosteron system (RAAS) and fibrinolytic system [29, 53-55]. The RAAS affects vascular fibrinolytic balance because angiotensin IV and aldosteron trigger PAI-1 production whereas its counterpart, bradykinin is probably the most important stimulus of production and secretion of t-PA. The RAAS together with bradykinin plays a major role in regulating fibrinolytic balance, blood pressure and vascular tone [34].

The primary precursor molecule of the RAAS is angiotensinogen which is converted to angiotensin I by the renal protease renin. Cleavage of angiotensin I by ACE yield angiotensin II which promotes aldosterone secretion and release of PAI-1.

ACE probably influences the fibrinolytic balance et a central point by converting angiotensin I to angiotensin II, which increases PAI-1 activity. RAAS may also contribute to a reduction in t-PA production by degradation of bradykinin which increases the release of prostacyclin, nitric oxide and t-PA from endothelial cells.

Another possible link between the RAAS and the fibrinolytic system is thought to be via the endothelium. Several studies have demonstrated that endothelial angiotensin II induced PAI-1 expression is enhanced by the presence of aldosteron. Correspondingly, angiotensin II is enzymatically converted to angiotensin IV resulting in an increase of endothelial PAI-1 expression. In 1998 *Brown at al.* found a very strong correlation between serum aldosteron and PAI-1 in healthy humans on a low salt diet [29].

The inhibition of ACE decreases PAI-1 antigen concentration and PAI-1 activity therefore ACE inhibitors also may influence fibrinolytic activity by reduction of PAI-1 level and by increasing of t-PA concentration. Recent studies suggest that elevated fibrinolytic activity is mainly related to the blockade of angiotensin II, resulting in reduction of PAI-1 rather than due to significant changes in t-PA plasma levels [53, 55]. It could explain positive effect of ACE inhibitors in thromboembolic cardiovascular problems. There are no evidence whether it could effect bleeding in patients undergoing cardiac surgery with CPB.

1.2.5. PAI-1 promoter - 675 (4G/5G) and -844 A/G polymorphisms

Basic plasma concentrations of PAI-1 are characterized by wide inter-individual variations. One explanation for this variability might be that PAI-1 displays different genotypes. The human PAI - 1 gene (*SERPINE 1*, Serpin Peptidase Inhibitor, Clade E Member 1) is located in chromosome 7 and contains nine exons and eight introns [56]. The PAI-1 promoter of the PAI-1 gene contains two common polymorphisms -675 (4G/ 5G) and -844 A/G which could effect the fibrinolytic balance.

Functional insertion/deletion of ***PAI-1 gene - 675 (4G/5G) polymorphism*** has been described in the promoter region in the 675 position of the PAI-1 gene (Figure 1.3.) [57]. PAI-1 -675 (4G/5G) genetic polymorphism strongly influences circulating

PAI-1 levels. A single guanine base pair deletion (4G/4G) located in the promoter region of the PAI-1 gene results in reduced binding of a protein that represses transcription of PAI-1 mRNS. Thus the deletion variant results in increased PAI-1 transcription and increased PAI-1 levels, which in turn decreases the effect of t-PA [58]. From another, insertion of a guanine base pair (5G/5G) is associated with decreased PAI-1 levels in the circulation and most likely with greater fibrinolysis [59].

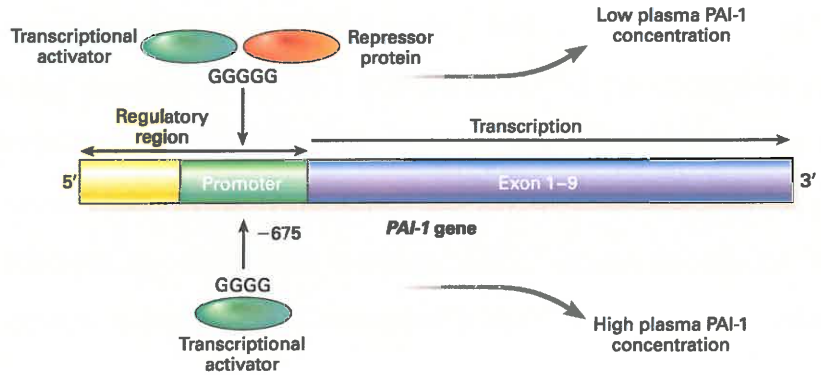

Figure 1.3. Plazminogen activator inhibitor type-1 (PAI-1) gene structure and the site of 4 Guanine/5 Guanine (4G/5G) polymorphism in the promoter region Reprinted from Kohler HP et al. *N Engl J Med.* 2000; 342: 1792–1801

The influence of patient PAI-1 genotype on blood loss after cardiac surgery and whether PAI-1 genotype affects the efficacy of antifibrinolytic drugs is unclear. Numerous of studies have been reported about the role of PAI-1gene promoter -675 (4G/5G) polymorphism and excessive bleeding after on-pump cardiac surgery [10, 11, 14, 17, 18].

A convincing study is published by *Iribarren* and coworkers (2008), who investigated the role of tranexamic acid in patients homozygous for the 5G/5G polymorphism of the PAI-1 gene [11]. In the study 50 patients undergoing elective CABG surgery were included. Patients were classified according to PAI-1 genotype. Twenty-four received 2 g tranexamic acid before and after CPB, whereas 26 received placebo. PAI-1 5G/5G homozygous who did not receive tranexamic acid showed significantly greater postoperative bleeding volume than patients with other PAI-1 genotypes (4G/4G and 4G/5G). Patients with the 5G/5G genotype receiving tranexamic acid showed significantly lower blood loss compared with the placebo group. *Iribarren* with co-authors concluded that 5G/5G carriers may be predisposed to greater bleeding

due to insufficient inhibition of fibrinolysis and thus could benefit the most from an antifibrinolytic prophylaxis.

Duggan and co-authors [14] published changes in PAI-1 gene expression after CPB. These authors noticed that PAI-1 gene expression decreased after CPB in all patients, but the largest reduction was observed in homozygous carriers of the 5G allele. Homozygous carriers of the 5G allele also were more likely to receive transfusion of coagulation blood products.

It has been suggested that ***PAI-1 gene - 844 A/G polymorphism*** reflected in Figure 1.4. acting together with PAI-1 gene -675 4G/5G polymorphism also may be relevant as fibrinolytic activity is regarded [36]. Another investigator has reported an association between 844 A/G polymorphism and myocardial infarction [35]. The - 844 A allele also has been associated with increased risk of venous thrombosis in carriers of the Factor V Leiden mutation [60]. Consequently, the G allele could be associated with a higher bleeding tendency. It has been shown *in vitro* that the - 844 A/G variation can affect the binding of nuclear proteins to the PAI-1 promoter as well [61].

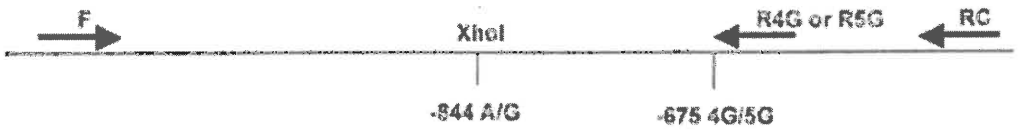

Figure 1.4. Plazminogen activator inhibitor type-1 (PAI-1) gene promoter -844 adenosine/guanine (A/G) polymorphism
Reprinted from Verschuur M*N et al. Atherosclerosis*, 2005; 181(2): 275-84
Abbreviations: Xhol, type II restriction enzyme; F, forward; R, reverse; 4G/5G, 4 Guanine/5 Guanine

So far there are not studies published concerning the PAI-1 gene -844 A/G polymorphism and postoperative bleeding due to increased fibrinolytic activity in patients undergoing elective cardiac surgery with CPB.

1.2.6. ACE intron 16 I/D polymorphism

As mentioned above there is a significant interaction between fibrinolytic system and RAAS. The ACE gene is located in chromosome 17. The gene comprises 26 exons and 25 introns. More than 160 ACE gene polymorphisms are listed.

ACE gene insertion/deletion I/D polymorphism in the intron 16 may play a role in fibrinolytic activity, and consequently, it might affect postoperative blood loss. Plasma ACE levels are stable, but large inter-individual differences have been observed [62]. I/D polymorphism of the ACE gene influences the concentration of circulating ACE as well as of endogenous inhibitors of fibrinolysis. The inserted allele is associated with approximately half of levels of ACE and PAI-1 levels and potentially an elevated fibrinolytic activity. Deletion of the allele is associated with elevated levels of both ACE and PAI-1 [63, 64]. In contrast, recent research has revealed that the presence of a deletion allele in homozygous state lower the activities of FX and FVII and lowers the plasma concentrations of fibrinogen, thus predisposing to anticoagulant effects [65].

Numerous studies in non-cardiac surgical populations have shown that ACE intron 16 I/D gene polymorphism is associated with a wide range of cardiovascular diseases and that the D/D genotype may influence the plasma concentration and activity of PAI-1 and activity and should be considered as a potent thrombophylic factor [66-68].

Conversely, there are only few studies that have examined the effect of ACE genotype variations upon bleeding after cardiac surgery [12, 37].

1.3. Cardiopulmonary bypass

Almost all cardiac surgery is performed under extracorporeal circulation. The first known operation involving open cardiotomy with temporary mechanical takeover of both heart and lung functions was done on April 5, 1951 at the University of Minnesota hospital by dr. *Clarence Dennis* [69]. The patient did not survive due to complex congenital heart defect. The first successful open hear procedure on a human

using bypass machine was performed by *John Gibbon* on May 6, 1953 in Philadelphia [70]. The operation was correction of an atrial septal defect on an 18 years-old girl.

CPB involves an extracorporeal circuit that adequately supplies the body with oxygenated systemic blood flow when the heart and lungs are not functional. Over the past 50 years, extracorporeal circulation has evolved into a remarkably safe means of providing systemic perfusion during open-heart surgery. The oxygenator - an artificial device that substitute for anatomical lungs by delivering oxygen to, and extracting carbon dioxide from, blood - was first conceptualized in the 17th century by *Robert Hooke* and developed into practical extracorporeal oxygenators by French and German experimental physiologists in the 19th century. Indeed, most of the oxygenators used until the late 1970s were derived from *von Schroder's* 1882 bubble oxygenator and *Frey* and *Gruber's* 1885 film oxygenator. As there is no intervening barrier between blood and oxygen, these are called "direct contact" oxygenators. The length of time of which either bubble or film direct contact oxygenators could be used without causing serous complications did not extend much beyond 4 hours. The principal limiting factor was damage of blood constituents - damage and destruction of red blood cells and platelets, coagulation disorders and protein denaturation as well as causing capillary leakage, poor peripheral perfusion, acidosis and progressive organ failure. They contributed significantly to the development and practice of cardiac surgery till the 1980s. The idea of a protective membrane between blood and air to decrease the problem of blood trauma began with observations by *Kolff* and *Berk* in 1944 [71]. Membrane oxygenators introduce a gas-permeable membrane between blood and oxygen. Much work since the 1960s focused on overcoming the gas exchange handicap of the membrane barrier, leading to the development of high-performance microporous hollow-fibre oxygenators that eventually replaced direct-contact oxygenators [72]. The membrane materials that have been found to provide the best gas transfer with minimal cellular trauma have been silicone and polypropylene and have become the standard materials used for oxygenators, currently. Silicone membranes continue to be the membrane of choice for long-term procedures The micropores provide conduits through to the polypropylene membrane that gives sufficient capability, however, after several hours of use the functional capacity of micropore membrane oxygenators decreases because of evaporation and subsequent condensation of serum that leaks through the micropores.

The blood drains by gravity from the right atrium into a heart-lung machine, where it is oxygenated and cooled or warmed, as appropriate, and than returned to the patient through an arterial cannula usually placed in the ascending aorta (Figure 1.5.) [73].

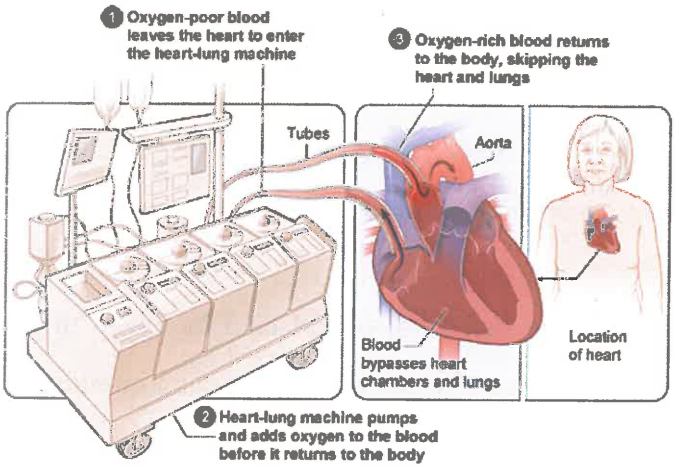

Figure 1.5. Cardiopulmonary bypass system
Reprinted from www.cardiachealth.org

1.3.1. Cardiopulmonary bypass effects on hemostasis

During CPB blood is pumped continuously over an oxygenator membrane making out a non-biological surface of 1,5-2 m^2. The greatest activation of circulating coagulation proteins occurs within the oxygenator, where the blood flow is non-laminar to maximize oxygen (O_2) transport. The venous reservoir and arterial line filter also represent large blood contact areas [16, 74]. During CPB various alterations have been observed in the hemostatic system.

Thrombin generation and activation of coagulation system

Conventional CPB leads to thrombin activation, unrelated to the surgical wound itself. Normally, thrombin does not circulate in plasma due to its rapid inactivation by ATIII [15]. After thoracotomy, there is a small increase in the total amount of thrombin but already 5 minutes after the start of CPB, there is an approximately 20-fold increase in the formation of thrombin and fibrin. During the period of CPB, the patient is exposed to administration of heparin, which lowers the thrombin activity and the fibrin

generation [9, 75]. Normally fibrin does not circulate in the blood. Fibrin is present only et the site of the wound. Only approximately 1% of the fibrin formed circulates as soluble fibrin in plasma. Soon after CPB is started, total fibrin formation is reduced because of the exposure to heparin whereas the soluble fraction of fibrin is increased by 35% of the total [9]. This indicates that much of the thrombin being formed during CPB is non-hemostatic thrombin, which in turn produces soluble fibrin. Non-hemostatically generated thrombin and formation of soluble fibrin continue to be 5 to 10-fold increased throughout the period of CPB. After reversing the effects of heparin with protamine, another peak in the generation of thrombin is observed because more thrombin and fibrin are produced at the site of the wound [75]. The increase in postoperative thrombin generation lasts hours to days and than begins to decline towards normal levels [9]. Figure 1.6 depicts thrombin production as a function of time during cardiac surgery with CPB is.

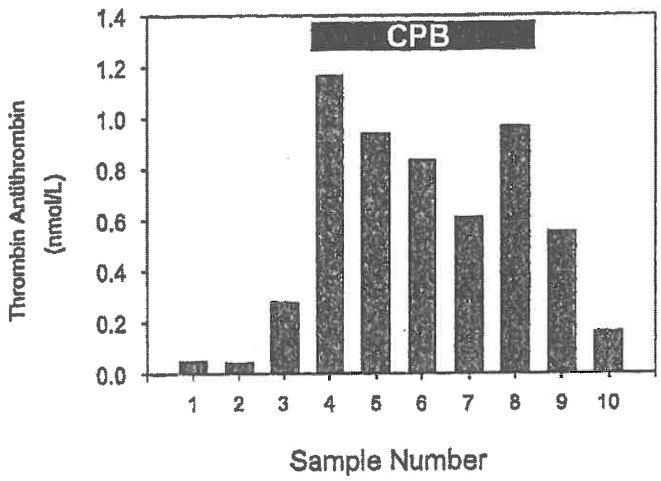

Figure 1.6. Original Thrombin marker data of *in vivo* thrombin production Reprinted from Chandler WL et al. *Blood* 2003;101:4355-4362

During CPB the hemostatic system is activated by the contact system causing enhanced activation of kinin-kallikrein system. Consequently, fibrinolysis, coagulation and inflammation cascades and platelets are activated [9, 16]. Additionally, coagulation also is altered by hemodilution and hypothermia [76].

The contact system

There is normally a low level of kallikrein activity on endothelial cell surfaces promoting the production of kinins. Once CPB is started, there is a significant increase in the contact system activity as blood touches the artificial material comprising the CPB circuit. The intrinsic coagulation pathway is triggered when FXII autocleaves itself upon contact with glass and others anionic surfaces and produces FXIIa, which converts pre-kallikrein to active kallikrein. Plasma kallikrein creates bradykinin from high molecular weight kininogens. Bradykinin levels increase 10-fold due to increased high molecular weight kininogens as well as reduced lung clearance because the pulmonary blood flow is reduced to a minimum mainly through the bronchial circulation [9]. These changes have important implication for fibrinolysis since bradykinin induces secretion of t-PA [34, 77]. Experiments *in vitro* have shown that FXIIa activate plasminogen to plasmin suggesting its possible role in inducing fibrinolysis during CPB. Additionally, FXIIa activates the classic complement cascade [78].

The fibrinolytic system

During CPB endothelial cells release t-PA stimulated by bradykinin. The plasma levels of t-PA increase 5 - 6-fold although one-third of the patients show no changes [16, 75]. Increased t-PA alone does not account for the increases fibrinolytic activity if no fibrin is present. In CPB soluble and circuit-bound fibrin provide a huge surface for plasminogen activation to occur [78]. There is a 10 to 100-fold increase in plasmin generation shortly after the initiation of CPB. Plasmin generation, along with fibrin degradation, remain increased by a 10 to 20-fold throughout the CPB. Normally only 1% of fibrin is degraded by the fibrinolysis. During CPB, fibrin formation and degradation rates are nearly equal [9]. This hyperfibrinolytic state consumes fibrinogen, thus leaving less available for coagulation postoperatively. The inhibitor protein of fibrinolysis, PAI-1 does not increase until the end of CPB and t-PA begins to fall with the release of the aortic cross clamp [15, 79]. PAI-1 secretion increases a 15-fold 2 hours after the cardiac surgery as a part of the "fibrinolytic shut down" causing a hypofibrinolytic state postoperatively, which concentration peaks on the first or second postoperative day and slowly decreases during the subsequent days or weeks [9, 80].

The D-dimer concentrations, which reflects fibrinolysis, increases modestly after the start of CPB, and than increases gradually until the release of the aortic cross clamp, upon which it accelerates to a peak with the administration of protamine (Figure 3.2.). D-dimer remains grossly increased as compared to its baseline value several hours after the cessation of CPB [80]. See figure 1.7.

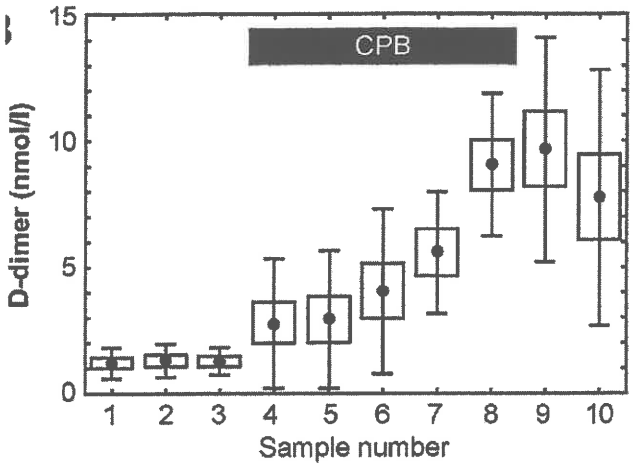

Figure 1.7. Ten serial measurements of D-dimer during cardiac surgery with cardiopulmonary bypass
Reprinted from Chandler WL et al. *Blood Coag Fibrinoly* 2004;15(26):583-91

Inflammation

The surface of the bypass circuit activates leukocytes, including neutrophils and monocytes that lead to an increase in the expression of TF (extrinsic coagulation pathway), procoagulant activation and thrombin generation on these cells. Additionally, blood contact with the artificial surface of the CPB, activates the intrinsic coagulation system. Thereby, FXIIa promotes an increased production of bradykinin, which activates the complement system. CPB also alters protein C, which is converted to APC by the thrombin/thrombomodulin complex. APC binds with endothelial protein C receptors to downregulate cytokine production and reduce the vascular permeability. Systemic inflammatory response syndrome caused by CPB increases TF expression, thereby favoring the production of thrombin [9].

Platelets (PLT)

Fibrinogen bound to the CPB circuit provides a large binding site for PLT through their GPIIb/IIIa receptors. Once bound to the vascular system or to the circuit

the PLT become activated, spread their pseudopods, express their receptors and release granules and micro-particles and support thrombin formation [81]. During CPB the platelets are activated and the platelet thrombin receptor responsiveness is reduced. Therefore, thrombin generation mainly occur through platelet protease-activated receptors 1. CPB increases platelet activation markers including gamma - thromboglobulin, platelet factor 4 and P-selectin and platelet granule membrane protein 140. PLT count typically remains depressed for 7-10 days postoperatively, and than gradually increases above the preoperative levels by 14 days after surgery [9, 74, 82].

Hemodilution

Immediately after connecting the patient to CPB, hemodilution due to the priming fluid in the bypass circuit reduces all factors in blood including coagulation factors, inhibitors and activation markers, by 30% to 40% [16, 76]. Hemodilution has a similar effect on the concentrations of all stable factors in blood such as antiplasmin, plasminogen, fibrinogen, ATIII and prothrombin (Figure 1.8.). The levels of ATIII, fibrinogen and coagulation factors II, VII, IX, X and XII are known to decrease by 50% initially due to hemodilution by a non-blood prime [83]. Hemodilution reduces erythrocyte aggregation and provokes immediate endothelial cell activation, with vWF.. In a large prospective cohort study of 613 patients undergoing CABG the use of higher total fluid volumes in the circuit were associated with higher blood loss [84].

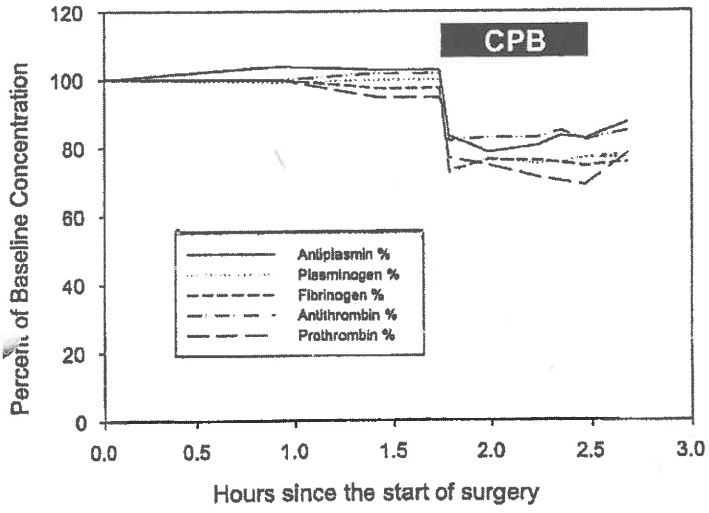

Figure 1.8. Effect of hemodilution on stable factor levels.
Reprinted from Chandler WL. *J Cardiothorac Vasc Anesth* 2005; 19:459-67

1.3.2.Anticoagulation during cardiopulmonary bypass

Anticoagulation is essential during CPB in order to prevent the production of thrombin and fibrin monomers caused by interaction of blood with a synthetic interface [73]. Heparin is used for providing necessary anticoagulation. Heparin inhibits the coagulation system by making a complex with ATIII. It also contributes to platelet dysfunction and induces a fibrinolytic state [85]. Heparin in a dose 300-400 units/kg must be given prior to cannulation for CPB. Heparin administration is monitored by measuring the activated coagulation time (ACT), which qualitatively assesses the anticoagulant effect of heparin. Determination of ACT helps to detect the required dose of heparin more precisely, and ACT usually is maintained above 480 seconds (s) during CPB. The ACT should be monitored every 20-30 minutes during CPB and additional heparin administered as necessary. Although a standard dose of heparin is given there is a great individual variability in the response to heparin.

Heparin resistance

In some patients, it is not possible to raise ACT to therapeutic levels after administration of heparin. Acquired resistance can occur in patients who have been treated with heparin. These patients traditionally require larger doses of heparin to achieve an appropriate level of anticoagulation. Possible causes for resistance include ATIII deficiency or abnormality of the activity, enhanced FVIII activity, platelet activation, or any combination of these factors [86].

Because of individual patient response to heparin and the effects of hypothermia and hemodilution on the ACT, anticoagulation can also be assessed by the *Medtronic Hepcon* system. This directly measures circulating heparin levels and also allows for determination of a neutralizing dose of protamine to return ACT to baseline [73].

Heparin neutralization

Protamine is a polycationic peptide that counteracts the effect of heparin. After separation from CPB, protamine in a dose of 1 mg per 100 units of heparin should be administered initially, followed by additional doses until ACT had returned to baseline

or less than 130 s. Despite complete neutralization of heparin ACT may remain elevated in patients with significant thrombocytopenia or coagulopathies. Protamine has some adverse reactions. These are unusual but unpredictable and may present with systemic hypotension and pulmonary vasoconstriction secondary to anaphylactic reactions, especially after, rapid administration of the drug.

Elevated ACT in later postoperative period may be caused by:

Residual heparin effects may be due to transfusion of *Cell saver* blood containing excessive amounts of heparin. Additional protamine may be useful to counteract its effect [73].

"Heparin rebound" may occur when heparin reappears in the circulation after neutralization with protamine. This is more likely to occur in patients who have received large doses of heparin and is more common in obese individuals [87]. This may occur because the half-life of protamine is about 5 minutes. An elevated ACT reflects this phenomena and additional dose of protamine should be given.

1.4. Preoperative considerations of hemostasis

1.4.1. Preoperative evaluation of bleeding risk

It would be desirable to be able to identify groups of patients who are at an increased risk of severe postoperative bleeding. Such information can be helpful for the decision as to whether prophylactic interventions might become necessary. Assessment of the bleeding history still remains the best tool for identifying patients at increased risk for perioperative bleeding complications. If bleeding history is positive, a comprehensive assessment of possible reasons is indicated [88].

Additionally, risk factors associated with bleeding after cardiac surgery with CPB should be considered in conditions of:

- Increased age
- Non-elective surgery
- Low body surface area
- Prolonged CPB time (>120 minutes)

- Combines intra cardiac - and coronary artery bypass surgery
- Number of coronary artery bypass grafts (>5)
- Redo surgery
- Preoperative antiplatelet agents or anticoagulants
- Renal and/or hepatic insufficiency

Vuylsteke and coworkers [89] developed a simple bleeding risk evaluation score for patients undergoing cardiac surgery that would allow straightforward preoperative stratification of patients into different risk categories. The score is based on variables routinely available to the clinical team before the operation.

The score including variables of *Papworth* bleeding risk stratification is presented in Table 1.1.

Table 1.1.

The *Papworth* bleeding risk stratification score table

Risk factor	Value = 0	Value = 1
Surgery priority	Elective	Urgent or emergency
Surgery type	CABG or single valve	All other surgery types
Aortic valve disease	None	Stenosis, regurgitation, both
BMI	BMI greater/equal to 25	BMI less than 25
Age	Younger than 75	75 years or older

Abbreviations: CABG, coronary artery bypass grafting; BMI, body mass index

Patients consequently, can be divided into three risk groups: scoring ≥ 3 high risk, the scoring 1 or 2 as medium risk and the scoring 0 as low risk. *Vuylsteke et al.* evaluated the patients using the recommended *Papworth* bleeding risk stratification score and showed that 21% of the patients in the highest bleeding risk group had excessive postoperative bleeding, 8% of the patients at medium risk group and only 3% of the patients belonging to the lowest risk group [89].

1.4.2. Laboratory assessment of standard coagulation tests

The minimal preoperative assessment of the patient's coagulation system should entail measurements of PI, APTT and platelet count [73]. However, this recommendation has not reached general consensus. Thus, the European Society of Anaesthesiology (ESA) in its latest guidelines do not recommend these tests without a history of bleeding [88] because they were originally designed to indicate coagulation factor deficiencies, not to assess clinical risk of bleeding [90]. Each of the standard coagulation tests reflects a certain stage of hemostasis. Nowadays coagulation monitoring can be classified as standard coagulation tests and acute care laboratories or point-of-care (POC) coagulation measurements [91].

Following coagulation assessment of the standard coagulation tests are available:

Skin bleeding time

It is used to screen for defects of platelet-vessel wall interaction and should, in theory, detect inherited or acquired disorders of platelet function, thrombocytopenia and VWD. The test can be influenced and prolonged by the use of aspirin and other non-steroidal anti-inflammatory drugs (NSAID) and by reduced Ht [92, 93]. Skin bleeding time can be detected by Duke's test with a punction of an earlobe. It is not a precise method because of non-standardized technique. Normal range of bleeding time is 2-3 min. The skin test also can be made by a method suggested by *Ivy* using standardized technique with a small cut in an elbow joint internal surface. Normal range of bleeding time by *Avy* is less than 10-12 minutes [23].

The limitations for the test are:

The test has to be performed in a standardized and reproducible manner for the results to be meaningful. The test has limited sensitivity and may be within the normal range in some subjects with the most prevalent heritable disorder, VWD.

Activated partial thromboplastin time

APTT is a test of the intrinsic- and common pathways of coagulation. The APTT is measured *in vitro* after adding partial thromboplastin and activator (kaolin) to decalcified citrated plasma. The APTT detects deficiencies or inhibitors of the intrinsic

and common pathway (factors II, V, VIII, IX, X, XI, XII and fibrinogen). The APTT should be designed to detect bleeding disorders due to hemophilia, VWD, including lupus anticoagulant and therapeutic anticoagulants [92, 93].

Some factor deficiencies causing prolongation of the APTT are clinically irrelevant and do not cause bleeding. This is important because FXII deficiency is among the commonest causes of unexpected prolongation of the APTL, being present in 2% [94]. Low values of APTT can be detected in cases of chronic disseminated intravascular coagulopathy (DIC).

The prolongation of APTT can be detect only when the FVIII concentration is < 30%. Mild but clinically significant hemophilia A or VWD may be missed, resulting in false reassurance because clinically important diseases may be modified or masked by physiological response. For example, FVIII rises markedly in pregnancy but in response to physical stress or trauma resulting APTT can be shorter. Furthermore, the sensitivity of the APTT to the common pathway factor deficiencies such as fibrinogen and prothrombin, is low.

Prothrombin index and international normalized ratio

PI (also expressed as prothrombin time) **and INR** assess the integrity of the extrinsic and the common pathways. The PI is measured *in vitro* after addition of the reagent, which contains thromboplastin and calcium to citrated plasma. Expectedly, PI detects important deficiencies of factors II, V, VII and X and is affected by the fibrinogen level as well. According to the World Health Organization and the International Committee on Thrombosis and Haemostasis, measurements of PI in blood for patients treated with oral anticoagulants should be reported as international normalized ratio (INR) values [95]. Reported INR results are independent of the reagent and methods used and are specially intended for assessing patients stabilized on long-term oral anticoagulant therapy.

INR is mainly used for anticoagulant monitoring but PI for detection of acquired bleeding disorders like DIC, liver disease, vitamin K and VII factor deficiency that are rare [93].

Prolongation of prothrombin time or shortening of PI can be detected only when the relevant factor level drops to less than 30%.

Fibrinogen

Fibrinogen is a soluble plasma glycoprotein, which is synthesized by the liver. During blood coagulation, it is converted by thrombin into fibrin. The plasma concentration is 1.5-4 g/L measured by the method suggested by *Clauss* [96] although in literature are data showing that fibrinogen levels measured by *Clauss* often have higher values and disagree with real values of fibrinogen concentration in the blood [97]. Low fibrinogen levels can indicate a systemic activation of the clotting system, with consumption of clotting factors faster than synthesis, for example, if the DIC is present, in the case of long lasting period on CPB. Low levels of fibrinogen also can be found in cases of congenital fibrinogen deficiency. Acquired hypofibrinogenemia also can be detected in cases of sepsis or trauma or massive bleeding. Recent studies have focused on the role of fibrinogen in the coagulation cascade. Fibrinogen is important because it is the first clotting factor to fall to critically low levels during massive bleedings [97]. It plays a fundamental role in maintaining hemostasis, serving both as the precursor of fibrin, which is cross-linked to form blood clots, and as a mediator of platelet aggregation [98]. A low level of fibrinogen is considered as a predictors of bleeding [91, 99] and correlates with increased bleeding [100, 101] and increased mortality [102].

Platelets

PLT are small irregularly shaped clear cell fragments of a diameter of 2-3 μm. The average lifetime of platelets is 5 to 9 days. A normal PLT in the blood is between 150 - 450 $\times 10^9$/L. Disorders leading to a decrease in PLT are idiopathic thrombocytopenic purpura, thrombotic thrombocytopenic purpura, heparin-induced thrombocytopenia (HIT), aplastic anemia. Preoperative thrombocytopenia < 100 x 10^9L is a serious risk factor for bleeding and requirement of massive blood transfusions postoperatively [103]. PLT and fibrinogen have been shown to decrease first because of platelet consumption in cases of severe bleeding

Normal PLT count is not a guarantee of adequate function. In some cases, PLT can be dysfunctional, for instance under exposure to aspirin and other antiaggregants, NSAID, uremia and after CPB. Platelet adhesion and aggregation will be impaired in

cases of VWD. PLT function is influenced by low Ht (< 25%) and low fibrinogen levels in plasma. PLT function decreases fundamentally when PLT < 50 $\times 10^9$/L.

D-dimer

Fibrin degradation product (D-dimer) is a small protein fragment, which is presented in blood after a blood clot is degraded by fibrinolysis. D-dimer testing is clinically when there is a suspicion of hyperfibrinolysis in cases of deep venous thrombosis, pulmonary embolism and DIC. After cardiac surgery with CPB, the D-dimer level usually is greatly increased as compared to baseline because of hyperfibrinolysis [80]. However, in cases of liver diseases, inflammation, malignancy, trauma and surgery, the D-dimer level may display false positive results.

POC coagulation monitors include some of the standard coagulation tests as ACT and those that evaluate the viscoelastic properties of blood. In bleeding patients these tests can be helpful to evaluate coagulopathy, bleeding causes and therapeutic options [86, 91]. In a recent review POC monitoring was shown to reduce mortality compared with 'usual care' [104]. POC tests are used to guide administration of coagulation factor concentrates. These tests have been shown to decrease the requirements of allogeneic blood product transfusion and are associated with improved outcomes [105, 106]. In conditions of postoperative bleeding, the POC platelet assays (TEG®, ROTEM®, Sonoclot®, Platelet function analyzer-100, aggregometry etc.) are useful tools for differentiating between patients with residual antithrombotic therapy and those who have a CPB- induced bleeding [86].

Activated coagulation time (ACT)

ACT is being routinely used in cardiac surgery to guide heparin-induced anticoagulation and its reversal [107]. The ACT is influenced by many factors besides heparin, including hypothermia, hemodilution, and, to a lesser degree, by thrombocytopenia [73].

Thromboelastography *(TEG® Hemoscope Corporation, Niles, USA)* and ***Rotation thromboelastometry*** *(ROTEM® Pentapharm GmbH, Germany)*

Both measure *in vitro* viscoelastic properties of the developing clot in whole blood. Specific patterns characterize the presence of clotting factor deficiencies, platelet dysfunction and/or thrombocytopenia, hypofibrinogenemia and fibrinolysis [108]. The overall coagulation profile can be qualitatively or quantitatively interpreted. Both devices operate on similar principles. Clinically, these techniques are particularly useful after cardiac surgery as well as after liver transplantation, in obstetrical patients and for evaluation of the efficacy of treatment in bleeding patients [86].

For ***quantitative analysis*** following parameters are monitored (Figure 1.9.):

- *Coagulation initiation*: recorded as reaction time (R) or clotting time (CT). CT is the time required to reach an amplitude of 2 mm, which represents the initiation of clot formation. The clot formation is partially dependent on thrombin generation.
- *Clot formation* time (K) or (CFT) time required for the amplitude to increase from 2 to 20 mm. Alpha (α) represents kinetics of clot development.
- *Clot strength* as maximum amplitude (MA) or maximum clot firmness (MCF), both measured in mm, represent the effect of platelet aggregation and fibrin polymerization.
- *Clot stability* is measured as a reduction of clot strength after MA or MCF. It is expressed as clot lysis (CL) or lysis index (LY) and (CL30) measures the clot lysis remaining 30 min after MA or MCF. Low lysis index indicates hyperfibrinolysis [88].

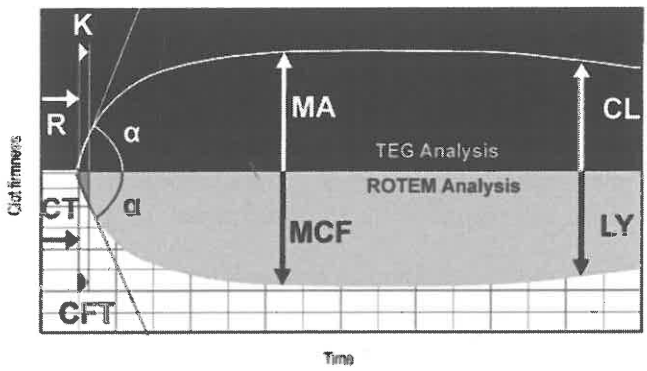

Figure 1.9. Thromboelastography (TEG®) and rotation thromboelastometry (ROTEM®) tracing parameters of quantitative analysis
Reprinted from Michael TG et al. *Anesthesia Analgesia*, 2008;106(5):i1366-i1375
Abbreviations: R, reaction time; CT, clotting time; K, clot formation time; CFT, clot formation time, MA, maximum amplitude; MCF, maximum clot firmness, CL; clot lysis, LY; lysis index

Qualitative analysis represents graphic image, which can be interpreted in terms of the hypo-, normal, or hypercoagulable state of the sample and the degree of lysis (Figure 1.10.).

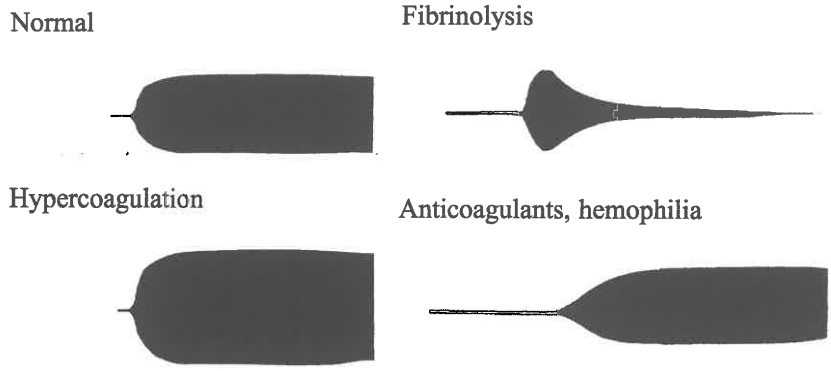

Figure 1.10. Thromboelastography (TEG®) and rotation thromboelastometry (ROTEM®) qualitative analysis
Reprinted from *TEG® 5000 User Manual*, 2007: 14

Various sample preparations can be used with the TEG® and ROTEM® analyzers. Native sample type for global evaluation of coagulation takes approximately 50 minutes until data are generated. Activation of the blood sample is essential to maintain TEG® and ROTEM® as a POC tests, deriving all of the main parameter in 30 minutes. Different TEG® and ROTEM® tests are available [88]:

- *Intrinsic activation* (kaolin activated TEG (kTEG) or INTEM assay): addition of a contact activator stimulates intrinsic activation, providing an assay analogous to APTT
- *Extrinsic activation* (rapid TEG or EXTEM assay): addition of TF activates coagulation via the extrinsic pathway, providing an assay analogous to PT
- *Heparin anticoagulation* (heparinase modified kaolin activated (hep.kTEG) or HEPTEM assay): addition of heparinase to an intrinsically activated assay degrades heparin in the blood, enabling identification of coagulopathy caused by heparin
- *Fibrin clot quality* (functional fibrinogen (FF) or FIBTEM assay): addition of a platelet inhibitor to an extrinsically activated assay. This test measures strength of the fibrin-based clot. Low FF/FIBTEM usually indicates fibrinogen deficiency. Adequate FF/FIBTEM clot strength in bleeding patients may indicate platelet deficiency.

- *Hyperfibrinolysis* (APTEM): addition of the antifibrinolytic agent aprotinin to an extrinsic activation assay. Improvement of coagulation with aprotinin indicates hyperfibrinolysis

- *Platelet dysfunction* due to antiplatelet therapy (TEG®$_{\text{platelet mapping}}$) shows MA reduction with antiplatelet therapy. The assay uses arachidonic acid or ADP agonists to generate adjusted MA that measures the inhibiting effect of antiplatelet agents such as TxA2 inhibitors (aspirin), ADP inhibitors (clopidogrel, ticlopidine), GPIIb/IIIa inhibitors (abciximab, tirofiban, eptifibatide). Preoperative platelet function testing with TEG®$_{\text{platelet mapping}}$ is associated with reduced bleeding, reduced transfusion requirements, reduced costs and improved outcomes in cardiac surgery [109].

TEG® and ROTEM® has been described as a useful POC monitoring during cardiac surgery [110, 111]. TEG® guided hemostatic therapy significantly reduces the requirements to allogenic blood products for trauma patients [112, 113] and after cardiac surgery [114].

Platelet function analyzer - 100 (PFA-100)

PFA-100 measures the complex process of primary hemostasis and help in the rapid detection of platelet dysfunction. The PFA-100 measures platelet response to agonists in citrated whole blood. It helps detect inherited, acquired and drug-induced platelet dysfunction (VWD, evaluates the efficacy of desmopressin therapy, dysfunction due to the effect of aspirin, clopidogrel and other antiplatelet drugs)[115]. However, the PFA-100 has demonstrated a relatively low predictive value for bleeding risk [88, 111].

Sonoclot®Analyzer

The Sonoclot®Analyzer was introduced by *von Kaulla* et al. in 1975 [116]. It detects viscoelastic changes of a whole blood sample and displays a qualitative graph known as the Sonoclot Signature and a quantitative results of ACT, the clot rate (CR) and the platelet function (PF) [107]. It has been shown to predict abnormal postoperative bleeding after surgery employing CPB [117].

Multiple electrode whole-blood aggregometry

It is considered as a clinical standard for platelet function testing. It correlates with platelet transfusion requirements, and performed 0.5-1 days before surgery it can predict postoperative bleeding in patients undergoing CPB treated with thienopyridines [88, 118]. However, platelet aggregometry is labor-intensive, costly, time-consuming, and requires a high degree of experience to perform and interpret [107].

Endogenous thrombin potential test (ETP)

ETP reflects *in vitro* thrombin generation, also called calibrated automated thrombography. The method adds TF and phospholipids to the patient's platelet poor plasma and monitors the cleavage of a fluorogenic substrate, producing a curve, the area under which represents ETP. Low ETP, occuring after CPB, may indicate hypocoagulable state and bleeding risk [119] whereas high ETP may predict possible thrombotic risk [120]. Two limitation of the ETP are that it measures the ability of plasma to produce thrombin *in vitro* and it is not the same as *in vivo*. Second is that the test cannot be run in the presence of heparin or direct thrombin inhibitors (DTIs)[9].

1.4.3. Anticoagulants and antiplatelet medications

ANTIPLATELET MEDICATIONS

Antiplatelet agents can be divided into 3 groups: aspirin, GPIIb/IIIa inhibitors and P2Y12 antagonists [25].

Aspirin

It is irreversibly acetylates the catalytic site of cyclooxygenase-1, the enzyme necessary for the processing of TxA2 [25]. There are different opinions about administration of aspirin before surgery. One opinion is that it has not been associated with increased postoperative bleeding and may improve outcome after cardiac surgery. It should be continued preoperatively [121, 122]. At the same time, some authors [123] recommend that Aspirin should be stopped 5-7 days before elective surgery. ESA guidelines recommend withdrawal of Aspirin therapy 7 days before cardiac surgery [88]. Aspirin-clopidogrel combination therapy has additional antiplatelet efficacy,

compared with aspirin monotherapy, but at additional risk of major bleeding. Thus aspirin monotherapy should be confined of those with excessive bleeding risk before surgery [124].

GPIIb/IIIa inhibitors

Reopro (abciximab), integrilin (eptifibatide) and aggrastat (tirofiban) are intravenous antiplatelet drugs that block aggregation of activated platelets. Tirofiban and integrilin are short-acting GPIIb/IIIa inhibitors that allow for recovery of 80% of platelet function within 4-6 hours of withdrawal. They are commonly used for acute coronary syndromes and percutaneous coronary intervention [25]. Abciximab is a long-acting GPIIb/IIIa inhibitor and after withdrawal, recovery of platelet function takes up to 48 h [73].

P2Y12 antagonists

Plavix (clopidogrel), ticlid (ticlopidine) and more recently effient (prasugrel) as well as brilinta (ticagrelor) are oral antiplatelet drugs that prevent platelet thrombus formation by inhibiting the ADP receptors P2Y12 [25] Prasugrel appears to be more potent and is associated with more surgical bleeding than clopidogrel, whereas ticagrelor is associated with less surgical bleeding [125]. Although *Chen* with coworkers [126] report that clopidogrel increases bleeding risk, platelet and red blood cell (RBC) transfusions if it is discontinued 6 days before CABG. Ticagrelor is cleared mainly through the liver but it has been associated with an increase in creatinine and should be avoided in patients with renal dysfunction [127]. Summary of important antiplatelet agents is presented at Table 1.2.

Table 1.2.

Characteristics of important antiplatelet agents

Antiplatelet agent	Group	Platelet inhibiton	Route	Half-life	Discontinued before surgery
Aspirin	COX inh.	irreversible	PO	3 (2-15) h	0-7 days
Abciximab	GPIIb/IIIa	irreversible	IV	12 h	1-2 days
Eptifibatide	GPIIb/IIIa	reversible	IV	2 h	4-6 h

Antiplatelet agent	Group	Platelet inhibiton	Route	Half-life	Discontinued before surgery
Tirofiban	GPIIb/IIIa	reversible	IV	2 h	4-6 h
Clopidogrel	P2Y12	irreversible	PO	8 h	3-7 days
Ticlopidine	P2Y12	irreversible	PO	12 h	7 days
Prasugrel	P2Y12	irreversible	PO	7 (2-15) h	7 days
Ticagrelor	P2Y12	reversible	PO	7-8 h	2-3 days

Abbreviations: COX, cyclooxygenase inhibitors; GPIIb/IIIa, glycoprotein IIb/IIIa inhibitors; P2Y12 antagonists; PO per-oral; IV, intravenously; h, hours

ANTICOAGULANTS

Anticoagulants could be divided: coumadin group (vitamin K antagonists), heparin and derivatives substances {standard heparin, low molecular weight heparins (LMWH)}, synthetic penta-saccharide inhibitors of FXa (fondaparinux), direct FXa inhibitors (rivaroxaban, apixaban) and direct thrombin inhibitors (DTIs) (hirudin, lepirudin, bivalirudin, argatroban). See Table 1.4.

Vitamin K antagonist

Coumarin is a warfarin derivate. It inhibits γ-carboxylation of the serine protease zymogens, such as prothrombin, factors VII, IX, X, protein C and protein S. During warfarin treatment, the function of FVII and protein C are rapidly lost because of their short half-life (6-9 h), but prothrombin and FX continue their function for 2-3 days (half-life 42-72 h and 27-48 h, respectively) [128]. Although INR and prothrombin time may be prolonged by the decreased of FVII, thrombin generation *in vivo* from residual FX and prothrombin may last during the induction of warfarin therapy. However, the induction of warfarin should be overlapped with heparin or DTIs for 2-3 days to suppress residual thrombin activity. Warfarin is used for patients with a heart valvular prosthesis, atrial fibrillation and stroke. Therapeutic range of INR should be maintained between 2-4 [129].

The management of warfarin therapy in patients undergoing surgery is complicated because restoring of hemostatic function requires a few days. Warfarin is usually discontinued 4 -5 days before surgery to obtain normal INR on the day of surgery [73, 130].

If acute reversal of the warfarin effect is necessary, vitamin K - 5 mg x 2 intravenously (IV) is usually not sufficient due to its slow onset of action (4-6 h after IV administration). Therefore, fresh frozen plasma (FFP) is frequently used in doses of 10-30 ml/kg IV. Prothrombin complex concentrate (PCC) is also available in most European countries and has been used successfully for emergency reversal of coumarin therapy [88, 131]. Because it has been shown to be more efficient as compared to FFP for reversal of the effects of oral anti-coagulants [132], the ESA guidelines recommend administration of PCC for rapid and effective reversal of warfarin before cardiac surgery [88].

Heparin and its derivatives

Include standard heparin, which is a mixture of glycosaminoglycans. The latter substance provides anticoagulation by binding to ATIII. High molecular weight fractions of heparin catalyze in a 1:1 ratio the interaction between ATIII and thrombin, whereas low molecular weight fractions catalyze the interaction between ATIII and FXa [133]. Thus, heparin accelerates by a 1.000-fold the ATIII-inactivation of thrombin [15]. Heparin has relatively short half-life (30-90 min) after IV injection and its pharmacokinetics is unpredictable after SQ (SQ) administration, and hypersensitivity and HIT may occur.

Heparin induced thrombocytopenia (HIT)

Up to 8% of heparinized patients develop the antibodies associated with HIT and approximately 1–5% of them progress to HIT. More frequent HIT appears with standard heparin average 6.5% whereas the incidence is < 1% in those treated with LMWH. In the case of HIT there is a general reduction in PLT count due to increased adhesiveness of the platelet membrane that occurs when platelets are exposed to heparin. This is a benign situation and is characterized by an early and mild decrease in PLT count. HIT is a pathological condition that most often occur after more than 5 days of heparin administration (average onset time 9 days). The condition is mediated by antibody binding to a complex formed between heparin and platelet factor 4 [86, 134]. In patients developing HIT with a thrombosis (HITT), the incidence of thrombotic complications are 20%, which in turn may carry a mortality rate as high as 40%

The efficacy of heparin is reduced when the activity of ATIII is low (< 60%) [135]. Congenital ATIII deficiency is not frequent. Acquired ATIII deficiency has been noticed in 10-20% of patients undergoing cardiac surgery. However, the condition is more common in individuals receiving heparin preoperatively. In most patients, heparin can be continued up to the time of surgery and its effect can be reversed with protamine [73].

LMWF is more selective antagonist to FXa as compared to heparin. Fondaparinux is a synthetic LMWH composed of five saccharide chains.The compound is effective in inhibiting procoagulant FXa [129]. LMWH and fondaparinux have excellent bioavailability and long half-lives (3-6 h). Moreover, they are less frequently causing HIT and major bleedings [136]. PT and APTT are not sensitive to LMWH and fondaparinux because the production of FXa reaches supraphysiological levels. A specific anti-Xa activity assay is therefore needed for controlling anticoagulant effect.

Major bleeding complications are less likely with LMWH and fondaparinux as compared to heparin, although their anticoagulant effect are not completely reversible with protamine [129]. The bleeding risk might increase if these drugs are not discontinued within 12-24 hours of surgery [137]. On the other hand, in the study of *Medalion* and coworkers [138], administration of LMWH more than 8 h before surgery was not associated with increased postoperative bleeding or blood transfusion requirements. LMWH given at a dose of 1 mg/kg subcutaneous (SQ) should be stopped at least 8-12 h prior to surgery [73, 88].

Direct thrombin inhibitors (DTIs)

DTIs make anticoagulant activity independent of endogenous ATIII. They have much smaller molecular size in comperition with heparin. DTIs inhibit thrombin binding to fibrin, whereas a heparin/ATIII complex does not [86, 139]. DTIs are particularly indicated for use in individuals at increased risk of developing HIT and in patients undergoing percutaneous coronary interventions and for those in whom contraindications against heparin exist [129]. Comparison between DTIs and heparin are shown in Table 1.3.

Table 1.3.

Comparison between DTIs and heparin

Characteristics	DTIs	Heparin
Mode of action	Direct	Indirect
Catalyst needed	No	Yes: ATIII
Inhibits clot-bound thrombin	Yes	No
Activates platelets	No	Yes
Antigenicity	No: bivalidurin Yes: lepirudin	Yes
Antidote drug	No	Yes: protamine

Abbreviations: DTIs, direct thrombin inhibitors; ATIII, antithrombin III

Lepirudin with a half-life 1-2 h after IV or SQ administration may bioaccumulate in the kidneys and antibodies against the drug have been reported in more than 80% of the patients receiving it more than 6 days. Such antibodies may reduce lepirudin metabolism, prolong APTT and increase the risk of bleeding.

Bivalirudin has a shorter half-life and rapid clearance by a combination of renal and proteolytic mechanisms [19].

For a variety of reasons (monitoring, lack of antidote, cost, toxicity, safety) DTIs have not displaced standard heparin under the use of extracorporeal circulation [15].

Table 1.4.

Characteristics of anticoagulants

Drug	Mechanism of action	Route	Half-life	Elimination	Monitoring
Heparin	inhibit thrombin/FXa	IV, SQ	1-2,5h	hepatic	APTT/ACT
Warfarin	reduce vit.K factors	PO	36-42h	hepatic	PT/INR
LMWH	inhibit FXa> thrombin	SQ	3-5h	renal	Anti-Xa
Fondaparinux	inhibit FXa	SQ	17h	renal	Anti-Xa
Rivaroxaban	inhibit FXa	PO	5-10h	renal	Anti-Xa
Apixaban	inhibit FXa	PO	5-18h	renal	Anti-Xa
Argatroban	inhibit thrombin	IV	40-50min	hepatic	APTT/ACT

Drug	Mechanism of action	Route	Half-life	Elimination	Monitoring
Lepirudin	inhibit thrombin	IV, SQ	1-2 h	renal	APTT/ACT
Bivalirudin	inhibit thrombin	IV	25min	plasma/renal	APTT/ACT
APC	EPCR modulation FVa/FVIIIa	IV	13min	plasma	-

Abbreviations: FXa, coagulation factor Xa; FVa, FVIIIa, coagulation factors Va, VIIIa; APC, activated protein C; EPCR, endothelial protein C receptor; SQ, subcutineous; PO per oral; IV, intravenously; h, hours; APTT, activated partial thromboplastin time; ACT, activated coagulation time

1.5. Mediastinal bleeding

1.5.1. Etiology of mediastinal bleeding

Bleeding after cardiac surgery can be broadly divided into two groups: surgical or non-surgical bleeding.

Surgical bleeding is the major cause of postoperative bleeding after cardiac surgery employing CPB. Previous investigators have reported that surgical causes of bleeding necessitating re-exploration occur with a frequency ranging between 35 and 85% [7, 19, 140]. Surgical postoperative bleeding is usually related to suturing of sites of aortotomies or pericardium and cannulation places and anastomotic sites, like side branches of arterial or venous conduits and grafts. Furthermore, substernal soft tissues, sternal suture sites, bone marrow, periosteum and raw surfaces caused by previous surgery or pericarditis may become sources of surgical bleeding.

Non-surgical bleeding usually occurs after complex operations with long duration of CPB and is frequently associated with abnormal coagulation. It is often puzzling to find out whether the increased bleeding, which is usually monitored as chest tube drainage, is due to a coagulopathy. Patients with non-surgical bleeding, are likely to have a higher hourly blood loss. The latter condition is often combined with a greater incidence of low cardiac output syndrome and the use of greater amounts of inotropes and alpha-receptor agonists.

Factors influencing both surgical and non-surgical bleeding can be broken down into those occurring preoperatively and those that occur intra- and postoperatively.

Preoperative risk factors of increased bleeding are presented in chapter 1.4. These factors include increased age, low BMI, emergency surgery, pharmacological agents (antiplatelet drugs, anticoagulants), inherited disorders of coagulation such as VWD and hemophilia. Decreased production of coagulation factors is often seen in association with chronic renal/hepatic disease, uremia, amyloidosis, malignant diseases and autoimmune disorders such as systemic lupus erythematosus and vitamin K deficiency due to impaired absorption (ulcerative colitis, gallstones etc.).

Intra-operative risk factors include duration of CPB, alterations in PLT count and PLT dysfunction caused by CPB, hypothermia and by hemodilution. Intra-operative risk factors also encompass reduced concentrations of coagulation factors due to hemodilution, activation of fibrinolysis and a declining pro-coagulant activity due to hypothermia and to heparin administration [19]. During CPB hemodilution reduces most factors by 50% [76, 83] and factor V may be reduced by 80%. Use of *cell saving* devices also may be cause the loss of clotting factors.

CPB is the main source of intra-operative bleedings. A prolonged period of CPB is an independent risk factor of increased morbidity and mortality after cardiac surgery and it is the best predictor of microvascular bleeding. The risk increases if the CPB lasts for more than 120 minutes [141].

Hypothermia may reduce the PLT and enzyme functions. A drop in the body temperature by 1 degree ^{o}C decreases the coagulation function by 10% [142]. Hypothermia impairs platelet adhesion and aggregation, inhibits enzymes promoting fibrinolysis and decreases the generation of thrombin. The most pronounced anti-coagulant effects of hypothermia have been noticed at body temperatures $< 34\ ^{0}C$ [143].

Thrombocytopenia can be due to hemodilution and consumption during CPB. The extracorporeal circuit reduces the PLT count approximately by 30-50% and thrombocytopenia will be progressive with the duration of CPB. Protamine administration transiently reduces the PLT count by about 30% [73].

Platelet dysfunction may be caused during exposure of platelets to the surface of the CPB circuit with release of alpha granule and alteration of platelet membrane

receptors. The degree of the PLT dysfunction is associated with the CPB duration and the depth and extent of hypothermia and a correlation is observed between PLT dysfunction and postoperative blood loss.

Anticoagulant effects related to heparin also can be observed as residual effects, that may result from inadequate neutralization with protamine or from *cell saver* blood transfused after protamine.

Fibrinolysis causes clotting factor degradation and platelet dysfunction. The fibrinolytic system may be activated by thrombolytic agents used preoperatively, by t-PA during CPB and by administering heparin, which might induce a fibrinolytic state on its own right [73].

The most common ***postoperative risk factor,*** which causes bleeding in the post bypass period is considered to be the „heparin rebound" phenomena [19]. This phenomenon is best defined as a return of blood hypocoagulability after adequate neutralization of heparin has been accomplished [144]. Reappearance of heparin usually occurs in the circulation between 1 and 8 hours after its neutralization with protamine. The incidence of this rebound phenomenon has been investigated by several investigators. An heparin effect was detected in 43% of the patients studied at 2h, in 31% at 4h, and in 37% at 8h after the administration of protamine [145, 146]. Numerous reasons have been attributed to the re-appearance of heparin in the circulation. It may either be due to reabsorption of heparin from extravascular depots into the blood stream or to a faster degradation of protamine. Application of *cell saver and* re-transfusion of saved blood after protamine administration may also reintroduce heparin that is not deactivated by protamine. Although several studies have reported that *cell savers* with RBC that are separated and washed in physiologic saline, small remains of heparin still could be found.

1.5.2. Antifibrinolytic therapy

According to randomized trials and meta-analyses, antifibrinolytic drugs have been demonstrated to reduce perioperative blood loss after cardiac surgery [86]. These drugs should be used for all on-pump cardiac operations. Most protocols include

administration of a first dose at the time of skin incision, or before heparinization, and a second dose in the pump prime followed by a constant infusion during the operation [73].

Aprotinin

A serine protease inhibitor. Since the first publication by *Royston* and coworker [147] in 1987, aprotinin gained wide acceptance as an antifibrinolytic drug, which was considered to reduce blood loss and transfusion requirements, and to provide anti-inflammatory effects during cardiac surgery [148, 149]. Aprotinin preserves adhesive platelet receptors during the early period of CPB and exhibits antifibrinolytic properties by inhibiting activation of plasmin and kallikrein. Finally, it blocks the contact phase of coagulation and inhibits the intrinsic coagulation cascade [73].

For almost 20 years, aprotinin has been widely used as a blood saving drug in cardiac surgery and more than 400 articles have been published elucidating its effects [149]. However, in 2006 *Mangano* and coworkers [150] published an observational, multi-center study including 4.374 patients undergoing coronary surgery. These authors demonstrated that patients who received aprotinin had a double risk of acute renal failure, a 55% increased risk for myocardial infarction and an 18% higher risk of getting stroke or encephalopathy. In 2007 the investigators reported that [151] 5 year mortality after cardiac surgery was 50% higher in patients who received aprotinin. As a result on 2007 November aprotinin was decided to stop for the routine use in cardiac surgery [149].

ε – aminocaproic acid (amicar)

A synthetic lysine analogue, which may preserve platelet function by inhibiting the conversation of plasminogen to plasmin. It is effective in reducing blood loss but less effective as aprotinin and because of its low cost it can be the drug of choice for first time uncomplicated cardiac operations [73]. Doses of 5 g may be given prior to heparinization followed by 5 g in pump prime and 1 g/h during the surgery.

Tranexamic acid (cyclokapron)

A synthetic lysine analogue, which has similar properties to ε – aminocaproic acid: it inhibits fibrinolysis at a serum concentration of 10 μg/mL [73]. Moreover tranexamic acid is at least 7-10 fold more potent than ε – aminocaproic acid and has a longer half-life (2 hours) [15, 19]. It has also been shown to reduce blood loss by 43% and 27%, respectively, in on-pump and off-pump surgery and it reduced total postoperative blood loss [152, 153]. There are reports that tranexamic acid is less effective as compared to aprotinin [154] and does not affect the ACT. The appropriate dosing of tranexamic acid is not well defined. A common recommendation is 10 mg/kg over 20 minutes followed by an infusion of 1 mg/kg/h, whereas other workers have suggested injection of a 1 g bolus followed by 500 mg in the pump prime and infusion of 400mg/h IV [73, 155].

Topical use of tranexamic acid at a dose of 1 g in pericardial space also has been shown to significantly reduce perioperative bleeding [15, 156]. It also has been recommended to apply it in the chest cavity [88].

Postoperative thrombotic complications such as myocardial infarction, acute renal failure, stroke, pulmonary artery thromboembolic disorders have not been noticed under exposure to tranexamic acid [157, 158].

1.5.3. Assessment of bleeding in the ICU

Excessive bleeding can be defined as blood loss greater than 100 mL/h or greater than corresponding to a transfusion requirement of 5 units of RBC within 24 h [19] or more than 2ml/kg/h in the first 4 h after surgery [89].

The appropriate assessment of bleeding in the intensive care unit (ICU) requires the following steps [73]:

Monitoring of ***amount and color of chest tube drainage (CTD)***. The chest tubes must be kept open without blood clots inside. In the latter case, bleeding may be masked and blood can drain into the pleural space. Ongoing bleeding without drainage leads to tamponade. Determination of color (arterial, venous, dark or light), Hb and Ht

measurements in the chest tube blood and the pattern of drainage (sudden dump or continuously slowly) can help differentiating surgical - from medical bleeding.

Hemodynamic by using a Swan-Ganz catheter. Maintenance of adequate filling pressures and cardiac output is essential. If filling pressures are decreasing and crystalloid or colloid solution is administrated, decreased Ht can be from hemodilution but more likely from ongoing bleeding. Evidence of rising filling pressures and decreasing cardiac output can suggest the development of cardiac tamponade. Echocardiography is desirable in making the correct diagnosis.

Review of the ***standard coagulation tests*** and serial Ht measurements should be carried out if the patient is bleeding after arrival to the ICU . The coagulation tests may be helpful in assessing whether a coagulopathy is contributing to a mediastinal bleeding. PI, APTT and INR may be useful for assessment of the extrinsic and the intrinsic coagulation cascades, APTT and ACT should be taken to exclude the possibility of a rebound heparin effect. Fibrinogen and D-dimer measurements can help detecting fibrinolysis. More precise tests, that can show alterations in hemostasis, are TEG® or ROTEM® and these should therefore be performed in the case of increased bleeding. Moreover, TEG® has been shown to contribute for assessment of surgical or non-surgical bleeding [159].

Review of ***chest x-ray***. Undrained clotted blood can accumulate in the pleural space or in the pericardial cavity. A chest x-ray may show an expansion of the mediastinum as compared with the preoperative x-rays. A hydrothorax may appear on the chest radiogram together with elevated inspiratory pressures and weak breathing sounds when auscultating the lungs.

1.5.4. Management of mediastinal bleeding

Persisted bleeding must be treated immediately and aggressively based on the suspected cause of hemorrhage. The most benign and least invasive treatments should be considered first. Management of mediastinal bleeding requires the following steps [73]:

Early ***exploration for ongoing bleeding*** or tamponade and understanding whether it is due to surgical or non-surgical reasons. If coagulopathy is present with unexpected high CTD, the goal is to normalize the patient's coagulation profile within 4 hours [7]. If the bleeding is suspected to be of surgical origin and re-operation is necessary, it should be carried out as fast as possible. It is well established that delayed re-exploration for bleeding is associated with adverse outcomes and the risks are increased if time to re-exploration is prolonged by 12 hours or longer [4]. Re-explorations very often is delayed. Recently, *Choong* and coworkers [4] showed that delaying surgical re-exploration by more than 12 hours from the end of operation results in a longer stay in ICU, a higher need for intra-aortic balloon pump support, and increased mortality. *Ranucci* and coworkers [3] also demonstrated that patients who underwent re-exploration had a higher mortality rate 14,2% *vs*. 3,4% in uncomplicated cases.

Hypothermia inhibits thrombin generation and fibrin synthesis and impairs platelet function and increases fibrinolysis. Patient should be warmed up to 36.5 - 37^0C.

Vasodilators or beta blockers should be used to ***control hypertension,*** and ***agitation and shivering*** should be prevented by using short acting sedatives.

The standard coagulation tests are not always immediately available at the beginning when bleeding is presented. Use of blood components to manage early significant bleeding should be based on the patient's history, suspecting a hemostatic defect, for instance, due to long duration of CPB, prior surgery, uremia or use of aspirin. When coagulation studies became available the treatment should become more appropriate. Elevated PT implies the need for FFP and/or CRIO, elevated APTT – requires additional protamine at a dose of 25-50 mg, FFP or/and CRIO may also be indicated. Such POC tests as ACT and TEG® could be useful in more precisely assessment of alterations in hemosthasis. *N. Porite* with co-authors have been demonstrated that TEG® reduces the need for hemostatic products in cardiac surgery [160]. Abnormal results in a patient who has minimal or no bleeding need not to be treated.

Correction of PH and Ca. Acidosis when pH ≤ 7.1.inhibits thrombin generation and platelet function, while accelerating fibrinogen degradation. The positively charged Ca^{2+} enhances fibrin polymerization, coagulation factor - and platelet activities.

Hypocalcaemia can increase the mortality; therefore Ca levels should be maintained > 1.15 mmol/L [88].

Use of ***blood components, medical agents*** *and* ***individual coagulation factors.***

Blood components

Should be administered based on the suspicion of a hemostatic defect. Transfusions of allogeneic blood products are associated with many adverse effects. Patients in need of re-operation have a significantly higher blood loss and need significantly higher amounts of FFP, packed RBC and PLT concentrates as compared to those with a smooth postoperative course [3].

Red blood cell transfusion is indicated if the patient is anemic (Ht 22-24%). Borderline Ht for bleeding patient is 26-28% to maintain satisfactory tissue oxygenation. One unit will raise the Ht of a 70 kg man by 3%. At least 70% of transfused cells will survive 24 hours.

Platelets should be given to the bleeding patient if the PLT count is less than 100 $\times 10^9$/L. Transfusion is not indicated in the non-bleeding patient until the count approaches 30.000, although most of patients in the immediate postoperative period tend to bleed at PLT count less than about 60 $\times 10^9$/L. Each unit transfused should increase the platelet count by 7-10 $\times 10^9$/L [73].

Fresh frozen plasma contains all clotting factors including fibrinogen (2 g/L) and numerous other proteins [161]. Only 30% of the normal level of most clotting factors is essential to provide hemostasis. It would be desirable to administer FFP to improve hemostasis, even if the INR is normal due to hemodilution, effects of CPB and a progressive loss of coagulation factors during ongoing bleeding. A usual dose is 10-15 ml/kg, but there is a risk for fluid overload, transfusion- related acute lung injury (TRALI) and transmission of pathogens.

Cryoprecipitate is a good source of factor VIII and vWF, fibrinogen and FXIII. One bag of CRIO contains 350 mg fibrinogen. Therefore it should be transfused in cases of VWD and when the fibrinogen level is < 1.5-2 g/L. Therefore CRIO is not used anymore in acquired or congenital hypofibrinogenemia in most of the European countries because fibrinogen concentrate (FC) is now generally available [161, 162]. Therefore indication for CRIO is lack of available FC [88].

Medical agents

Protamine should be given in a dose 25-50 mg if the ACT is elevated due to "heparin rebound". Excess protamine should be avoided because it also impairs coagulation, possibly due to antiplatelet activity [163].

Tranexamic acid is recommended if the bleeding is due to hyperfibrinolytic activity as demonstrated with the ROTEM® technique. Tranexamic acid can be given in a dose of 10-25 mg/kg as a bolus injection with repeated administration [88].

Desmopressin can be given in a dose of 0.3 - 0.4 µg/kg IV over 20 minutes to avoid peripheral vasodilatation and hypotension. The effect is seen within 30-60 minutes. On the other hand, desmopressin has not been found to have much benefit in reducing bleeding in routine cardiac surgery [158, 164]. However it may be beneficial in patients with VWD and other disorders affecting platelet function like uremia and when antiplatelet medications are used.

Individual coagulation factors

Recombinant factor VIIa - Novoseven (rFVII) induces hemostasis directly at the site of bleeding by binding to locally expressed TF and by activating the extrinsic coagulation pathway via FX. This results in generation of thrombin and prompt correction of PT [73]. FVIIa has been given in a dose of 90-120 µg/kg [88]. A bolus dose of 90 µg/kg acutely increases FVIIa from 1% to approximately 15% [15]. Originally, rFVII was approved for use in hemophiliacs but it is not the first choice of drug for severe uncontrollable bleeding. Administration of rFVII can be considered if sever bleeding is going on despite the use of conventional, surgical therapy in the presence of PH 7.34 - 7.35, body temperature 37 ± 0.5 ^{0}C, Ca^{2+} > 1 mmol/L, Ht > 25%, PLT count > 100 x 10^{9}/L and fibrinogen > 1.5 g/L [88, 165]. A small randomize controlled trial in 20 cardiac surgical patients demonstrated significantly fewer transfusions in the rFVII group [86]. However there are data reporting an increasing risk for arterial thromboembolic events (5%-10%) after rFVII has been used to control sever bleeding [166, 167], including patients undergoing cardiac surgery [86].

Prothrombin complex concentrate - Octaplex (PCC) is a combination of blood clotting factors II, VII, IX, X and proteins C and S. PCC is efficacious and safe in immediate correction of dosage-dependent INR in patients who need rapid reversal of anticoagulant effect from the use of vitamin K antagonists [88, 131, 168]. If

uncontrolled bleeding is present PCC is recommended when coagulopathy is proven by TEG® or EXTEM. Dosage depends on INR. A mean dose of 30 IU/kg bodyweight normalized PI/INR in patients with reduced coagulation activity [169]. *Schochl* et al report that PCC and/or FC administration for hemostatic therapy proven by TEG® in trauma patients significantly reduce allogenic blood product requirements [113]. After administration of PCC there is theoretically a risk for thrombosis in 1-1.5% and DIC [170]. Antithrombotic prophylaxis should be started as early as possible [165].

Fibrinogen concentrate is a pure preparation of fibrinogen, containing fibrinogen 20 g/L, which provides more precise dosing of fibrinogen and coagulation management. Comparing with FFP there is a low risk of allergy, lower volume needed (for 70 kg patient, only 245 ml *vs.* 1.8 L of FFP), ABO compatibility is not needed and it is no risk of TRALI or virus transmission and it is ready for use in 5-10 minutes. Numerous retrospective and prospective clinical studies of cardiothoracic surgery patients had been shown that FC improves clotting function and reduces blood loss and reduce mortality [101, 105, 161]. A prospective randomized pilot study (n=20) demonstrated that prophylactic FC infusion is potentially useful for reducing bleeding after CABG [101]. Fibrinogen supplementation is recommended in all cases when significant bleeding is accompanied by TEG® or FIBTEM signs of a functional fibrinogen deficit, or a plasma fibrinogen level of < 1.5-2 g/L with an initial dose of 25-50 mg/kg [88]. Importantly reduced fibrinogen levels are shown to correlate with increased bleeding [101]. In Europe FC is well established for treatment of congenital fibrinogen deficiency and is increasingly used for acquired fibrinogen deficiency [162] especially in trauma patients with massive bleeding [112, 113], but is not yet a standard component of many transfusion protocols [171].

Factor XIII plasma transglutaminase that promotes fibrin stabilization by forming multiple cross-links between fibrin monomers and protects them from fibrinolysis. A decrease in FXIII concentration leads to reduction of clot firmness in TEM®. FXIII efficiently inhibits hyperfibrinolysis [172]. FXIII is recommended in cases of on-going or diffuse bleeding and low clot strength despite adequate fibrinogen levels [88]. An initial dose is 30 IU/kg. Clinical studies have shown increased bleeding tendency in surgical patients with FXIII activity < 60% [173, 174].

2. MATERIALS AND METHODS

2.1. The study design

Between March 1st 2010 and July 30th 2011, 90 adult patients scheduled for cardiac surgery by the use of CPB, were enrolled into a prospective observational study in the Center of Cardiac surgery, Department of Anesthesiology and Cardiac surgery of Pauls Stradins Clinical University hospital. For every patient, the predicted operative mortality was calculated using the European System for Cardiac Operative Risk Evaluation (EuroSCORE I) [175].

Each patient was informed about the study and written informed consent was obtained from every patient. The study protocol and the informed consent form were approved by the Ethics Committee of Development Society (approval Nr. 151209-4L) of Pauls Stradins Clinical University hospital. Separate consent approved by Latvian Biomedical Research and Study Center was given to inform about investigation of genome data and about patient inclusion in Latvian genome database.

Inclusion criteria:

- more than 18 years of age;
- first-time CABG and/or valve replacement under CPB;
- predicted operative mortality calculated using the EuroSCORE I < 10%;
- anticoagulants, antiplatelets and NSAID drugs were withdrawn at least five days prior to surgery, in order to disclose drug-induced platelet dysfunction. The last dose of LMWH was administered the evening before surgery or 12 hours before surgery.

Exclusion criteria:

- emergency or urgent heart surgery;
- redo operation;
- preoperative hemostatic disorders with a history of hemorrhagic events or coagulopathy (PI < 50% or INR greater than 1.5, fibrinogen plasma concentration below 1.5 g/L, PLT count < 100 x 10^9/L);
- severe renal failure;
- hepatic dysfunctions or failure;
- autoimmune disorders.

2.2. Methods

2.2.1. Perioperative management

Anesthesia

The same anesthetic procedure was used in all patients. Anesthesia was induced with fentanyl (Fentanyl-Kalceks 0.05mg/ml, A/S Kalceks, Latvia) 0.2-0.3 mg, etomidate (Etomidate, Sagent Agila, India) 0.1-0.3 mg/kg. Cisatracurium (Nimbex, GlaxoSmithKline Manufacturing S.p.A, Italy) 0.2 mg/kg was used for muscle relaxation. All patients received tranexamic acid (Rottapharm, SL, Spain) 2-4 g during the surgery. Anesthesia was maintained with sevoflurane (Sevoflurane Piramal, Piramal Healthcare Ltd, United Kingdom) administered at MAC 0.8-1.2. During CPB, anesthesia was maintained with fentanyl 0.03-0.06 μg/kg/min, propofol 3-5 mg/kg/h (Propofol-Lipuro® 10mg/ml, B. Braun Melsungen AG, Germany) and cisatracurium 0.1 mg/kg/h. Before the start of CPB, heparin (Pan-Heparin Sodium®, Panpharma S.A./ Rotexmedica Gmbh, Germany) was administered in a dose of 300 to 400 units/kg initially followed by 5.000 to 10.000 units to achieve and maintain ACT above 480 s during CPB. Standard pulsatile CPB with an extracorporeal circuit consisting of a polypropylene membrane oxygenator (Admiral®, Eurosets TM, Italy) with moderate hypothermia (bladder temperature 34-35 ^{0}C) in combination with hemodilution was used. Extracorporeal circuit was filled up with the constant volume of 1400 ml solution of deltajonin (AlleMan®, Pharma GmbH, Germany) for all the patients. Deltajonin is isotonic saline solution, consisting of natrium acetate - 6.124 g/L, natrium chloride - 25.5 g/L, potassium chloride - 0.298 g/L, calcium chloride - 0.308 g/L, magnesium chloride - 0.208 g/L, with osmolarity of 299 mosm/L and PH 6.7-7.7. Myocardial protection was achieved by St. thomas 4:1 cardioplegia (AlleMan®, Pharma GmbH, Germany) and volume used for cardioplegia dependent on anatomical features of the heart. Weaning off CPB after the surgical procedure was performed after rewarming the patient to a bladder temperature of at least 36 ^{0}C. After separation from CPB, protamine (Protamin "Meda"-Ampullen, Meda Pharma, Wien, Austria) in a dose of 1 mg per 100 units of heparin was administered initially, followed by additional doses until ACT had returned to baseline, or less than 130 s.

2.2.2. Data collection and analysis

During the study demographic data, type of surgery and surgical data were noticed for each patient. Blood samples for genetic analysis, fibrinolysis parameters and coagulation variables were taken at different time points: before surgery (T0), upon admission to the ICU (T1), six hours after surgery (T6) and twenty-four hours after surgery (T24). Upon admission to ICU (T1) TEG® was performed. Moreover, 24-hour postoperative bleeding volume at three time points: (T1) one hour after surgery, (T4) four hours after surgery and at (T24) twenty-four hours after surgery was monitored. A graphical image of the investigated parameters is featured in Figure 2.1.

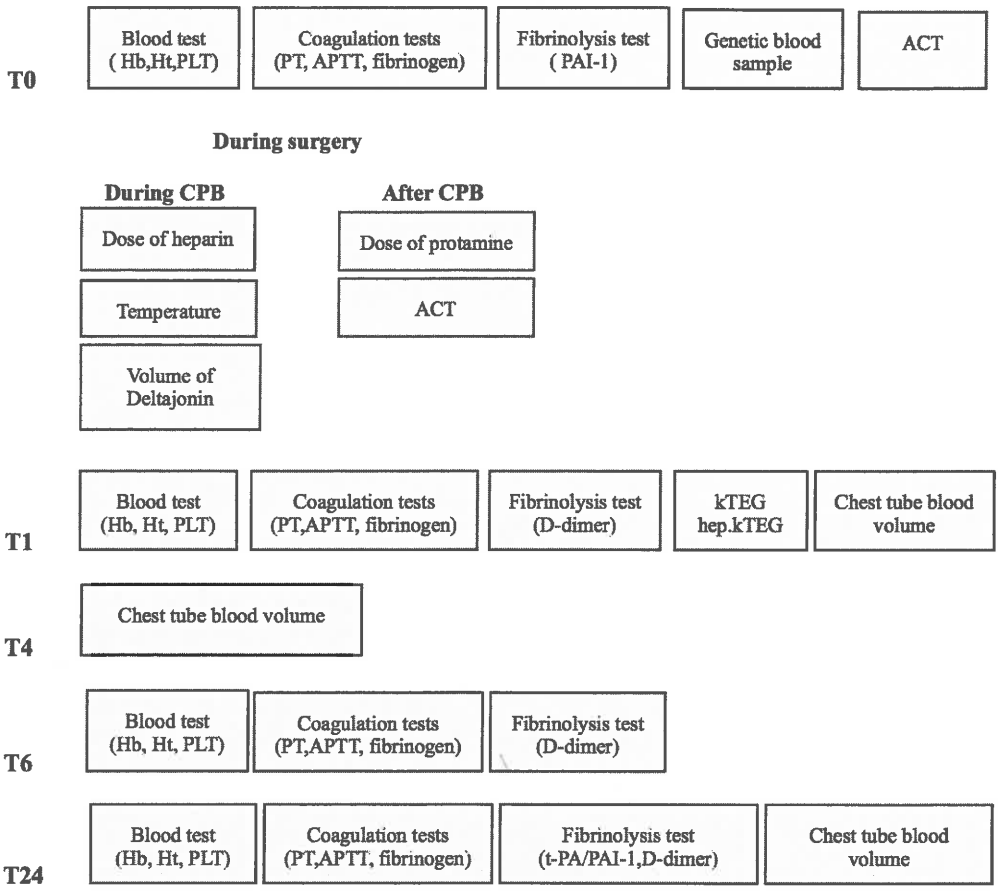

Figure 2.1. Investigated variables obtained at different time points of the study

Abbreviations: Hb, hemoglobin; Ht, hematocrit; PLT, platelet count; PT, prothrombin time; APTT, activated partial prothrombin time; t-PA, tissue plasminogen activator; PAI-1, plasminogen activator inhibitor type-1; kTEG, kaolin activated thromboelastography; hep.kTEG, heparinase modified kaolin activated thromboelastography; ACT, activated coagulation time; T0; day before operation; T1, on admission in ICU; T4, 4 hours after surgery, T6, 6 hours after surgery; T24, 24 hours after surgery

Demographic data of patient

The following data were noticed: age (years), sex (male/female), BMI and body surface area (BSA), predicted operative mortality calculated by the EuroSCORE I, ejection fraction (EF), patient co-morbidities and preoperative medications like antiplatelet drugs and anticoagulants (aspirin, clopidogrel, LMWH, warfarin).

Body mass index

Was devised between 1830 and 1850 by the Belgian polymath *Adolphe Quetelet* during the course of developing social physics [176]. BMI is defined as the individual's body mass divided by the square of his or her height. BMI is categorized as underweight less than 18.5 kg/m^2, normal weight 18.5-25 kg/m^2 and overweight from 25-30 kg/m^2.

Body surface area

Is considered to be a better predictor of metabolic mass than BMI because it is less affected by abnormal adipose mass. Various calculations have been published. One commonly used formula is the *Mosteller* formula published in 1987 [177].

$$BSA(m^2) = \sqrt{\frac{weight\ (\mathrm{kg}) \times height\ (\mathrm{cm})}{3600}} = \frac{weight\ (\mathrm{kg})^{0.5} \times height\ (\mathrm{cm})^{0.5}}{60}$$

Average BSA is generally taken to be 1.73 m^2 for an adult (for men 1.9 m^2, for women 1.6 m^2).

EuroSCORE is ***European System for Cardiac Operative Risk Evaluation***

The evaluation score include patient-related, cardiac-related and operation-related factors (Table 2.1.). In the study were included patients who had predicted operative mortality calculated using the EuroSCORE I < 10%.

Table 2.1.

European System for Cardiac Operative Risk Evaluation I

Factors	Description	Score
Patient-related factors:		
1. Age	1. Per 5 years or over 60 years	**1**
2. Sex	2. Female	**1**
3. Chronic pulmonary disease	3. Long-term use of bronchodilators or steroids for lung disease	**1**
4. Extracardiac arteriopathy	4. Claudication, carotid occlusion or > 50% stenosis, previous or planned intervention on the abdominal aorta, limb arteries or carotids	**2**
5. Neurological dysfunction disease	5. Severely affecting ambulation or day-to-day functioning	**2**
6. Previous cardiac surgery	6. Requiring opening of the pericardium	**3**
7. Serum creatinine	7. > 200 mmol/L preoperatively	**2**
8. Active endocarditis	8. Still antibiotic treatment for endocarditis at the time of surgery	**3**
9. Critical preoperative status	9. One or more of: ventricular tachycardia or fibrillation or aborted sudden death, preoperative cardiac massage, preoperative ventilation before arrival in the anesthetic room, preoperative inotropic support, intra-aortic balloon counter pulsation or preoperative acute renal failure	**3**
Cardiac-related factors:		
1. Unstable angina	1. Rest angina requiring IV nitrates until arrival in the anaesthetic room	**2**
2. LV dysfunction	2. Moderate or LVEF 30-50%, poor or LVEF < 30%	**1** **3**
3. Recent myocardial infarct	3. < 90 days	**2**
4. Pulmonary hypertension	4. Systolic PA pressure > 60 mmHg	**2**
Operation-related factors:		
1. Emergency	1. Carried out on referral before the beginning of the next working day	**2**
2. Other than isolated CABG	2. Major cardiac procedure other than or in addition to CABG	**2**
3. Surgery on thoracic aorta	3. For disorder of ascending, arch or descending aorta	**3**
4. Postinfarct septal rupture		**4**

Abbreviations: IV; intravenous, LV, left ventricular; LVEF, left ventricular ejection fraction; PA, pulmonary artery; CABG, coronary artery bypass grafting. Table is copied from www.euroscore.com

Type of surgery

In the study were included patients undergoing elective CABG, valve replacement or mixed (CABG + valve replacement) heart surgery employing CPB.

Surgical parameters

The following surgical parameters were noticed: CPB time (min), aortic clamp and reperfusion times (min), doses of heparin administrated before and during CPB and dose of protamine used after separation from CPB, value of ACT (sec) at baseline and after protamine was given, temperature on CPB and at ICU (^{0}C), extracorporeal priming volume of deltajonin (ml) and volume of St.thomas solution used for cardioplegia.

Parameters of fibrinolysis

Following fibrinolysis parameters were analyzed in peripheral venous blood samples: PAI-1 preoperatively (T0) and t-PA/PAI-1 complex 24 hours after the surgery (T24). In order to assess the fibrinolytic activity, D-dimer concentrations were determined at three time points after the surgery: upon admission to the ICU (T1), as well as 6 hours and 24 hours postoperatively (T6, T24).

Assessment of plasminogen activator inhibitor type -1

PAI-1was quantitatively assessed by means of an enzyme-linked immunosorbent assay (ZYMUTEST®, HYPHEN BioMed, France). The assay included immunoconjugate, which contains specific monoclonal antibodies for PAI-1: antigen coupled to horse radish peroxidase. It is introduced into microwells coated with another monoclonal antibody specific for PAI-1 antigen. The diluted tested sample is immediately introduced and the immunological reaction starts. When present, PAI-1 antigen binds onto a monoclonal antibody coated with a solid phase through one epitope. Than, the antigen-antibody complex binds the second monoclonal antibody coupled to horse radish peroxidase by another epitope. Following a washing step, the peroxidase substrate, tetramethylbenzidine is introduced in the presence of hydrogen peroxide, and a blue color develops. When the reaction is stopped with sulfuric acid, a yellow color occurs. The amount of color developed is directly proportional to the concentration of human PAI-1 antigen in the tested sample. Normal range 1-25 ng/mL.

Determination of tissue-plasminogen activator/plasminogen activator inhibitor - 1 complex

Enzyme-linked immunosorbent assay (ZYMUTEST®, HYPHEN BioMed, France) was used to quantificate t-PA/PAI-1 complex. The assay contained purified monoclonal antibodies that are specific for human t-PA. When present, t-PA/PAI-1 complexes were captured onto the solid phase through the t-PA moiety. Following a washing step, the immunoconjugate, which is an anti-PAI-1 monoclonal antibody coupled to horse radish peroxidase, was introduced. The latter conjugate was bound to its specific epitope on PAI-1 present in t-PA/PAI-1 complexes. Following a new washing step, the peroxidase substrate, tetramethylbenzidine in the presence of hydrogen peroxide, was introduced and a blue color is displayed. The color turns yellow when the reaction is stopped with sulfuric acid. The amount of color developed is directly proportional to the concentration of human t-PA/PAI-1 complexes in the tested sample. Normal range < 5 ng/mL.

Determination of D-dimer

The immunoturbidimetric test (D-dimer PLUS®, Dade Behring, Marburg, Germany) was used for quantitative determination of cross-linked fibrin degradation products (D-dimer). Principally, the procedure is based on polystyrene particles, that are covalently coated with a monoclonal antibody (8D3) and becomes aggregated when mixed with samples containing D-dimer. The D-dimer cross-linkage region has a stereo-symmetrical structure. Consequently, one antibody suffices in order to trigger an aggregation reaction, which is than detected turbidimetrically via the increase in turbidity. Normal range < 300 ng/mL.

Assessment of standard coagulation tests: fibrinogen, PI, INR, APTT and of Hb and PLT count

Blood samples were tested for standard coagulation variables such as PI, INR, APTT and fibrinogen. In addition, Ht, Hb and PLT count also were taken from a peripheral venous catheter at the data collection points at T0, T1, T6 and T24.

Hb and PLT count were determined using Sysmex® CA-1600 analyzer (Siemens Healthcare Diagnostics, Marburg, Germany) [178].

Hb and PLT count

Hb concentration and PLT count were analyzed by means of a Beckman Coulter LH 750 Hematology Analyzer. The Coulter LH 750 uses impedance technology to measure PLT count (normal range 150 - 450 x 10^9/L). A hemoglobin cyanide method was used to measure Hb concentration. Normal range Hb 120 - 160 g/L.

Fibrinogen

Fibrinogen plasma concentration was determined as described by *Clauss* [96]. In short, citrated plasma was brought to coagulation by administration of an excessive amount of thrombin 50 IU/mL (Multifibren U reagent, Siemens Healthcare Diagnostics, U.S.A.). Here the coagulation time depends largely on the fibrinogen content of the specimen. The reference value is 1.8-3.6 g/L. The measurement range lies between 0.8 and >12 g/L.

Prothrombin index and International Normalized Ratio

PI was analyzed with a prothrombin complex assay (Lyophilized Dade® and Innovin® reagent, Siemens Healthcare Diagnostics, U.S.A.). The method is based on the fact that the coagulation cascade is activated by incubating plasma with the optimal amount of thromboplastin and calcium. PI normal range is 70 - 120%.

The INR is determined according to the following equation [179]:

$$INR = R^{ISI}, \text{ where } R = \text{patient PT/mean normal PT}$$

ISI - the International Sensitivity Index of the reagent.

The INR values were computed automatically by coagulometer. Normal range for INR 0.2-1.8

Activated Partial Thromboplastin Time

APTT was analyzed in citrated human plasma (Pathrombin*SL reagent, Siemens Healthcare Diagnostics, U.S.A.). Pathrombin*SL reagent enable rapid screening for disorders of the intrinsic coagulation system and sensitively defects of FVIII and FIX.

In addition it can be used for monitoring heparin therapy [180]. Incubation of plasma with the optimal quantity of phospholipids and a surface activator leads to activation of factors of the intrinsic coagulation cascade. The addition of calcium ions triggers the coagulation process; the time to formation of a fibrin clot is measured. APTT normal range 26 - 36 s.

Thromboelastography

TEG®5000 (TEG® Haemoscope Corporation, Niles, U.S.A.), presented in Figure 2.2., was performed once immediately after surgery on admission in ICU (T1). According to the manufacturers instructions [181] blood samples from the radial artery catheter were collected by aspirating 20 ml of blood to perform heparinase-modified kaolin activated thromboelastography (hep.kTEG) and non-heparinase modified kaolin-activated thromboelastography (kTEG).

Figure 2.2. Thromboelastography (TEG®) 5000 Hemostasis Analyzer System Reprinted from www.haemonetics.com

The TEG® 5000 analyzer measures the clot's physical property by the use of a special stationary cylindrical cup that contains the blood sample and is oscillated through an angle of 4^0 45 (Figure 2.3.). Each rotation cycle lasts 10 sec. A spin is suspended in the blood by a torsion wire and is monitored for motions. The torque of the rotating cup is transmitted to the immersed pin only after fibrin-platelet binding has linked the cup and the pin together. The strength of these fibrin-platelet bonds affects the magnitude of the pin motion, such that strong clots move the pin directly in phase with cup motion. Thus, the magnitude of the output is directly related to the strength of the formed clot. As the clots retract or lyse the bonds are broken and the transfer of cup

motions are diminished. The rotating movements of the pin are converted by a mechanical-electrical transducer to an electrical signal, which can be monitored on the screen of a computer [181].

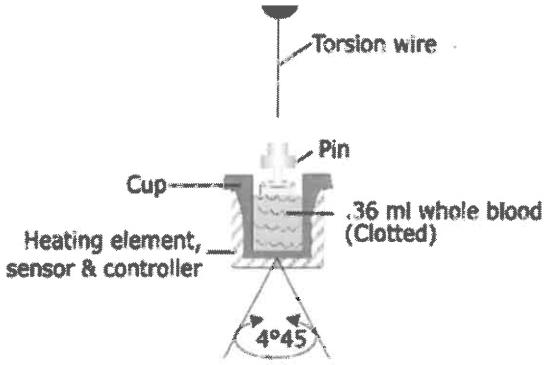

Figure 2.3. Thromboelastography (TEG®) 5000 sample cup design
Reprinted from TEG® 5000 User Manual, Design and Principles of operation, 2007: 10

Hep.kTEG has heparinase containing cups containing 2 IU of lyophilized heparinase-1 enzyme from *Flavobacterium heparinum*, which is sufficient to rapidly and specifically reverse 6 U/ml of heparin in whole blood. Heparinase acts by cleaving the heparin molecule into small inactive fragments without affecting the function of other blood components involved in coagulation.

kTEG contains kaolin (hydrated aluminum silicate) which activates the intrinsic pathway via factor XII.

The following TEG® parameters were recorded: reaction time – R (normal range 4-8 minutes), clot formation time - K (normal range 1-4 min), the alpha angle – A (normal range 47^0-78^0) and maximum amplitude – MA (normal range 55-73 mm).

Gene determination

Blood samples were taken preoperatively from a central vein at time point T0 into EDTA vacutainers, which consist sodium citrate and heparin as anticoagulants. Gene determination consist of 5 steps: genomic deoxyribonucleic acid (DNA) extraction, polymerize chain reaction (PCR), electrophoresis of agarose gel, purification of PCR products and sequencing.

Genomic DNA extraction

DNA was extracted using standard fenol-chlorophorm extraction method, which consists of 4 stages:

1. Distribution and purification of white blood cells

Is performed by addition of- 10 ml of RBC Lysis Al solution into 50 ml tubes. Than, the white blood cells can be carefully suctioned from the top of the tube and subsequently transferred to another 50 ml tube. Once more 10 ml of RBC Lysis Al solution is added to the cell suspension in the 50 ml tubes. Slowly white blood cells are mixed together with the solution by turning the tube around three times. Thereafter, the cell suspension is placed in a refrigerator (FR240-1501A+, Snaige, Lithuania) for 15 minutes of incubation at a temperature of + 4 ± 2 °C. Subsequently, the samples are cool- centrifuged at + 4°C (Universal 32, Hettich, Germany) with 4000 rotations for 15 minutes. After centrifugation, the supernatant is removed. The precipitate together with the remaining buffer is suspended by using a dropper (Teccan Freedom Evo, Tecan group Ltd, Switzerland) and carefully avoiding damage to the white blood cells.

2. The lysis of white blood cells

Cells are transferred into 15 ml tubes. Than, 5 ml of a cell suspension solution is added to the cell precipitate and mixed thoroughly until the white cells are suspended in the solution. After addition of 0.4 ml 10% sodium dodecyl sulfate solution and turning the tube around three times for mixing, 5 µl Proteinase K is added and mixed together by repeating the three times turning of the tube. After this, the sample tube is ready for incubation after an overnight incubation in a thermostated cabinet (TC-80 M-2, Med Labor Technique, Russia) keeping a temperature of + 50 °C.

3. DNA extraction from lysate

Blood samples were incubated after addition of 5 ml Phenol solution (Stanlab, Poland) . Samples were shaken for at least15 minutes in a special mixing device (Minigen type 4TEC, Biosan, Latvia) and than centrifuged at + 20 °C (Universal 32, Hettich, Germany) at 4000 rotations/min for 10 minutes. The surface coat was

transferred into a new 15 ml tube. Than, 5 ml of chloroform (Rigas Chemistry, Latvia) was added and mixed together for 5 minutes in a mixing device and subsequently centrifuged at + 20 °C, with 4000 rotations/min for 10 minutes. Once more, the surface layer was transferred into new 15 ml tubes. Slowly 5 ml of isopropylspiritus was added, carefully and mixed together until a precipitate of DNA appeared. Again, the samples were centrifuged at + 20 °C using 4000 rotations/min for 10 minutes. As soon as the centrifuge stopped, a part of the liquid was removed from the tube and replaced by 5 ml 70% ethanol solution,mixed together and incubated for 2 minutes. Again the samples were centrifuged at + 20 °C with 4000 rotations/min for 10 minutes. After centrifugation accurately 70% ethanol solution is spilled off from sample tubes. The tubes are inverted and leaved on filter paper. Precipitate was left for drying for 10 minutes at room temperature.

4. Dissolving of DNA

1ml of DNA Hydration solution (TE buffer) was added to the precipitate and left on a stirrer for slowly stirring for one night. In the next morning the samples were put into a refrigerator at + 4 ± 2 °C and is kept there for one week. Afterwards, the tubes with the DNA were shaken for 10 sec and centrifuged for a short time (until 1 minute).

After dissolution of DNA its concentration was measured with nanodrop. Consequently, DNA was distributed in polymerize chain reaction (PCR) plates and diluted in water – final concentration - 7 ng/μL, 200. Later, 4 μL of this solution was transferred to a different PCR plate which will be directly used for PCR. For single PCR reaction 28 ng of genomic DNA was used. This dilution distribution is done by automatic pipetting device Tecan Freedom Evo (Tecan Group Ltd, Switzerland). Plated are than dried overnight and stored in refrigerator at - 20 °C until used.

Polymerize chain reaction

Region harboring depending of analyzed gene polymorphism was PCR amplified by using sequence specific primers (Metabion, Germany). DNA synthesis is a cyclic process, which is repeated 20 - 40 times using special PCR system (ViiA7, Life technologies, U.S.A.). In every cycle DNA is doubled.

During the PCR synthesis of DNA can be divided into three stages: DNA denaturation, hybridization of oligonucleotides and DNA synthesis.

1. DNA denaturation - samples are heated till the temperature of 94 - 98 C^0 until the hydrogen bonds between the DNA complementary threads are degraded. As a result, single DNA is made. It can be used for synthesis of a complementary thread with the help of enzyme polymerize.

2. Hybridization of oligonucleotides - the oligonucleotides bind with complementary DNA regions when the temperature is decreased till 50 - 65 C^0.

3. DNA synthesis - samples are heated till the temperature of 72 C^0, which is an optimale temperature for adequate activity of Tag polymerize. Enzyme polymerize binds with a complex of oligonucleotides and DNA matrix and makes synthesis of DNA.

Electrophoresis of agarose gel

The agarose gel electrophoresis technique is used for identification of DNA fragments to confirm the results obtained with the PCR technique. Gel is colored with ethidium bromide (Invitrogen, U.S.A.). When ethidium bromide binds with DNA, it makes the complex which fluorescens in ultra-violet light. To be able to follow up the process of electrophoresis samples are mixed together with coloring substance before they are carried up on the gel. To determine the length of the sample fragments, a length marker (Ladder DNA marker, Fermentas, Lithuania) is carried up on the gel. In the present study, a 100 base pairs (bp) marker was used and electrophoresis was made at 90-100 miliampers (mA) power.

Purification of PCR products

PCR products were purified using Shrimp Alkaline Phosphatase/Exonuclease I (Fermentas, Lithuania). Shrimp Alkaline Phosphatase makes the dephosphorilization of oligonucleotides and Exonuclease I split single DNA.

Sequencing

Sequencing is a method of base pair sequence determination in specific regions of the DNA. DNA is like a matrix, which consists of fragments that differ from each other by only one base length. Before sequencing, DNA was cleaned out from fluorescent nucleotides with sephadex G-50 (Sigma Aldrich, U.S.A.), the sequencing reaction was done using Abi Prizm 3130xl Genetic Analyzer (Applied Biosystems, U.S.A.). Sequence analysis was done with Finch TV software. The following reagents were used for one sample: 5 μL dH_2O, 2 μL 5 x Seq buffer, 0,5 μL BigDye, 0,5 μL the oligonucleotide (Applied Biosystems, U.S.A.).

For ***determination of PAI-1 gene polymorphisms*** the Sanger sequencing was used. In short – PCR amplification of the region harbouring polymorphism of interest was done using sequence specific primers (see table 2.2). After detection of amplified fragments by Agarose gel electrophoresis, PCR fragments were sequenced, sequences were analyzed and polymorphism was detected as described above.

In table 2.2. we present the amplified regions of the PAI-1 gene investigated in the study.

Table .2.2.

Synthetic oligonucleotides and amplified regions of PAI gene

SNP	olygo-nucleotide	sequence	amplified region
rs1799768	PAI-IF	5'-ATCCCTTTTCCCCTTGTGTC-3'	chr7:100,769,460-100,769,948
	PAI-IR	5'- AACCTCCATCAAAACGTGGA-3'	
rs2227631	PAI-844-F	5'-GGACCACTGCTCCACAGAAT-3'	chr7:100,362,793-100,363,117
	PAI-844-R	5'-CCTGAGGGCTCTCTTGTGTC-3'	

Abbreviations: SNP, single-nucleotide polymorphism; PAI, plasminogen activator inhibitor gene; F, forward primer; R, reverse primer; rs, analyzed polymorphism ; chr, chromosoma

Graphic images - sequence chromatograms - presenting detection of PAI-1 gene -844 A/G polymorphism are shown in the Figure 2.4.

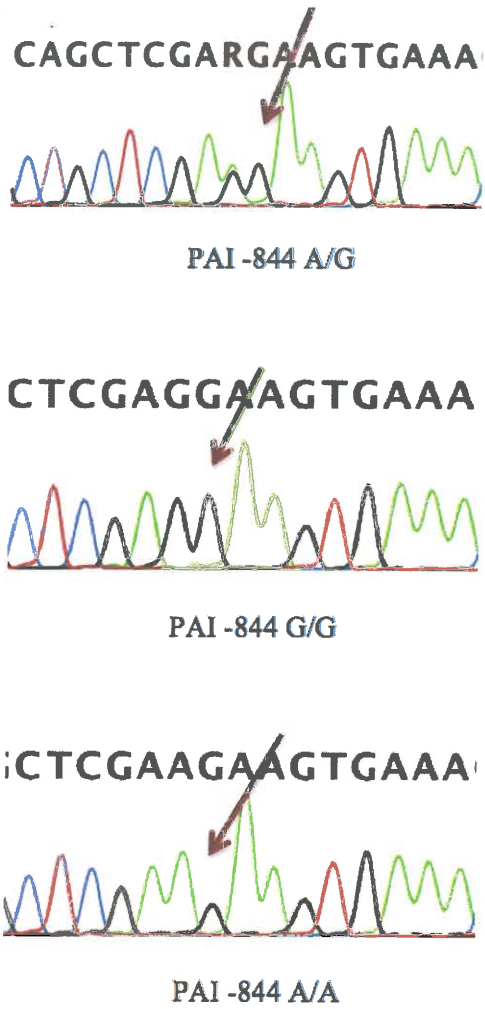

Figure 2.4. Sequence chromatogram of plasminogen activator inhibitor type-1 (PAI-1) gene -844 adenosine/guanine (A/G) polymorphism
Chromatogram made by dr.med. Liene Ņikitina-Zaķe

For ***determination of ACE I/D polymorphism*** approach as described by *H. Tomita et al.* [182] was used. In short: insertion and deletion alleles were identified using PCR amplification of respective fragments from intron 16 in the ACE gene and fragment size determination by agarose gel electrophoresis. PCR was performed using primers ACE-ID-forward/reverse (F/R) (table 2.3.). The amplification products were determined in agarose gel. Fragment size harboring delation allele visualized at 191bp, and insertion allele I at 478 bp (presented in figure 2.5.). Because deletion allele is

shorter and, thus, is preferentially amplified in heterozygous, we used separate primer pair (ACE-IN-F/R) for all samples which were identified as DD homozygous. This PCR reaction yields amplification at 335 bp only if Allele I is present. No products in homozygous for DD are visualized.

In table 2.3. we present the primers used in ACE gene analysis.

Table .2.3.

Primers used in ACE I/D analysis

SNP	olygo-nucleotide	sequence	amplified region
rs1799752	ACE ID F	5'- CTGGAGACCACTCCCATCCTTTCT-3'	chr17:61,565,843-61,566,033
	ACE ID R	5'- GATGTGGCCATCACATTCGTCAGAT-3'	
rs1799752	ACE IN F	5'- TGGGACCACAGCGCCCGCCACTAC-3'	
	ACE IN R	5'- TCGCCAGCCCTCCCATGCCCATAA-3'	

Abbreviations: SNP, single-nucleotide polymorphism; ACE, angiotensin converting enzyme gene; I/D, insertion/delation; ID, deletion; IN, insertion; F, forward primer; R, reverse primer; rs, analyzed polymorphism ; chr, chromosome

In Figure 2.5. we present the analysis of ACE gene Intron 16 I/D polymorphism and display the following: 1A is a carrier of D/D homozygosity; 1B is the same patient as in 1A, where analyzed gene region does not contain the insertion; 2A is a carrier of I/I homozygosity; 2B shows gene region with positive insertion; 3A is a carrier of I/D heterozygosity; 3B shows positive insertion in the analyzed gene region

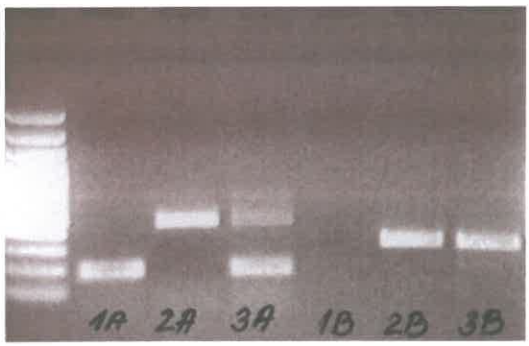

Figure 2.5. Angiotensin converting enzyme (ACE) gene Intron 16 insertion/delation I/D polymorphism analysis

1A, 2A, 3A - amplification of the region with I/D, 1B, 2B, 3B - the same samples amplified for specific insertion fragment

Postoperative bleeding volume

Was recorded as chest tube drainage (CTD) in milliliters at the time points T1, T4 and T24. Indication for reoperation because of suspected surgical bleeding was based on evaluation of clinical and hemodynamic changes. More precisely a surgical bleeding was diagnosed at the time of re-exploration if one or more specific bleeding sites were identified. If the patient was re-operated, the CTD volume until reoperation, and 24 hours afterwards was registered. Patients with diagnosed surgical bleeding during reoperation were excluded from the further study. If no specific site was located, the bleeding was registered as a hemostatic disorder and the patient was included in the study group.

2.2.3. Methods of statistical analysis

The statistical analysis was performed using Statistical Package for the Social Sciences (SPSS® version 20.0) and Microsoft Excel programs. In the present study continuous variables were described as the mean ± standard deviation (SD) and categorical variables as percentages (%). Statistical significance was defined as a $p < 0.05$. The sample size was determined on the basis of the number of cases according to inclusion criteria hospitalized during the study period.

In the study the following statistical tests were used:

- Descriptive statistics
- Chi-square test
- Pearson correlation coefficient
- Mann-Whitney U test
- Kruskal - Wallis H test
- Student t-test

Descriptive statistics

Is the discipline of quantitatively describing the main features of a collection of data. Descriptive statistics provides simple summaries about the sample and about the

observations that have been made. Such summaries may be either quantitative, i.e. summary statistics, or visual, i.e. simple-to-understand graphs. One of the main aims of descriptive statistics is to facilitate the comparison of different populations [183].

In the present study descriptive statistics were used for continuous variables, which were described as mean and standard deviation (SD).

Chi-square test

A Chi-square (X^2) statistic was used to investigate whether distributions of categorical variables differ from each other. The Chi-Square statistic compares categorical responses between two (or more) independent groups. Different Chi-square tests are available: Pearson's Chi-squared test, Yates's Chi-squared test, Cochran-Mantel-Haenszel Chi-squared test, McNemar's test and others.

In the present study, the chi-square test was used to compare baseline categorical characteristics between patient groups.

Pearson correlation coefficient (PCCoef.)

PCCoef. is a measure of the correlation (linear dependence) between two variables X and Y, given a value between +1 and −1. A value of 1 implies that a linear equation describes the relationship between X and Y perfectly, with all data points lying on a straight line for which Y increases as X increases. A value of −1 implies that all data points lie on a line for which Y decreases as X increases. A value of 0 implies that there is no linear correlation between the variables. Correlation can be defined as negative or positive in a range from none to strong (Table 2.4.). PCCoef is widely used in science as a measure of the strength of linear dependence between two variables. It was developed by *Karl Pearson* from a similar but slightly different idea introduced by *Francis Galton* in the 1880s [184, 185].

Several authors have offered guidelines for the interpretation of a PCCoef. [186, 187]. However, all such criteria are in some ways arbitrary and should not be observed too strictly [187].

Table 2.4.

Interpretation of the correlation coefficient.

Correlation	Negative	Positive
None	- 0.09 to 0.0	0.0 to 0.09
Small	- 0.3 to - 0.1	0.1 to 0.3
Medium	- 0.5 to - 0.3	0.3 to 0.5
Strong	- 1.0 to - 0.5	0.5 to 1.0

Pearson's distance can be measured, which is a metric distance for two variables X and Y. It can be defined from PCCoef. as

$$d_{x,y} = 1 - p_{x,y}$$

Considering that the PCCoef. falls between -1 and 1, the Pearson distance lies in 0 to 2 [188].

In the present study PCCoef. was calculated between hematologic (fibrinolytic and coagulation variables) and demographic data, surgical parameters and the volume of 24-hour postoperative bleeding.

Mann-Whitney U test

It is also called the Mann–Whitney–Wilcoxon or Wilcoxon rank-sum test. It is a non-parametric statistical hypothesis test for assessing whether one of two samples of independent observations tends to have values that are either larger or smaller than that of the other group. It is one of the most well known non-parametric significance tests. It was proposed initially by the *German Gustav Deuchler* [189] in 1914 and later, independently, by *Frank Wilcoxon* [190] in 1945 for equal sample sizes, and extended to arbitrary sample sizes and in other ways by *Henry Mann* and his student *Donald Ransom Whitney* in 1947 [191].

Mann Whitney U test is considered to be more robust and more efficient than the Student t-test, it remains the logical choice when the data are ordinal but not interval scaled, so that the spacing between adjacent values cannot be assumed to be constant.

In the present study Mann-Whitney U test was used to compare non-parametric variables between two groups of patients.

Kruskal - Wallis H test

The Kruskal–Wallis H test is another non-parametric method for analyzing whether samples originate from the same distribution. It is used for comparing two or more samples that are independent, or not related. The parametric equivalent to the Kruskal-Wallis test is the one-way analysis of variance (ANOVA). The factual null hypothesis is that the populations from which the samples originate have the same median. When the Kruskal-Wallis H test leads to significant results, at least one of the samples differs from the other samples. The test does not identify where the differences occur or how many differences actually occur. It is an extension of the Mann-Whitney U test to three or more groups. The Mann-Whitney may help to analyze the specific sample pairs for significant differences [192]. Since it is a non-parametric method, the Kruskal–Wallis H test does not assume a normal distribution. However, the test does assume an identically shaped and scaled distribution for each group, except for any difference in medians.

In the present study, Kruskal - Wallis H test was used for non-parametric variables to compare and find significant differences between three or more patient groups.

Student t-test

The Student t-test was introduced in 1908 by *William Sealy Gosset* [193, 194]. *Gosset* devised the t-test as a cheap way to monitor the quality of stout [194]. It is most commonly applied statistical method for testing of normally distributed quantitative data sets. The t-test can be performed knowing just the means, standard deviation, and number of data points.

In the present study, Student t-test was performed to compare parametric variables with normal distribution between two patient groups.

3. RESULTS

3.1. Clinical results

Totally 90 consecutive adult cardiac surgical patients (47 men and 43 women) 65 ± 11 years (mean ± SD) of age were considered for inclusion in the study. Seven patients (7.8%) required re-operation due to hemipericardium or excessive surgical bleeding between 10 minutes and 32 hours after the surgery, as assessed by the CTD system, but none of them died because of bleeding or re-operation. The seven patients were discarded from further data analysis. As surveyed in Table 3.1., 83 patients (42 men and 41 women) were subjected to further analysis.

Table 3.1.

Characteristic of demographic data and preoperative parameters of 83 patients subjected to study

Demographic data	Mean ± SD	Range (min.-max.)
Age, years	65 ± 11	28 - 82
BSA (m^2), *Mosteller*	1.9 ± 0.2	1.6 - 2.5
BMI (kg/m^2)	28 ± 5	18 - 40
EF (%)	56 ± 8	35 - 74
EuroSCORE I (%)	4.8 ± 1.8	1.2 - 8.4
Preoperative parameters	Mean ± SD	Range (min.-max.)
Hemoglobin, g/dL	136 ± 15	90 - 166
Hematocrit, %	40 ± 4.8	26 - 49
APTT, s	34.4 ± 6.9	26 - 75
Platelet count, x 10^9/L	216 ± 58	120 - 450
Prothrombin index, %	89 ± 14	52 - 129
Fibrinogen, g/L	4.6 ± 1.3	1.8 - 10

Abbreviations: BSA, body surface area; BMI, body mass index; EF, ejection fraction; EuroSCORE, European System for Cardiac Operative Risk Evaluation; APTT, activated partial thromboplastin time; SD, standard deviation

Two patients out of the 83 had postoperative complications. One patient had pneumonia from the fifth postoperative day, and the second had hepatorenal syndrome from the third day after surgery. Two patients died in hospital, one from acute heart failure the second postoperative day and another from ileuss because of mesenteric thrombosis five days postoperatively.

Type of surgery

All the patients included in the study underwent elective cardiac surgery. The distribution of patients according to type of surgery (Figure 3.1) was as follows:

- 34 patients (41%) underwent CABG
- 31 patients (37%) had valve replacement surgery
- 18 patients (22%) had mixed (CABG + valve) surgery

Cell saver was used in 76% of the cases.

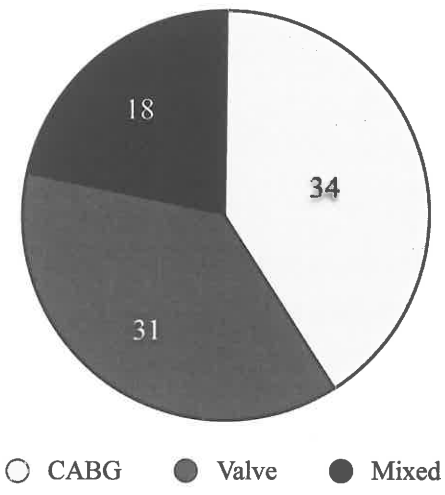

Figure 3.1. Division of patients according to type of surgery

Abbreviations: CABG, coronary artery bypass grafting surgery

Preoperative medications

Preoperatively 59 (71%) patients were treated with one or more antiaggregants or anticoagulants. Fifty-six patients received aspirin within 7 ± 2 days before surgery, 16 patients were treated with clopidogrel within 8 ± 2 days and 59 patients received LMWH – with the last injection 12 hours before surgery. Four patients were treated with warfarin until 5 days before surgery (Figure 3.2.). There were not statistically significant differences in 24-hour blood loss between patients treated preoperatively with various antiaggregants and anticoagulants.

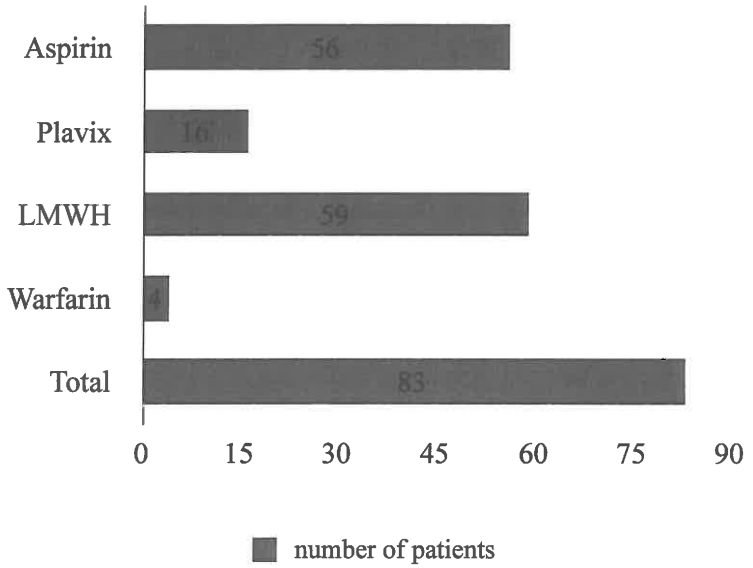

Figure 3.2. Preoperative medications

Abbreviations: LMWH, low molecular weight heparin ;

From 83 patients requiring open heart surgery (32.5%) had history of myocardial infarction, (20.5%) hypercholesterolemia and (15.7%) chronic angina pectoris. Most often observed co-morbidities were primary hypertension (52%), diabetes mellitus type 2 (25.3%) and chronic obstructive pulmonary disease (COPD; 13.3%).

Most often the patients had combinations of primary hypertension and history of myocardial infarction or primary hypertension with hypercholesterolemia and diabetes mellitus type 2.

Surgical parameters and blood loss after surgery (Table 3.2).

Regarding times spent on surgical details, the following apply: mean duration of CPB 105 ± 40 minutes and mean times of aorta occlusion and reperfusion 66 ± 27 minutes and 34 ±15 minutes, respectively.

The mean bladder temperature during CPB was 35.3 ± 0.4 C^0. On admission to the ICU practically all the patients had already normal axillar temperature 36 ± 0.4 C^0 range from 35 - 37 C^0. Therefore we speculate that body temperature should not influence coagulation system after admission to ICU.

Table 3.2.

Surgical parameters and blood loss after surgery

Surgical parameters	Mean ± SD	Range (min.-max.)
CPB duration (min)	105 ± 40	52 - 252
Aorta occlusion time (min)	66 ± 27	17 - 175
Reperfusion time (min)	34 ± 15	12 - 83
Heparin dose, mL	7.6 ± 1.4	5 - 13
Protamine dose, mg	302 ± 66	200 - 500
Baseline ACT , s	141 ± 23	95- 202
Postprotamine ACT, s	130 ± 13	92 - 180
Temp. on CPB, ^0C	35.3 ± 0.4	34 - 36.2
Temp on ICU, ^0C	36 ± 0.4	35 - 37
Deltajonin, mL	1503 ± 516	600 - 3200
Cardioplegia, mL	1785 ± 584	524 - 3900
Blood loss, mL T1	60 ± 32	15 - 150
Blood loss, mL T4	208 ± 120	55 - 510
Blood loss, mL T24	569 ± 270	80 - 1250

Abbreviations: CPB, cardiopulmonary bypass; ACT, activated coagulation time; s, seconds; temp, temperature; T1, on admission in ICU; T4, four hours after surgery; T24, twenty-four hours after surgery. Data are given mean ± standard deviation (SD) and min.-max. range.

Surgical parameters such as durations of CPB and aorta occlusion, time to reperfusion, temperature during CPB and upon admission to the ICU as well as postoperative blood loss after different types of open heart surgery (CABG, valve or mixed surgery) were analyzed. Statistically significant differences between the various categories of surgery were found in duration of CPB, aorta occlusion- and reperfusion times and in body temperature during CPB. However, we noticed no significant difference in postoperative blood loss at 1, 4 and 24-hours after surgery (Table 3.3).

Table 3.3.

Surgical parameters and blood loss after surgery depending on type of surgery

Type of surgery	CABG, n = 34	Valve, n = 31	Mixed, n = 18	p value
CPB duration (min)	90 ± 25	90 ± 25	146 ± 50	< 0.001*
Aorta occlusion time (min)	54 ± 18	54 ± 17	94 ± 31	0.01*
Reperfusion time (min)	32 ± 15	33 ± 15	42 ± 16	< 0.001*
Temp. on CPB, C^0	35.4 ± 0.4	35.4 ± 0.5	35.0 ± 0.3	0.03*
Temp on ICU, C^0	36.0 ± 0.4	35.7. ± 1	36.0 ± 0.4	0.2
Blood loss, ml/1h	56 ± 30	61 ± 29	65 ± 30	0.7
Blood loss, ml/4h	195 ± 106	203 ± 112	241 ± 154	0.75
Blood loss, ml/24h	570 ± 289	552 ± 238	597 ± 297	0.9

Abbreviations: CPB, cardiopulmonary bypass; ICU, intensive care unit; temp., temperature; SD, standard deviation
Data are given mean ± standard deviation (SD), * statistical significant difference $p < 0.05$

3. 2. Coagulation tests in association with bleeding

3.2.1. APTT, PI, PLT and Fibrinogen

Activated Partial Thromboplastin Time

All patients had preoperative APTT values above the lower limit of 26 s. Higher or equal APTT values as compared to the normal value of 36 s preoperatively were registered in 27 patients and 21 of them received LMWH, and the last dose was administered 12 hours before surgery.

At T1 mean APTT was 34 ± 4.8 s There is a general tendency towards higher APTT values since 25 out of 83 patients had values that were equal to - or higher in comparison with normal.

At T6 the highest APTT values were detected, which statistically differed as compare to APTL values at T0. The mean values were increased by about 18%. 57 patients had APTT values that were equal to or higher than 36 sec., with a mean value of 47 ± 13 s.

At T24 mean APTT was 38 ± 7 s Also showed significant difference if compare with APTL at T0. Thus, still 51 patients had APTT values that were equal to or higher than normal. APTT mean values at different time points are shown in Table 3.4.

Table 3.4.

Mean APTT values at different time points

Time point	Mean ± SD	Range (min.-max.)
APTT, s T0	34.4 ± 6.9*≠	26 - 75
APTT, s T1	34 ± 4.8	25 - 48
APTT, s T6	42 ± 13*	29 - 104
APTT, s T24	38 ± 7≠	28 - 74

Abbreviations: APTT, activated partial thromboplastin time; s, seconds; T0, preoperatively one day before surgery; T1, on admission in ICU; T6, six hours after surgery; T24, twenty-four hours after surgery. Data are given mean ± standard deviation (SD) and min.-max. range.
*$p < 0.05$ between APTT at T0 and T6; ≠$p < 0.05$ between APTT at T0 and T2

Only APTT determinate preoperatively at T0 showed medium positive correlation with 4-hour postoperative blood loss ($r = 0.3$, $p = 0.01$).

Regarding postoperative blood loss at T1, T4 and T24 time points after surgery, it did not differ statistically between patients with normal or higher APTT values.

Prothrombin Index

Preoperatively, most of the patients had PI within the normal range and only 7 patients were observed with lower PI values as compared with normal (70%).

AT T1, there was a minimal tendency towards higher than normal PI values, but already at T6 and at T24 PI returned to baseline. PI values postoperatively did not differed significantly as compared with PI at T0. Mean values of PI at different time points are shown at Table 3.5.

We found no correlation between PI and postoperative blood loss analyzed at three time points.

Table 3.5.

Mean PT values at different time points

Time point	Mean ± SD	Range (min.-max.)
PI, % T0	89 ± 14	52 - 129
PI, % T1	90 ± 12	62 - 125
PI, % T6	87 ± 12	57 - 116
PI, % T24	88 ± 12	61 - 125

Abbreviations: PI, prothrombin index; T0, preoperatively one day before surgery; T1, on admission in ICU; T6, six hours after surgery; T24, twenty-four hours after surgery. Data are given mean ± standard deviation (SD) and min.-max. range.

Platelet count

Mean PLT count at T0 was 216 ± 58 $\times 10^9$/L and 9 patients preoperatively had lower values of PLT as compared with normal, with the lowest value of 120 $\times 10^9$/L. However, these values were not associated with a greater bleeding tendency after surgery.

During the first 24 hours, there is an obvious tendency for PLT count to decrease after surgery. At T1 50 and at T6 49 out of 83 patients had PLT counts less than the

normal value. The lowest mean PLT count 140 ± 47 $\times 10^9$/L was observed 24 hours after surgery. Correspondingly, the range was from 48 - 324 $\times 10^9$/L. It decreased to approximately by 35.2% of baseline and 51 patients had PLT count values below normal. Mean PLT counts at different time points are shown at Table 3.6.

Table 3.6.

Mean PLT values at different time points

Time point	Mean ± SD	Range (min.-max.)
PLT, $\times 10^9$/L T0	216 ± 58*	120 - 450
PLT, $\times 10^9$/L T1	144 ± 47*	75 - 346
PLT, $\times 10^9$/L T6	146 ± 49*	56 - 345
PLT, $\times 10^9$/L T24	140 ± 47*	48 - 324

Abbreviations: PLT, platelet count; T0, preoperatively one day before surgery; T1, on admission in ICU; T6, six hours after surgery; T24, twenty-four hours after surgery. Data are given mean ± standard deviation (SD) and min.-max. range.
*$p < 0.05$, when compare with PLT count at T0

At T6 and T24 significant difference ($p = 0.03$; $p = 0.01$) between the patients with normal and low PLT counts (< 150 $\times 10^9$/L) in postoperative blood loss was found (Figure 3.3.).

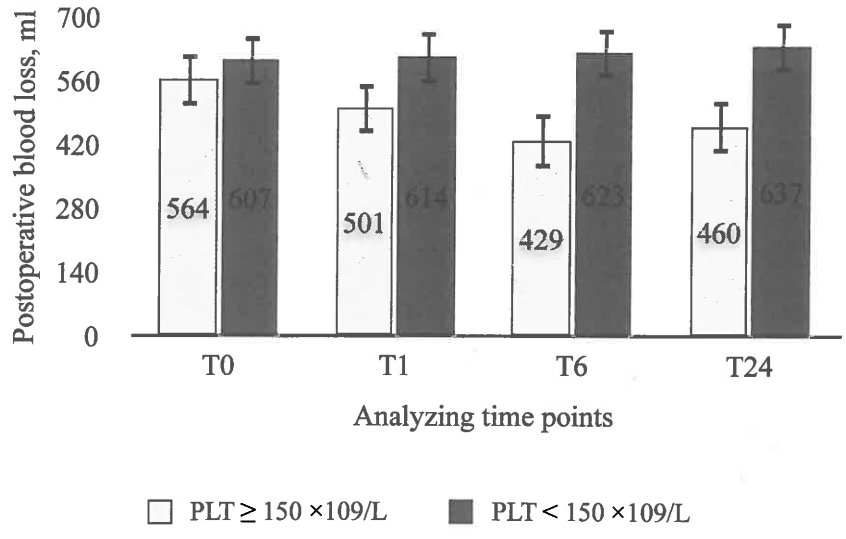

Figure 3.3. Postoperative blood loss comparing patients with platelet (PLT) count ≥ or < 150 $\times 10^9$/L at four time points

As expected, we found a correlation between PLT count and postoperative blood loss. The highest correlation was observed between T24 PLT count and T24 blood loss ($r = -0.3$, $p = 0.01$) as well as T6 PLT count and T24 blood loss ($r = -0.25$, $p = 0.02$).

Fibrinogen

All patients had a preoperative fibrinogen level within the normal range with a mean value of 4.1 ± 1.3 g/L. It decreased by 22% from baseline at T1 and only 4 patients at this time point had a fibrinogen level less than normal of 1.8 g/L. Fibrinogen level started to increase after 6 hours and continued to raise 24 hours after surgery. Mean plasma levels of fibrinogen at different time points are shown in Table 3.7.

Table 3.7.

Mean levels of fibrinogen at different time points

Time point	Mean ± SD	Range (min.-max.)
Fibrinogen, g/L T0	4.1 ± 1.3*≠	1.8 - 10
Fibrinogen, g/L T1	3.2 ± 1.05*	1.5 - 7.3
Fibrinogen, g/L T6	3.5 ± 0.9≠	1.8 - 6.6
Fibrinogen, g/L T24	4 ± 0.9	2.2 - 6.4

Abbreviations: T0, preoperatively one day before surgery; T1, on admission in ICU; T6, six hours after surgery; T24, twenty-four hours after surgery. Data are given mean ± standard deviation (SD) and min.-max. range.
*$p < 0.05$ between fibrinogen at T0 and T1; ≠ $p < 0.05$ between fibrinogen at T0 and T6

As expected fibrinogen showed the highest correlation with postoperative bleeding. Preoperative level of fibrinogen correlated with postoperative blood loss at T1 ($r = -0.3$, $p = 0.01$), at T4 ($r = -0.4$, $p < 0.0001$) and at T24 ($r = -0.5$, $p < 0.0001$), as shown in Figure 3.4.

The fibrinogen level at T6 reached significant correlation with blood loss at T1 ($r = -0.3$, $p = 0.007$), at T4 ($r = -0.4$, $p = 0.001$) and at T24 ($r = -0.4$, $p < 0.0001$).

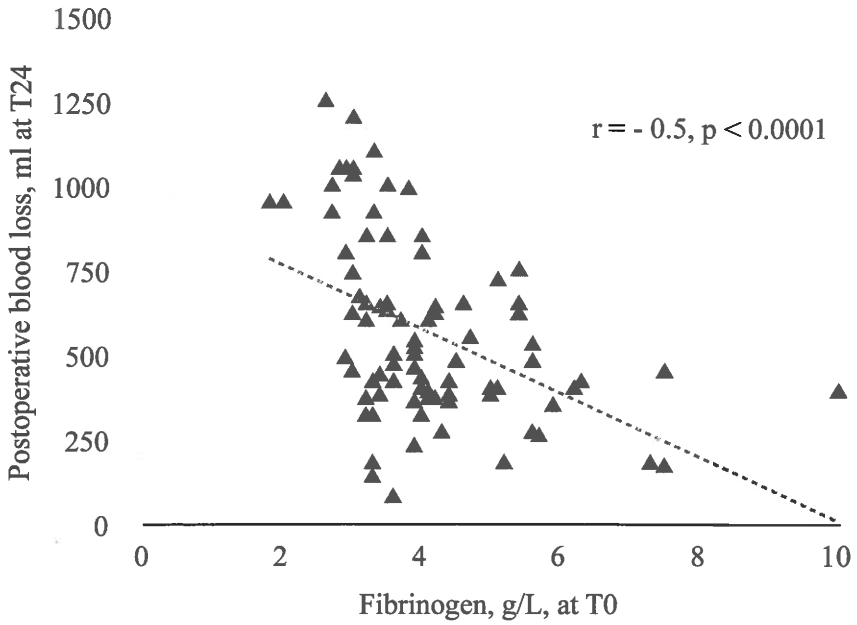

Figure 3.4. Preoperative (T0) fibrinogen plasma level correlation with postoperative 24-hour blood loss

3.2.2. Fibrinolysis parameters: PAI-1 and t-PA/PAI-1 complex

Plasminogen activator inhibitor type-1

Mean preoperative PAI-1 plasma concentration was 24 ± 12 ng/mL ranging from 3 to 50 ng/mL. PAI-1 values that are higher or equal to as normal of 25 ng/mL were noticed in 30 patients with a mean concentration of 38 ± 7.5 ng/mL. Out of the total of 83 patients, 53 had lower values than 25 ng/mL. The mean PAI-1 plasma concentration was 16 ± 6 ng/mL.

Patients with lower preoperative levels of PAI-1 than 25 ng/mL showed significantly greater postoperative bleeding volume: at T1 67 ± 31 ml *vs*. 47 ± 30 ml ($p = 0.008$) at T4 237 ± 118 ml *vs*. 159 ± 109 ml ($p = 0.003$) and at T24 635 ± 272 ml *vs*. 454 ± 227 ml ($p = 0.002$), as shown in Figure 3.5.

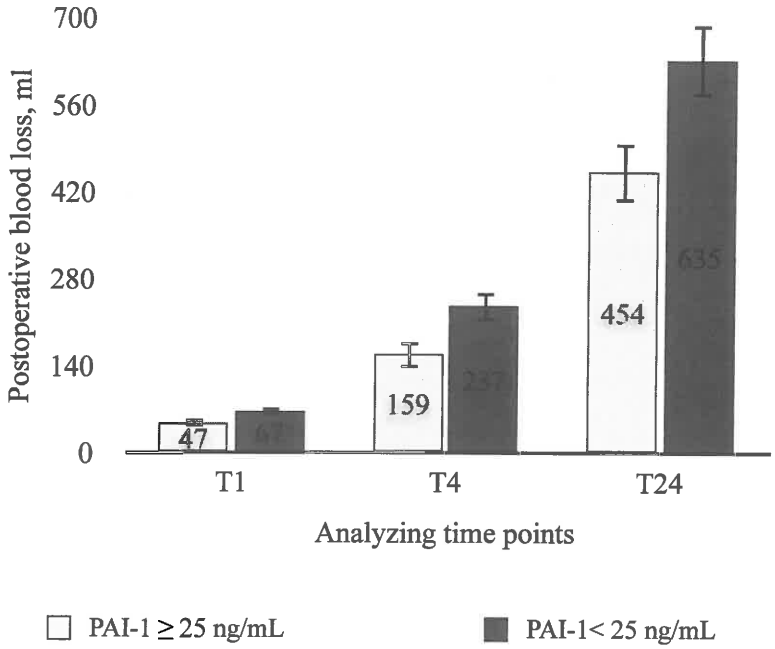

Figure 3.5. Preoperative plasminogen activator inhibitor type-1 (PAI-1) plasma concentrations and postoperative blood loss at three time points (T1, T4 and T24) between patients with preoperative PAI-1 levels ≥ or < 25 ng/mL

Preoperative PAI-1 showed correlation with postoperative blood loss at all three time points: T1, T4 and T24, when volume of blood loss was fixed. The highest association was found between preoperative PAI-1 plasma concentration and 24-hour blood loss (r = - 0.3, p = 0.01). Presented in Figure 3.6.

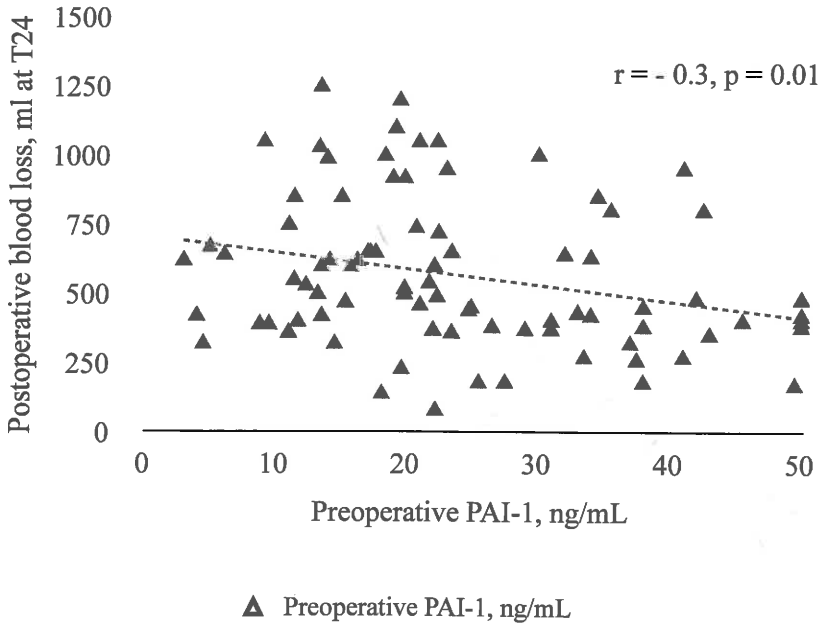

Figure 3.6. Preoperative plasminogen activator inhibitor type-1 (PAI-1) plasma concentration correlation with postoperative 24-hour blood loss

Complex of tissue plasminogen activator/plasminogen activator inhibitor-1

Mean postoperative t-PA/PAI-1 plasma concentration measured 24 hours after surgery was 3.6 ± 2.1 ng/mL with a range from 1 to 9.5 ng/mL. Eighteen patients had a complex concentration ≥ 5 ng/mL with a mean plasma complex concentration of 7.2 ± 1.3 ng/mL. Of the 83 patients, 65 had lower values than 5 ng/mL with a mean t-PA/ PAI-1 plasma concentration of 2.6 ± 0.9 ng/mL.

Patients with lower postoperative levels of t-PA/PAI-1 than 5 ng/mL showed significantly greater postoperative bleeding: at T1 63 ± 34 ml *vs*. 49 ± 16 ml (p = 0.02), at T4 220 ± 131 ml *vs*. 169 ± 59 ml (p = 0.02) and at T24 598 ± 287 ml *vs*. 465 ± 163 ml (p = 0.01). Figure 3.7.

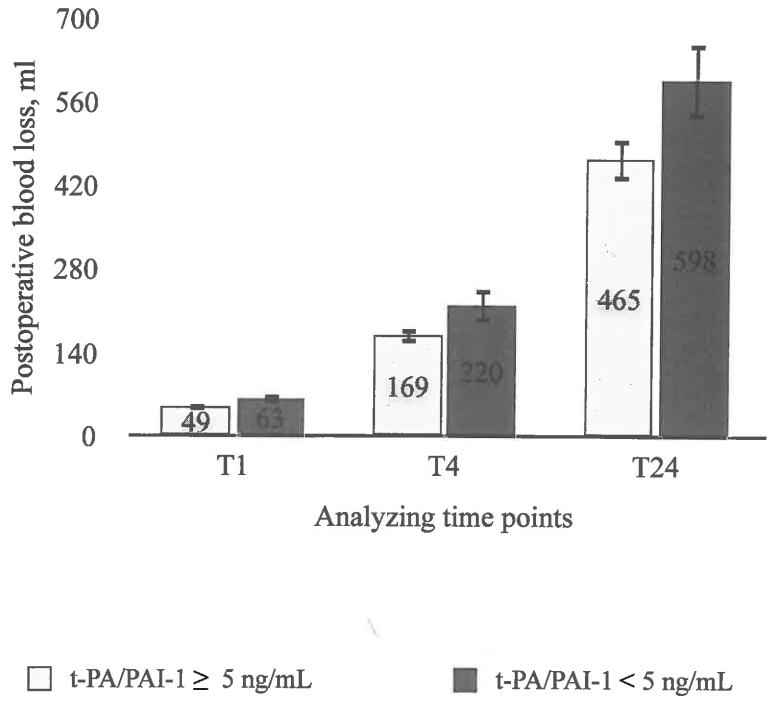

Figure 3.7. Postoperative plasma concentrations of tissue plasminogen activator/ plasminogen activator inhibitor type-1 complex (t-PA/PAI-1) and postoperative blood loss at three time points (T1, T4 and T24) between patients with postoperative t-PA/PAI-1 levels ≥ or < 5 ng/mL

Complex of t-PA/PA-1 did not show any significant correlation with postoperative bleeding volume determinated at three times points.

3.3. Genetic polymorphisms and fibrinolytic bleeding

3.3.1. PAI-1 gene -675 (4G/5G) polymorphism

Analyzing PAI-1 gene -675 (4G/5G) polymorphism 83 patients subjected to analysis were classified into three groups according to their PAI-1 genotypes:

- PAI-1 gene -675 **4G/4G** genotype group (n = 21)
- PAI-1 gene -675 **4G/5G** genotype group (n = 42)
- PAI-1 gene -675 **5G/5G** genotype group (n = 20)

Characteristics of patients according to PAI-1 gene promoter -675 (4G/5G) polymorphism are given in Table 3.8.

Table 3.8.

Characteristics of patients divided into three PAI-1 gene -675 (4G/5G) genotype groups

Characteristic	4G/4G n=21	4G/5G n=42	5G/5G n=20	p value
Demographic data				
Age, yr	65.2 ± 11	66 ± 10	64 ± 12	0.6
Male sex, n (%)	11 (52%)	19 (45%)	12 (60%)	0.2
BMI, kg/m^2	26.6 ± 4.5	28 ± 4.9	28 ± 5	0.5
EF (%)	56.7 ± 7	55 ± 7.8	57 ± 8	0.2
Type of surgery, n (%)				
CABG, n (%)	8 (38%)	17 (41%)	9 (45%)	0.1
Valve, n (%)	10 (47%)	14 (33%)	7 (35%)	0.3
Mixed, n (%)	3 (15%)	11 (26%)	4 (20%)	0.04*
Preoperative parameters				
Hemoglobin, g/dL	136 ± 18	136 ± 15	135 ± 15	0.9
Platelet count, x 10^9/L	218 ± 63	216 ± 53	215 ± 65	0.9
Prothrombin index, %	92 ± 13	90 ± 13	84 ± 16	0.08
Fibrinogen, g/L	4.5 ± 1.7	4.5 ± 1.3	4.2 ± 0.8	0.3

Characteristic	4G/4G n=21	4G/5G n=42	5G/5G n=20	p value
Surgical parameters				
CPB duration (min)	95 ± 36	106 ± 41	113 ± 43	0.2
Aorta occlusion time (min)	59 ± 27	66 ± 24	72 ± 32	0.2
Reperfusion time (min)	32 ± 15	34 ± 16	34 ± 12	0.7
Blood loss, mL T24	432±168*	568±192	609±321*	0.02*

Abbreviations: n, number of patients; BMI, body mass index; EF, ejection fraction; CABG, coronary artery bypass grafting; CPB, cardiopulmonary bypass
Data are given mean ± standard deviation (SD), * statistical significant difference $p < 0.05$

We did not find significant differences in demographic characteristics such as mean age, gender, BMI, EF and preoperative laboratory parameters (Hb, PLT, PT, fibrinogen level) between the three genotype groups. But in the 4G/5G genotype group, the number of patients who underwent mixed cardiac surgery was significantly higher.

PAI-1 -675 (4G/5G) polymorphism

The distribution of PAI-1 polymorphism was as follows: 21 patients (25%) in the 4G/4G genotype group, 42 (51%) in the 4G/5G group, and 20 (24%) of the 83 patients studied in 5G/5G group. All alleles were in the Hardy-Weinberg equilibrium.

Relation between genotype and plasma concentrations of PAI-1, t-PA/PAI-1 complex

Preoperative PAI-1 plasma concentrations differed according to the patient's genotype. In genotype group 5G/5G the mean concentration was 17 ± 10.8 ng/mL, and correspondingly, 27 ± 13 ng/mL and 24 ± 9.6 ng/mL in genotype groups 4G/5G and 4G/4G, respectively.

Preoperative PAI-1 levels differed significantly between carriers of genotypes 5G/5G and 4G/5G (17 ± 10.8 *vs.* 27 ± 13, $p = 0.004$) and of genotypes 5G/5G and 4G/4G (17 ± 10.8 *vs.* 24 ± 9.6, $p = 0.04$), respectively (Figure 3.8.).

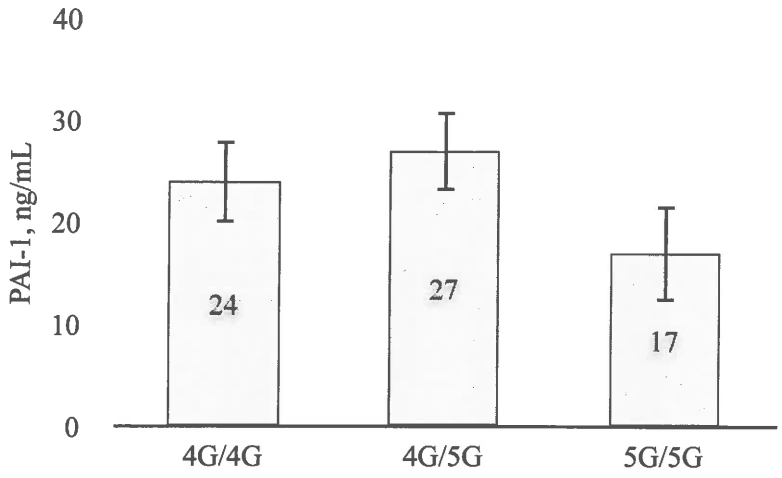

Figure 3.8. Preoperative plasminogen activator inhibitor type-1 (PAI-1) levels according to PAI-1 -675 (4G/5G) genotype

With respect to t-PA/PAI-1 complex measured 24 hours after the surgery, the mean plasma concentrations were: 5G/5G 3.6 ± 2.4 ng/mL, 4G/5G 3.9 ± 2.1 ng/mL and 4G/4G 3.1 ± 1.8 ng/mL. The levels of t-PA/PAI-1 complex did not differ statistically between the three genotype groups.

Relation between genotype and D-dimer levels

Genotype group 5G/5G displayed the highest postoperative D-dimer levels at all the three time points: at T1 334 ± 224 ng/mL, at T6 371 ± 226 ng/mL and at T24 326 ± 206 ng/mL.

D-dimer levels differed also statistically between genotypes 5G/5G and 4G/4G at T6 (371 ± 226 *vs.* 232 ± 185, p = 0.03) and at T24 (326 ± 207 *vs.* 209 ± 160, p = 0.04), as depicted in Figure 3.9.

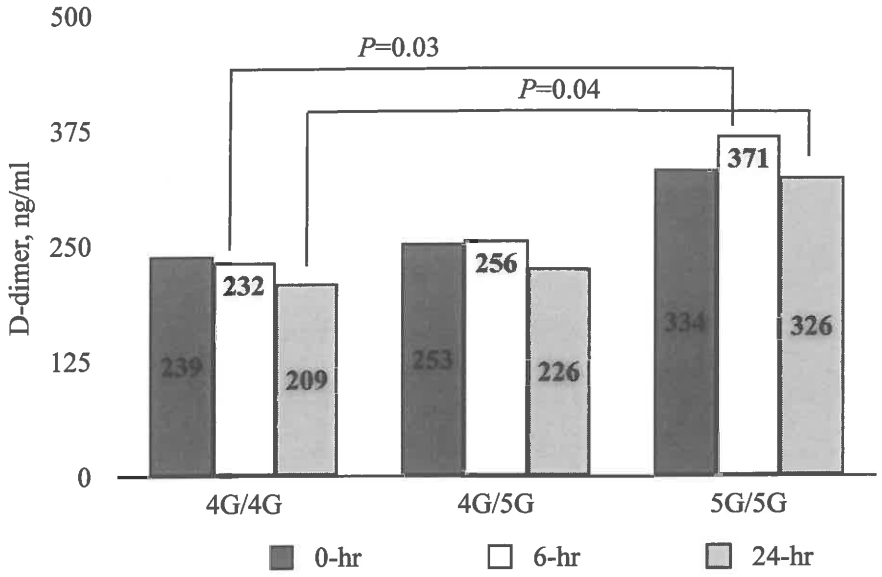

Figure 3.9. D-dimer levels at different time points (T1, T6 and T24) according to plasminogen activator inhibitor type-1 (PAI-1) -675 (4G/5G) genotypes

Relation between genotype and postoperative 24-hour blood loss

As expected, there were differences in postoperative 24-hour blood loss between the three PAI-1 genotypes. Statistically significant difference was found between genotypes 5G/5G and 4G/4G (609 ± 321 *vs.* 432 ± 168, p = 0.02).

3.3.2. PAI-1 gene -844 A/G polymorphism

Analyzing PAI-1 gene -844 A/G polymorphism 83 patients subjected to analysis were classified into three groups according to their PAI-1 genotypes

- PAI-1 gene -844 **G/G** genotype group (n = 22)
- PAI-1 gene -844 **A/G** genotype group (n = 38)
- PAI-1 gene -844 **A/A** genotype group (n = 23)

Characteristics of patients according to PAI-1 gene -844 A/G polymorphism are given in Table 3.9.

Table 3.9.

Characteristics of patients divided into three PAI-1 gene -844 A/G genotype groups

Characteristic	G/G n=22	A/G n=38	A/A n=23	p value
Demographic data				
Age, yr	61.5 ± 11	68 ± 10	67 ± 11	0.1
Male sex, n (%)	15 (68%)	16 (42%)	11 (48%)	0.7
BMI, kg/m^2	28.3 ± 5	28 ± 4.8	27 ± 5	0.6
EF (%)	56.7 ± 8	55 ± 8.1	56 ± 7.2	0.8
Type of surgery, n (%)				
CABG, n (%)	11 (50%)	15 (40%)	8 (35%)	0.4
Valve, n (%)	8 (36%)	13 (34%)	10 (43%)	0.5
Mixed, n (%)	3 (14%)	10 (26%)	5 (22%)	0.1
Preoperative parameters				
Hemoglobin, g/dL	136 ± 15	139 ± 13	131 ± 18	0.2
Platelet count, x $10^9/L$	221 ± 67	215 ± 50	215 ± 63	0.9
Prothrombin index, %	84 ± 15	90 ± 14	92 ± 12	0.1
Fibrinogen, g/L	4.4 ± 1.3	4.6 ± 1.2	4.7 ± 1.5	0.4
Surgical parameters				
CPB duration (min)	110 ± 42	106 ± 42	99 ± 37	0.2
Aorta occlusion time (min)	69 ± 30	65 ± 25	63 ± 11	0.5

Characteristic	G/G n=22	A/G n=38	A/A n=23	p value
Reperfusion time (min)	33 ± 12	33 ± 15	33 ± 16	0.7
Blood loss, mL T24	601 ± 221*	604 ± 308	436 ± 267*	0.03*

Abbreviations: n, number of patients; BMI, body mass index; EF, ejection fraction; CABG, coronary artery bypass grafting; CPB, cardiopulmonary bypass
Data are given mean ± standard deviation (SD), * statistical significant difference $p < 0.05$

We did not find significant differences in demographic characteristics such as mean age, gender, BMI, EF and preoperative laboratory parameters (Hb, PLT, PT, fibrinogen level) between the three genotype groups.

PAI-1 -844 A/G polymorphism

We found the following distribution of PAI-1 polymorphism among the 83 patients studied: 22 patients (26.5%) had genotype G/G, 38 patients (46%) had genotype A/G group, and 23 (27.5%) belonged in the A/A group .All alleles were in the Hardy-Weinberg equilibrium.

Relation between genotype and plasma concentrations of PAI-1, t-PA/PAI-1 complex

Preoperative PAI-1 plasma concentrations differed according to the genotype of the patient. Thus, in genotype G/G group, the PAI-1 plasma level was 18 ± 12 ng/mL, and correspondingly, 24 ± 13 ng/mL and 28 ± 12 ng/mL, in genotype groups A/G and A/A, respectively.

Moreover, preoperative PAI-1 levels differed significantly between carriers of PAI -844 G/G and A/A genotypes (18 ± 12 *vs*. 28 ± 12, $p = 0.004$).

Preoperative PAI-1 plasma levels according to PAI-1 -844 A/G genotype are presented in Figure 3.10.

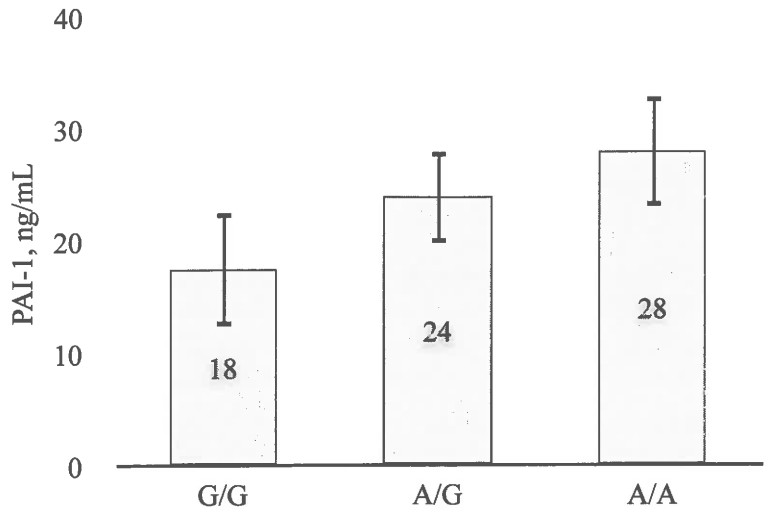

Figure 3.10. Preoperative plasminogen activator inhibitor type-1 (PAI-1) levels according to PAI-1 -844 A/G genotype

With respect to t-PA/PAI-1 complex measured 24 hours after the surgery, the mean plasma concentrations were 3.4 ± 2.4 ng/mL, 3.6 ± 2.1 ng/mL and 3.8 ± 1.8 ng/mL in genotype groups G/G, A/G and A/A, respectively.

We found no statistical differences in t-PA/PAI-1 complex concentrations between the three PAI-1-844 A/G genotype groups.

Relation between genotype and D-dimer levels

Analyzing the results, we found a tendency towards higher D-dimer levels postoperatively in the PAI-1-844 A/G genotype group particularly at T1 and T6. But A/G carriers had the lowest level of D-dimer at T24 if compare with G/G carries where D-Dimer level had tendency to stay et the same level or even to increase 24 hours after surgery.

We found that G/G carriers were tendended to have higher postoperative D-dimer levels at T24 in comparison with those of genotypes A/A and A/G. D-dimer levels differed significantly between carriers of the G/G and the A/G genotypes at T24 (Table 3.10.).

Table 3.10.

D-dimer levels at different time points according to PAI-1 -844 A/G genotype

	G/G	A/G	A/A	p value
D-dimer, ng/mL T1	287 ± 255	312 ± 213	251 ± 170	0.5
D-dimer, ng/mL T6	255 ± 203	312 ± 200	289 ± 210	0.5
D-dimer, ng/mL T24	267 ± 168*	184 ± 129*	234 ± 187	0.04*

Abbreviations: T1, on admission in ICU; T6, six hours after surgery; T24, twenty-four hours after surgery
Data are given mean ± standard deviation (SD), * statistical significant difference $p < 0.05$

Relation between genotype and postoperative 24-hour blood loss

There were differences in postoperative 24-hour blood loss between the three PAI-1-844 A/G genotypes. Patients with genotype A/A presented with the lowest 24-hour postoperative blood loss. Statistically significant differences were found between the G/G and A/A genotypes (601 ± 221 *vs.* 436 ± 266, $p = 0.03$) and the A/A and A/G genotypes (436 ± 266 *vs.* 604 ± 308, $p = 0.03$).

3.3.3. ACE gene Intron 16 I/D polymorphism

Analyzing ACE gene Intron 16 I/D polymorphism 83 patients subjected to analysis were classified into three groups according to their ACE genotypes:

- ACE gene Intron 16 **I/I** genotype group (n = 22)
- ACE gene Intron 16 **I/D** genotype group (n = 42)
- ACE gene Intron 16 **D/D** genotype group (n = 19)

Characteristics of patients according to ACE gene Intron 16 I/D polymorphism are given in Table 3.11.

Table 3.11.

Characteristics of patients divided into three ACE gene Intron 16 I/D genotype groups

Characteristic	I/I n=22	I/D n=42	D/D n=19	p value
Demographic data				
Age, yr	68 ± 10	66 ± 10	63 ± 14	0.3
Male sex, n (%)	9 (41%)	23 (55%)	10 (53%)	0.01*
BMI, kg/m^2	27 ± 5	28 ± 5	27 ± 4.3	0.8
EF (%)	58 ± 6	55 ± 9	55 ± 7.2	0.2
Type of surgery, n (%)				
CABG, n (%)	8 (36%)	18 (43%)	8 (42%)	0.05
Valve, n (%)	9 (41%)	13 (31%)	9 (47%)	0.6
Mixed, n (%)	5 (23%)	11 (26%)	2 (11%)	0.03*
Preoperative parameters				
Hemoglobin, g/dL	132 ± 17	137 ± 16	138 ± 12	0.3
Platelet count, x 10^9/L	211 ± 45	218 ± 65	220 ± 58	0.9
Prothrombin index, %	94 ± 15	89 ± 13	85 ± 14	0.3
Fibrinogen, g/L	4.5 ± 1.2	4.6 ± 1.2	4.7 ± 1.7	0.9
Surgical parameters				
CPB duration (min)	101 ± 47	105 ± 39	109 ± 37	0.3

Characteristic	I/I n=22	I/D n=42	D/D n=19	p value
Aorta occlusion time (min)	64 ± 27	65 ± 27	69 ± 29	0.9
Reperfusion time (min)	29 ± 13	36 ± 17	33 ± 9	0.7
Blood loss, mL T24	589 ± 262	546 ± 276	544 ± 331	0.65

Abbreviations: n, number of patients; BMI, body mass index; EF, ejection fraction; CABG, coronary artery bypass grafting; CPB, cardiopulmonary bypass
Data are given mean ± standard deviation (SD), * statistical significant difference $p < 0.05$

There were no significant differences in demographic characteristics such as mean age, BMI, EF, preoperative standard laboratory tests (Hb, PLT, PT, fibrinogen level) and surgical parameters between the three genotype groups. In the group of genotype I/D, there were significantly more males than females, and more patients underwent mixed heart surgery, as shown in Table 3.10.

ACE gene Intron 16 I/D polymorphism

The distribution of ACE gene Intron 16 I/D polymorphism was as follows: 22 patients (26.5%) presented with genotype I/I, 42 (51%) with genotype I/D and 19 (23.5%) of the 83 patients studied had genotype D/D. All alleles were in the Hardy-Weinberg equilibrium.

Relation between genotype and plasma concentrations of PAI-1, t-PA/PAI-1 complex

Preoperative PAI-1 plasma concentrations differed according to the patient's genotype. Carriers of genotype I/I presented with preoperative PAI-1 of 18.4 ± 10.7 ng/mL, which was the lowest registered. Correspondingly, the genotypes I/D and D/D displayed PAI-1 plasma concentrations of 24 ± 13 ng/mL and 27 ± 13 ng/mL, respectively. Preoperative PAI-1 levels differed significantly between carriers of ACE gene Intron 16 I/I and D/D genotypes ($p = 0.02$), as depicted in Figure 3.11.

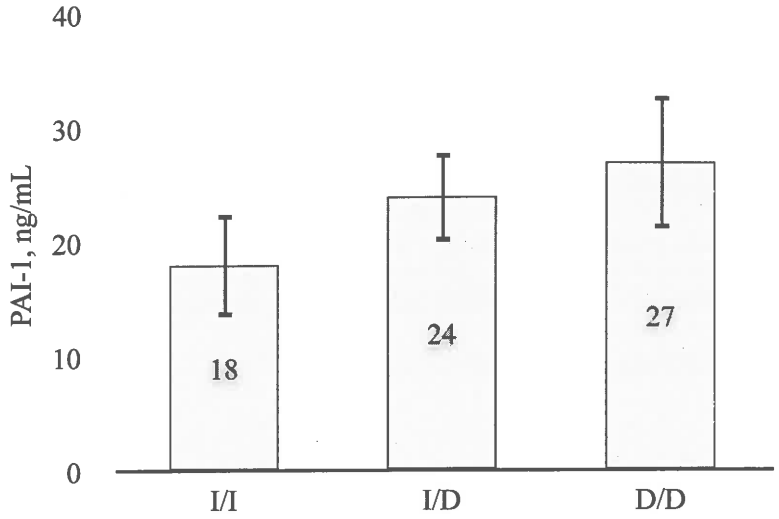

Figure 3.11. Preoperative plasminogen activator inhibitor type-1 (PAI-1) levels according to angiotensin converting enzyme (ACE) Intron 16 I/D genotype

ACE gene Intron 16 I/I carriers also presented with the lowest plasma levels of t-PA/PAI-1 complex. By analyzing the t-PA/PAI-1 complex determined 24 hours after the surgery, the mean plasma concentration in the I/I genotype group was 2.8 ± 1.7 ng/mL and in the I/D and D/D groups, 4 ± 2.3 ng/mL and 3.6 ± 2 ng/mL, respectively. Statistically significant difference was reached between the I/I and the I/D carriers ($p = 0.02$), as shown in Figure 3.12.

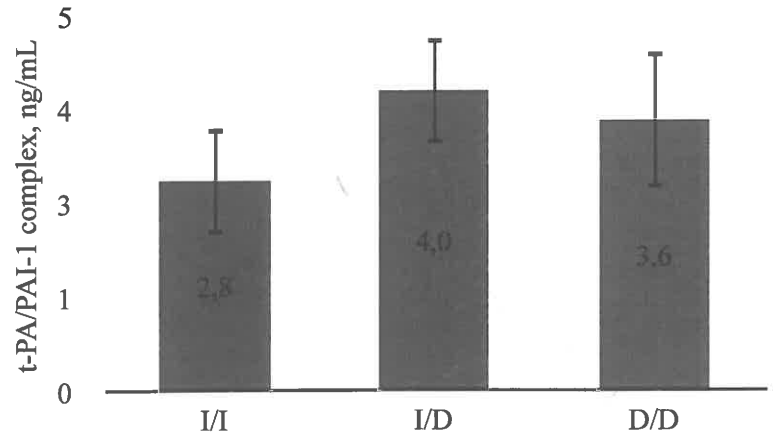

Figure 3.12. Postoperative plasma levels of tissue plasminogen activator/plasminogen activator inhibitor type-1 (t-PA/PAI-1) complex according to angiotensin converting enzyme (ACE) Intron 16 I/D genotype

Relation between genotype and D-dimer levels

ACE gene Intron 16 I/I genotype group presented higher D-dimer levels at all three time points after surgery showing tendency of enhanced fibrinolysis postoperatively. At the T1 time point I/I genotype group showed the highest D-dimer level and it differed significantly between I/I and D/D carriers (p = 0.03). Although there were not found statistically significant difference at T6 and T24 time points between I/I and I/A or D/D genotype groups comparing postoperative D-dimer levels (Table 3.12.)

Table 3.12.

D-dimer levels at different time points according to ACE gene Intron 16 I/D genotype

	I/I	I/D	D/D	p value
D-dimer, ng/mL T1	367 ± 203*	294 ± 238	234 ± 161*	0.03*
D-dimer, ng/mL T6	331 ± 218	291 ± 204	244 ± 182	0.4
D-dimer, ng/mL T24	274 ± 167	247 ± 165	208 ± 173	0.2

Abbreviations: T1, on admission in ICU; T6, six hours after surgery; T24, twenty-four hours after surgery
Data are given mean ± standard deviation (SD); * statistical significant difference $p < 0.05$

Relation between genotype and postoperative 24-hour blood loss

There were found similar 24-hour postoperative blood loss in all three ACE gene Intron 16 I/D genotype groups with a small tendency towards higher blood loss in the group of patients with genotype I/I. Data are presented in Table 3.10. We found no significant difference between the groups.

3.4. Changes of coagulation state after CPB detected by thromboelastography and standard coagulation tests

In the present study, we investigated the changes of coagulation state which can be influenced by hemodilution during CPB as assessed by TEG® after cardiac surgery employing cardiopulmonary bypass in 83 patients. The patients included in the study were allocated to two groups depending on the deltajonin volume used for priming of the CPB circuit. For all patients initial priming volume in the extracorporeal circuit was constant at 1400 mL. In addition, a volume of deltajonin was used as required to provide adequate priming of the circuit based on the individual BSA of the patient. This was the main reason why deltajonin volume differed between the patients at the end of operation. Considering the fact that the mean volume of deltajonin per patient was 809 ± 256 mL/m^2 (range 375 - 1500 mL/m^2), the patients were divided into two groups:

Group I (n = 40) was the most diluted group. The extracorporeal circuit priming volume of deltajonin was more than the calculated mean. The deltajonin volume was 1015 ± 200 mL/m^2 (range 800 - 1500 mL/m^2).

The BSA of these patients were 1.9 ± 0.2 m^2.

Group II (n = 43) was the less diluted group. The extracorporeal circuit priming volume of deltajonin was less than the calculated mean. The deltajonin volume was 620 ± 116 mL/m^2 (range 375 - 778 mL/m^2).

The BSA of these patients were 1.8 ± 0.2 m^2.

The groups were comparable regarding demographic characteristics such as mean age, gender, EuroSCORE, BSA, EF, type of surgery and surgical parameters. The hematologic variables were within normal range although baseline levels of Hb and Ht differed significantly between Group I and II. The volume of St.thomas solution used for cardioplegia was approximately the same, and did not differ significantly between the groups. The demographic characteristics of the patients are shown in Table 3.13.

Table 3.13.

Characteristic of all patients (total) and of the two groups

Characteristics	Total n = 83	Group I n = 40	Group II n = 43	p value
Demographic data				
Age, yr	65 ± 11	64 ± 12	67 ± 9	0.1
Male sex, n (%)	42 (51%)	20 (50%)	22 (51%)	0.8
EuroSCORE (%)	4.8 ± 1.8	4.8 ± 2	4.9 ± 1.7	0.2
BSA (m^2), *Mosteller*	1.9 ± 0.2	1.9 ± 0.2	1.8 ± 0.2	0.4
EF (%)	56 ± 8	55 ± 8	57 ± 8	0.2
Type of surgery, n (%)				
CABG, n (%)	34 (41%)	17 (42%)	17 (40%)	1.0
Valve, n (%)	31 (37%)	15 (38%)	16 (37%)	0.9
Mixed, n (%)	18 (22%)	8 (20%)	10 (23%)	0.6
Preoperative parameters				
Hemoglobin, g/dL	136 ± 15	140 ± 14*	131 ± 15*	0.01*
Hematocrit, %	41 ± 4.9	42 ± 4.6*	39 ± 4.9*	0.01*
APTT, s	34 ± 7	35.6 ± 9	33 ± 4	0.1
Platelet count, x 10^9/L	216 ± 58	216 ± 62	216 ± 54	0.9
Prothrombin index, %	89 ± 14	88 ± 15	90 ± 13	0.5
Fibrinogen, g/L	4.6 ± 1.3	4.4 ± 1.3	4.7 ± 1.4	0.3
Surgical parameters				
CPB duration (min)	105 ± 40	111 ± 42	99 ± 38	0.2
Aorta occlusion time (min)	66 ± 27	71 ± 29	61 ± 25	0.08
Reperfusion time (min)	34 ± 15	35 ± 15	33 ± 15	0.5
Heparin dose, ml	7.6 ± 1.4	7.8 ± 1.4	7.4 ± 1.3	0.2
Protamine dose, mg	302 ± 66	308 ± 67	296 ± 66	0.4
Baseline ACT, s	141 ± 23	141 ± 21	141 ± 24	0.9
Postprotamine ACT, s	130 ± 13	130 ± 15	129 ± 10	0.8

Characteristics	Total n = 83	Group I n = 40	Group II n = 43	p value
Temp on CPB, C^0	35.3 ± 0.4	35.3 ± 0.4	35.3 ± 0.4	0.97
Deltajonin, ml	809 ± 256	1015 ± 200*	620 ± 116*	<0.001*
Cardioplegia, ml	1785 ± 584	1781 ± 546	1789 ± 624	0.95

Abbreviations: n, number of patients; BSA, body surface area; EF, ejection fraction; CABG, coronary artery bypass grafting; APTT, activated partial thromboplastin time; CPB, cardiopulmonary bypass; ACT, activated coagulation time; temp, temperature
Data are given mean ± standard deviation (SD), *statistical significant difference $p < 0.05$

Comparison of kTEG, heparinase modified kTEG and standard coagulation tests

All kTEG parameters collected after CPB were out of the normal range in the more diluted patients of Group I where R was 12.3 ± 6.4 min and K was 5.1 ± 2.8 min and A and MA were 40 ± 12^0 and 56 ± 10, respectively. In contrast, in the less diluted Group II, kTEG parameters were found to be within the normal range except for R min (9.7 ± 4.9 min).

Values of kTEG differed significantly between Group I and II: R ($p = 0.04$), K ($p = 0.02$), A ($p = 0.001$) and MA ($p = 0.05$). Concomitantly, we noticed significant differences between Group I and II as hep.kTEG parameters were regarded with differences in K ($p = 0.02$), A ($p = 0.03$) and MA ($p = 0.04$) whereas R time did not reach statistical significance ($p = 0.1$). The hep.kTEG mean values in both groups were within the normal range; only the mean value of R time in Group I was higher than normal (8.2 ± 2.6 min).

In contrast, except for the plasma concentrations of fibrinogen in Group I and II (2.9 ± 0.8 *vs.* 3.5 ± 1.2, $p = 0.01$) we found no significant intergroup differences in the standard coagulation variables after CPB.

Comparison of TEG® values and standard coagulation tests for both groups of patients are listed in Table 3.14.

Table 3.14.

Results of kaolin activated thromboelastography (kTEG), heparinase modified kTEG (hep.kTEG) and standard coagulation tests of two patient groups

Analyzed parameters	Group I n = 40	Group II n = 43	p value
kTEG			
R, min	12.3 ± 6.4	9.7 ± 4.9	0.04*
K, min	5.1 ± 2.8	3.8 ± 2.5	0.02*
A, ^{0}	40 ± 12	50 ± 13	0.001*
MA, mm	56 ± 9.7	60 ± 10	0.04*
hep.kTEG			
R, min	8.2 ± 2.6	7.3 ± 2.7	0.1
K, min	3.5 ± 1.5	2.9 ± 1.1	0.02*
A, ^{0}	47 ± 11	53 ± 11	0.03*
MA. mm	59 ± 8	62 ± 6	0.04*
Standard coagulation tests			
APTT, s	34 ± 5	34 ± 4	0.9
Prothrombin index, %	85 ± 13	88 ± 10	0.2
Platelet count, x 10^9/L	148 ± 58	141 ± 32	0.5
Fibrinogen, g/L	2.9 ± 0.8	3.5 ± 1.2	0.01*

Abbreviations: n, number of patients; kTEG, kaolin activated thromboelastography; hep.kTEG, heparinase modified kaolin activated thromboelastography; R, reaction time (normal range 4-8 min); K, clot formation time (normal range 1-4 min); A, alpha angle (normal range 47^0-78^0); MA, maximum amplitude (normal range 55-73 mm); APTT, activated partial thromboplastin time

Data are given mean ± standard deviation (SD), * statistical significant difference $p < 0.05$

For comparison of qualitative and quantitative TEG® analysis between the more and less diluted patients depending on the priming volume of deltajonin used in the extracorporeal circuit during CPB as calculated on the basis of the patient's BSA (m^2) (Figures 3.13. and 3.14).

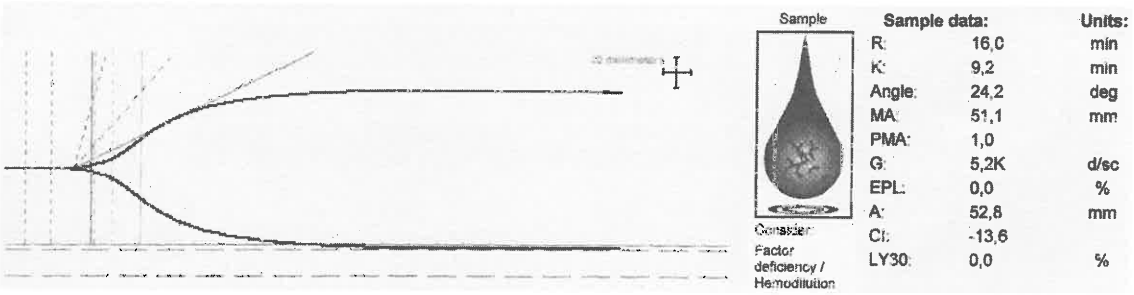

Figure 3.13. Thromboelastography data of qualitative and quantitative analysis. Patient from group I, extracorporeal circuit priming volume of deltajonin 1157ml/m^2, postoperative 24-hour blood loss 890 mL

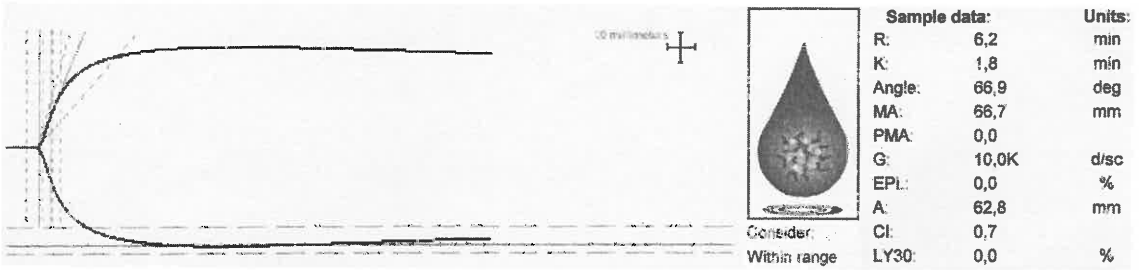

Figure 3.14. Thromboelastography data of qualitative and quantitative analysis. Patient from group II, extracorporeal circuit priming volume of deltajonin 500 ml/m^2, postoperative 24-hour blood loss 320 mL

Comparison of postoperative blood loss between two patient groups

As expected, there were differences in blood loss analyzed at three time points postoperatively. Comparison of the mean volume of postoperative blood loss during the first hour after surgery revealed no statistical difference between the groups ($p = 0.2$). Group I had significantly higher blood loss at 4-hour and 24-hour after surgery in comparison with Group II ($p = 0.04$ and $p = 0.01$, respectively), as evident from Figure 3.15.

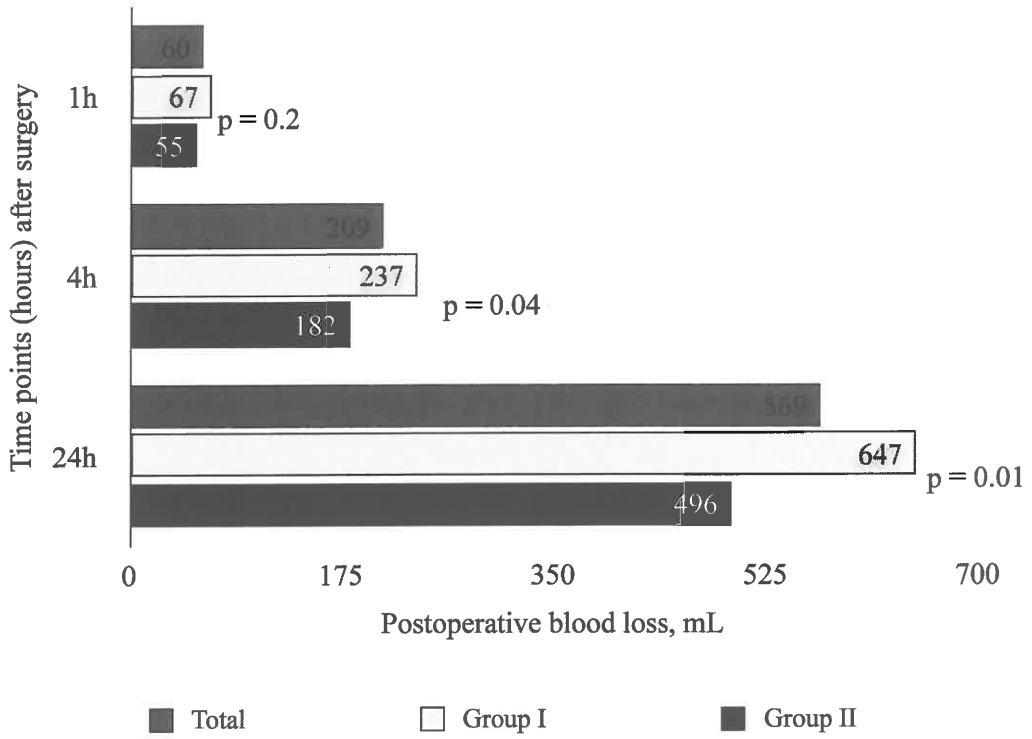

Figure 3.15. Postoperative blood loss at three time points (1h, 4h and 24h after surgery) between two groups: group I priming volume of deltajonin 800 - 1500 ml/m^2, group II - 375 - 778 ml/m^2

4. DISCUSSION OF THE RESULTS

Bleeding complications in cardiac surgery with CPB have multifactorial causes. Most frequent causes are dilution coagulopathy, enhanced fibrinolysis, platelet loss or dysfunction, and excessive hemostatic activation by the inflammation system [9, 195]. Numerous trials with different study design have been made to identify more precisely the biomarkers associated with bleeding after cardiac surgery with CPB. However, even today it is difficult to predict who is going to present with severe bleeding after cardiac surgery.

The present study focuses on the association between the standard coagulation tests (APTT, PI, PLT count and fibrinogen) and fibrinolysis markers (PAI-1 and t-PA/ PAI-1) with 24-hour postoperative blood loss after cardiac surgery with CPB. Moreover, genetic polymorphisms which could influence fibrinolytic balance and therefore bleeding tendency was analyzed and TEG was performed to determinate changes of coagulation state in patients undergoing heart surgery employing CPB.

4.1.Coagulation tests association with bleeding after CPB

The investigation revealed that preoperative ***activated partial thromboplastin time*** values most likely were affected by the use of LMWH before the surgery despite the fact that the last dose of LMWH was administered 12 hours before operation. Because 27 patients had higher or equal than normal APTT values at T0 and 21 patients of them received the last dose of LMWH 12 hours before surgery. In the literature there are data indicating that LMWH stopped more than 8 hours before surgery does not affect the bleeding volume after surgery [138]. *Medalion B.* and co-authors analyzed whether the use of LMWH before CABG could be associated with an increase in bleeding in parallel with increased transfusion of blood products after the operation. In that study 64 patients were included who were divided into three groups: an enoxaparin group (n=21), an IV heparin group (n = 20) and a placebo group (n=23). The last dose of enoxaparin 1 mg/kg SQ was administered 8-10 hours before the skin incision. There was no difference in CTD among the groups at 24 hour and in the amount of blood

products transfused. Our results were consistent with those of *Medalion B.* and coworkers. We also found that 24-hour postoperative blood loss did not differed between patients with higher or normal preoperative APTT values. After the surgery at time point T6 69% (n=57) of the patients had higher than normal APTT values and at time point T24 this was the casein 61% (n=51) of patients. This could be explained by the "heparin rebound phenomena" [87]. In this state ACT measurements should be done, and if necessary additional protamine should be given. The "heparin rebound " phenomenon usually occurs in 1-8 hours after heparin neutralization with protamine [146]. Higher APTT values after surgery also can be explained by hemodilution and reduction of the coagulation factors by 50% [9, 76, 83, 196]. It is well known that APTT reflects intrinsic and common coagulation pathways including factors II, V, VIII, IX, X, XI, XII and fibrinogen, that might remain reduced two hours after surgery due to hemodilution [196].

Despite the fact that more than a half of the patients had higher than normal APTT values after cardiac surgery at T6 and at T24, we did not find any APTT association with 24-hour postoperative blood loss There are several studies investigating the relationship between APTT and postoperative blood loss reporting similar results to ours [197-199]. *Ereth et al.* also were unable to find an association between APTT and 4 - and 24-hour postoperative blood loss in 200 patients who underwent cardiac surgery [197]. Recently *Carroll* and co-workers also showed the same tendency. In their study APTT did not correlate with CTD and transfused blood volume in 75 patients undergoing elective cardiac surgery with CPB [198]. Additionally, in a large study including 894 cardiac surgery patients APTT had a low predictive value as postoperative bleeding is regarded [199]. While *Nutall et al.* [197] found significant difference in APTT values between bleeders and non-bleeders, *Blome et al.* [99] report significant relationship between APTT and 24-hour CTB volume at two time points: termination of CPB and at 24 hours after surgery, when examined by multiple regression analysis.

Analyzing ***prothrombin index*** data, it showed the smallest changes after CPB in our results. Preoperatively, only 7 patients had PI lower than normal, and those did not show greater bleeding tendency after the surgery. After CPB, nobody had lower levels

of PI than normal. We speculate, that one of the explanation could be the administration of FFP or CRIO for patients with lower BMI or expected longer CPB time. In the present study PI values were analyzed on admission in ICU, when patients already had received coagulation product transfusions.

We were not able to find any correlation between postoperative PI analyzed at three time points and 24-hour postoperative blood loss and also preoperative PI did not show any association with CTD blood volume.

It is well known that PI (prothrombin time) reflects extrinsic and common coagulation pathways and should be affected by hemodilution as well. Results from the literature are controversial. *Blome et al.* [99] analyzing different coagulation factors and routine coagulation tests, such as APTT, PI, platelet count, D-dimer and postoperative bleeding in 98 elective cardiac surgery patients, found significant relationship between PI measured after termination of CPB with postoperative CTD. Although PI measured before surgery, 12 - and 24 hours after surgery did no showed significant correlation with blood loss. Contrary two more studies both demonstrate that all blood coagulation tests, including Prothrombin time, differed significantly between bleeders and non-bleeders [197, 200]. In the study reported by *Welsby* [110] prothrombin time also showed a correlation with postoperative bleeding ($r = 0.43$, $p = 0.02$). At the same time *Ti et al.* [201] in consistence with our study failed to find any difference in PI measurements and postoperative bleeding supporting our results.

In the present study ***platelet count*** showed a convincing tendency to decrease after CPB. At T24 more than a half of the patients presented with a reduced PLT count by 32.5%. We speculate that the main reasons could be hemodilution and consumption of PLT during CPB, when PLT count is known to decrease by about 30-50% [73]. The PLT count typically remains depressed for 7-10 days postoperatively after cardiac surgery with CPB [9, 74] and it is one of the factors influencing volume of postoperative blood loss [19]. Although previous studies have shown conflicting results, examining the relationship between PLT count and bleeding, most of the reports support the tendency, that PLT count predicts and influences postoperative blood loss after cardiac surgery [99]. Our results show similar finding, suggesting that the PLT count after CPB may affect the CTD volume 24 hours after surgery. In this study, we found a

correlation between PLT count and 24-hour postoperative blood loss. *Blome* with co-authors confirm our finding, demonstrating an association between PLT count measured after termination of CPB and 24 hours after surgery and postoperative CTD volume [99]. A recent publication assessing the impact of perioperative predictors including PLT count on postoperative bleeding, showed a similar tendency [202]. Eighty-seven consecutive patients who underwent heart surgery were studied. The percentage reduction in PLT count per – and postoperatively were significant predictors of postoperative bleeding

Since PLT count does not reflect platelet dysfunction, many investigators consider TEG®, especially the role of the maximum amplitude (MA) as a more specific and predictive parameter for postoperative bleeding. *Ereth et al.* [203] analyzed PLT count and TEG parameter MA in 200 patients undergoing cardiac surgery. They noticed that preoperative PLT count correlated non-significantly with 4- and 24-hour blood loss when compared to MA determinated by TEG®. A recent study of 30 adult cardiac patients showed a correlation between the PLT count and postoperative bleeding ($r = -0.45$), but by comparing the latter with TEG®MA, a still higher correlation was found ($r = -0.6$) [110]. Time on CPB also plays a role for postoperative bleeding. Lately, the MA has been demonstrated as the most sensitive parameter affected by CPB time [204].

Contradictory results also have been published concerning the suggested correlation between PLT count and increased postoperative bleeding. Thus, a study of 894 patients aimed at screening the relationships between standard blood clotting tests and postoperative bleeding in cardiac surgical patients found no correlation between PLT count and increased postoperative bleeding [199]. On the other hand, *Nutall et al.* [197] found a significant correlation between PLT count and intraoperative bleeding, but were unable to show the same association with 24-hour postoperative blood loss in patients undergoing elective cardiac surgery.

The role of ***fibrinogen*** plasma concentration as a predictor of postoperative bleeding after cardiac surgery has been investigated in many studies. However, the results of these investigations have been inconsistent [10, 82, 99, 197, 199, 205, 206]. Thus, five of the studies demonstrated a significant association between the fibrinogen level and postoperative blood loss [10, 99, 197, 199, 206] while two showed none such

association or were non-conclusive. Nowadays a multi-center trial [207] is going on investigating the role of prophylactic fibrinogen infusion to reduce postoperative bleeding and transfusion requirements after CABG in patients with endogenous fibrinogen levels in the lower normal range.

In a recent study the relationship between FXIII, fibrinogen, blood coagulation screening tests and postoperative bleeding in 98 patients undergoing cardiac surgery with CPB was investigated. These investigators found strong associations between preoperative and postoperative fibrinogen levels and postoperative bleeding [99].

Consistent with the findings presented in this thesis, *Karlsson* and his co-workers found a strong correlation between postoperative bleeding volume and preoperative fibrinogen concentration in the study of 170 patients. The investigators concluded that fibrinogen is an independent predictor of postoperative blood loss and transfusion requirements [101, 206, 208]. In a randomized placebo-controlled trial the same authors investigated the influence of fibrinogen concentrate infusion on postoperative bleeding in patients with preoperative fibrinogen level < 3.8 g/L [101]. In that study infusion of 2 g fibrinogen increased the plasma levels by 0.6 ± 0.2 g/L and reduced the postoperative blood loss by 32% [101]. The investigators did not find any adverse effects of fibrinogen infusion, only an increased release of fibrin degradation products [208].

The predictive values of blood clotting tests also have been subject to different opinions. In a large study consisting of 894 cardiac surgical patients the predictive value of blood clotting tests was analyzed. Authors demonstrated that only post-CPB fibrinogen differed significantly between bleeders and non-bleeders [199]. Their results were confirmed by *Nuttall et al.* [197], but other investigators have not been able to show such an association [205].

In the present study, lower fibrinogen levels correlated with greater postoperative blood loss and the highest correlation was found between preoperative (T0) fibrinogen and 24-hour postoperative blood loss. Our results are consistent with other above mentioned studies [197, 199, 206]. Although all the patients in our study had preoperative fibrinogen levels above the normal lower limit, it could well be that fibrinogen levels in the lower normal range, or below, could be too low to ensure normal coagulation capabilities when the hemostatic system is challenged by cardiac

surgery and the use of CPB. *Blome* and co-authors also report a study where all the patients had fibrinogen level within normal range or elevated before surgery. Never the less, a consistent finding was that higher blood loss was significantly associated with lower fibrinogen levels [99]. Our finding coincide with others researches showing that reduced fibrinogen levels are associated with increased bleeding [101, 206] and mortality [102] after surgery. Our observation that the fibrinogen levels decreased by 22% at T1 in comparison with baseline is most likely due to fibrin consumption due to enhanced fibrinolytic activity and hemodilution during CPB [9]. Correspondingly, *Blome* et al. presented an approximately 34 % reduction of the plasma fibrinogen concentration after CPB [99].

PAI-1 and t-PA/PAI-1 complex. The present study revealed that lower levels of PAI-1 preoperatively and of t-PA/PAI-1 complex postoperatively are associated with increased blood loss in the first 24 hours after the operation. Our patients were cooled on CPB to a bladder temperature of 34 - 35 °C with no intergroup difference. Some investigators claim that reduced temperature lowers endogenous production of PAI-1, resulting in enhanced fibrinolysis and increased per- and postoperative bleeding [142] whereas others refuse this idea [209]. Our patients were rewarmed to normal body temperature before transfer to the recovery. Therefore, it is unlikely that temperature had any influence on the formation of t- PA/PAI-1 complex.

Since PAI-1 is a more stable indicator of fibrinolysis, as compared to t-PA, whose concentration peaks during CPB, we determined PAI-1 before the operation and t-PA/PAI-1 complex after the surgery [52] and their associations with 24-hour postoperative blood loss after the surgery. Our findings indicated that those presenting with higher preoperative plasma concentrations of PAI-1 had less blood loss, and conversely, those with a lower preoperative plasma level had a larger blood loss at the data collection time points during the first 24 hours after surgery. Moreover, we found correlation between preoperative PAI-1 concentrations and 24-hour blood loss. Other investigators have noticed similar results [10, 45], showing that lower preoperative levels of PAI-1 are associated with excessive bleeding (>1L/24h) [10].

In an investigation of *Rivera* et al. [10] PAI-1 plasma concentrations were measured before and after cardiac surgery. Bleeders (> 1L/24h) had significantly lower

PAI-1 plasma levels before and immediately after surgery (25.5 ng/mL and 35.5 ng/mL) in comparison with non-bleeders (< 1L/24h) and the latter also presented with significantly higher PAI-1 plasma levels (43.5 ng/mL *vs.* 76.6 ng/mL).

In a recent investigation PAI-1 activity was studied in relation to the risk for perioperative bleeding complications in 62 patients scheduled for transurethral resection of the prostate [45]. Plasma concentration of PAI-1 was determined one week before the operation and the patients were divided into two groups with normal or low PAI-1 activity. In the group with low PAI-1 activity 75% of the patients had bleeding complications requiring reoperation against 28% in the group with normal PAI-1 activity. The group with normal PAI-1 activity had a mean of PAI-1 level of 21.9 ng/mL and of t-PA/PAI-1 complex of 6.12 ng/mL whereas that with low PAI-1 activity had concentrations of 8.4 ng/mL of PAI-1 and 2.34 ng/mL of t-PA/PAI-1 complex. Contrary to our results the investigators did not find any correlation between intra- and postoperative bleeding volume and PAI-1 plasma levels, but demonstrated that patients with lower PAI-1 activity, had lower levels of PAI-1 and of t-PA/PAI-1 complex and higher risks of bleeding, thus, confirming our findings

As to the best of our knowledge, the literature is scanty on reports focusing on the importance of t-PA/PAI-1 complex and its relationship with enhanced bleeding after CPB. Our notion that patients with lower levels of t-PA/PAI-1 complex after surgery tend to have greater blood loss during the first 24 hours after surgery is consistent with the findings of others investigators [10]. Thus, *Rivera* and coworkers determined t-PA/ PAI-1 complex concentrations in 26 patients immediately after cardiac surgery and showed that those with a lower level of complex have significantly higher 24-hour blood loss after surgery than those with a higher level (1190 *vs.* 605 ml/24h) [10].

Also in the study of patients undergoing prostatectomy mentioned above, the investigators observed a tendency for patients with lower levels of t-PA/PAI-1 complex to have a greater risk of bleeding complications as compared to those with high or normal complex levels. In the latter study the t-PA/PAI-1 complex concentrations were measured both before and after the surgery, and without exposure to CPB [45].

We were not able to find t-PA/PAI-1 complex correlation with postoperative blood loss at different time points. Data showing t-PA/PAI-1 complex correlation with bleeding were also lacking in the literature [10].

Recently, investigators reported favorable effects of administration of a modified PAI-1 protein with a very long half-life > 700 hours (VLHL PAI-1) on bleeding time and total blood loss after tail clip in PAI-1 deficient mice [210]. The experiments showed significant reduction in bleeding time and blood loss without side effects. However, so far, very long half- life PAI-1 is not available as a medicine to promote hemostasis after surgery, trauma, or PAI-1 deficiency in humans.

Most recently, a clinical application of VLHL PAI-1 has been patented with effects that are similar to native protein and highly specific to the proteolytic enzymes that are responsible for blood clot lysis and angiogenesis [211]. One of the indications for use of VLHL PAI-1 is bleeding related to hyperfibrinolysis, moreover, prevention of bleeding and topical application to control localized bleeding.

4.2. Genetic polymorphism and fibrinolytic bleeding

The plasma concentrations of PAI-1 and of t-PA/PAI-1 complex that are supposed to be the main regulators of fibrinolysis in humans, are both characterized by wide variations that may explain the large inter-individual differences in fibrinolytic activity [212]. Several recent studies have described the influence of genetic factors, such as PAI-1 promoter - 675 (4G/5G), PAI-1 - 844 (A/G) and ACE Intron 16 I/D polymorphisms, on the plasma levels of PAI-1, t-PA and t-PA/PAI-1 complex [10, 11, 18, 29, 35, 54].

The ***PAI-1 gene - 675 (4G/5G) polymorphism*** is known to influence plasma levels of PAI-1, the main regulator of fibrinolysis [10, 14, 17, 18, 36]. Much attention has has been paid to the 4G/4G polymorphism in association with coronary artery disease. However, the 5G/5G polymorphism and its influence on PAI-1 and t-PA/PA-1 complex plasma concentrations and their roles in fibrinolysis have been less focused on.

According to our data, PAI-1 gene 5G/5G polymorphism may be associated with lower preoperative plasma concentration of PAI-1, with higher levels of D-dimer postoperatively and larger bleedings after on-pump cardiac surgery. These results are consistent with several recent investigations showing that PAI-1 gene 4G/5G

polymorphism can affect fibrinolytic activity and be associated with increased postoperative bleeding [10, 11, 18, 37].

Regarding the preoperative PAI-1 plasma concentration, our study showed that PAI-1 plasma levels differed according to patient's genotype.The 5G/5G carriers had the lowest levels of PAI-1 before the operation. We noticed a 29% difference in preoperative PAI-1 levels between 4G/4G and 5G/5G genotypes. In the literature, the data are consistent with our results showing that the 4G/4G variant is associated with approximately 25% greater basal PAI-1 levels, therefore promoting inhibition of fibrinolysis and the 5G allele with lower levels of PAI-1 [58, 59]. Other recent studies have also observed such associations between 5G/5G genotype and lower PAI-1 plasma concentrations [18, 36, 45].

Burzotta et all. [18] investigated PAI-1 4G/5G polymorphism in association with basal PAI-1 levels in patients undergoing elective CABG surgery. The PAI-1 plasma levels were determined before surgery and subsequently daily up to 72 h and at discharge. They concluded that PAI-1 levels preoperatively and after surgery are approximately 20% higher in carriers of the 4G-allele as compared to 5G/5G homozygotes, mainly as a result of higher baseline values .

To the best of our knowledge, the literature is scanty on the reports focusing on the importance of the t-PA/PAI-1 complex and its relationship with PAI-1 gene - 675 (4G/5G) polymorphism. We expected to find a t-PA/PAI-1 complex association with PAI-1 genotype. Because the clearance rate of the t-PA/PAI-1 complex is slower than that of free t-PA and complex seems to be the main indicator of the PAI-1 concentration in the circulation. Consequently, it follows that the lower the plasma PAI-1 level, the smaller amount of t-PA/PAI-1 complex will be generated in the circulation [51]. Unfortunately, we could not find any statistically significant difference in the complex plasma concentrations comparing 3 PAI-1 genotypes. Even more, the 4G/4G carriers demonstrated the lowest plasma concentrations of the t-PA/PAI-1complex 24 hours after surgery, and 4G/5G carriers the highest. We speculate that the t-PA/PAI-1 complex level could be influenced by CPB as it is well known that the PAI-1 and t-PAI/PAI-1 concentrations are increased after CPB as part of the "fibrinolytic shut down". The latter concentrations start to rise immediately after surgery and slowly decrease in the following days [43]. In the present study, carriers of the 4G/5G genotype also showed

the highest preoperative plasma PAI-1 concentrations. In the latter genotype group, a significant correlation was found between the preoperative PAI-1 and the postoperative t-PA/PAI-1 complex levels and the blood loss at 24 hours after the surgery. We suggest that the lack of such a correlation could be explained by the fact that the 4G/5G genotype group had the biggest sample size.

The PAI-1 -675 (4G/5G) gene polymorphism affects PAI-1 plasma concentration in circulation and therefore fibrinolytic activity, which can be confirmed by higher levels of D-dimer [213]. According to our data, D-dimer reached higher postoperative levels in the 5G/5G group as compared with the 4G/5G and the 4G/4G genotype groups. Additionally, the 4G/5G and the 5G/5G carriers had a greater bleeding volume 24 hours after the surgery, as compared with the 4G/4G genotype group. It is difficult to isolate only one factor affecting blood loss after CPB. We speculate, whether the greater blood loss after surgery in the 4G/5G group could be influenced by hypothermia, hemodilution, or other factors, and not only by the PAI-1 genotype. Furthermore, only in the 5G/5G group, bleeding was confirmed with increased postoperative D-dimer levels indicating enhanced fibrinolysis. Our finding is consistent with recent researchers who also found an association between PAI gene -675 (4G/5G) polymorphism and increased postoperative bleeding volume [10, 12, 14, 37].

Jimenez Rivera with coworkers [10] studied genetic factors associated with excessive bleeding after CPB in a study on 26 patients. Excessive bleeding was observed in 1 (20%) of the 5 4G/4G genotype carriers, 5 (42%) of the 12 4G/5G carriers, and 7 (78%) of the 9 5G/5G genotype carriers. They concluded that excessive bleeding was significantly associated with the 5G homozygote for the PAI-1 polymorphism.

Iribarren et al. [37] investigated the possible role of PAI-1 -675 (4G/5G) polymorphism with coagulation, fibrinolysis and inflammation parameters in patients with excessive bleeding after elective CPB. The main conclusion agrees with our finding that 5G/5G carries show significantly greater blood loss 24 hours after surgery and lower levels of PAI-1.

So far, no studies have been published concerning ***PAI-1 gene -844 A/G polymorphism*** in patients undergoing cardiac surgery with CPB and its influence to

bleeding volume. A few investigators have reported the importance of PAI-1 gene -844 A/G polymorphism in patients undergoing myocardial infarction [35]. In the study where the association between PAI-1 -844 A/G and changes in PAI-1 and t-PA levels investigated in 305 patients with myocardial infarction in comparison with 328 healthy controls. Higher PAI-1 levels and reduced t-PA levels were observed in myocardial infarction patients in comparison with the healthy group. The elevation of PAI-1 levels was more pronounced in 844 A carriers. Regression analysis confirmed an independent association of -844 A/A genotype with myocardial infarction [35].

Additionally, the report published so far about PAI-1 -844 A/G polymorphism and fibrinolysis parameters showed that G-5G carriers had significantly lower PAI-1 concentrations [36]. Authors investigated contribution of PAI-1 promoter haplotypes to PAI-1 plasma levels analyzing together PAI-1 -675 (4G/5G) and PAI-1 -844 A/G polymorphisms in two independent populations each including 600 healthy causians. These results agree with our findings indicating that patients with PAI-1 -844 G/G genotype might have higher fibrinolytic activity due to lower levels of PAI-1 and t-PA/ PAI-1 complex. In our study PAI plasma levels were analyzed separately in both PAI-1 675 (4G/5G) and PAI-1 -844 A/G gene polymorphisms and the main limitation was a small sample size when comparing with the study by *Verschuur et al.* [36] on healthy volunteers.

We found no studies analyzing PAI-844 A/G polymorphism in association with bleeding after cardiac surgery with CPB. Therefore, we found it more challenging to investigate the influence of PAI-1 -844 A/G polymorphism to PAI-1 and t-PA/PAI-1 complex plasma concentrations and its association with bleeding after on-pump cardiac surgery. It was a trend to show that patients with PAI-1 -844 G/G genotype could have greater risk to bleeding because of enhanced fibrinolysis due to lower PAI-1 and t-PA/ PAI-1 plasma concentrations. According to our data, PAI-1 -844 G/G carriers demonstrated a significant 36% reduction in preoperative PAI-1 levels when compared to A/A carriers. Carriers of the G/G genotype showed lowest t-PA/PAI-1 levels 24 hours after the surgery, but without significant differences between the genotype groups.

Unfortunately we were unable to confirm increased fibrinolysis by higher D-dimer levels for PAI-1 -844 G/G carriers immediately after surgery (T1) and also at T6 time point. But 24 hours after operation the highest D-dimer level was demonstrated

by the G/G genotype group, possibly showing lower inhibitory potential of fibrinolysis in contrast A/G carriers showed the lowest D-dimer level at T24. Analyzing 24-hour postoperative blood loss, both G/G and A/G carries demonstrated higher postoperative blood loss, which significantly differed from A/A carriers that had the lowest blood loss after surgery. We speculate whether enhanced fibrinolysis might be one of the factors affecting the blood loss of G/G carriers, as assessed by the lower PAI-1 and t-PA/PAI-1 plasma concentrations. As noticed above there are no confirmatory data on PAI-1 gene -844 A/G polymorphism and postoperative bleeding after elective CPB surgery

The great challenge was to investigate ***ACE gene Intron 16 I/D polymorphism*** and its association with fibrinolysis and postoperative blood loss in patients undergoing cardiac surgery with CPB. Still this topic has been paid little attention to, and the few reports are conflicting [12, 37, 66-68].

In the present study, where PA-1 was determined preoperatively, and t-PA/PAI-1 complex and D-dimer levels 24 hours postoperatively in three ACE gene Intron 16 I/D genotype groups, we observed statistical significant difference with regard to preoperative PAI-1 between I/I and D/D carriers. The I/I genotype group had approximately a 33% lower PAI-1 plasma concentration before surgery as compared with the D/D genotype group. Carriers of I/I showed significantly lower levels of t-PA/ PAI-1 complex in comparison with I/D carriers. However, we detected higher D-dimer levels after surgery in the I/I genotype group and lower in D/D carriers, but 24-hour postoperative bleeding volume was not different in the three genotype groups.

Our study agrees with a previous report from Japan [67] evaluating the association between ACE I/D polymorphism and plasma PAI-1 levels in healthy volunteers found that those with the D-allele had higher levels of PAI-1, supporting our findings that carries of ACE intron 16 D/D genotype have higher plasma concentrations of PAI-1 when compare with I/I carriers.

There are few publications showing ACE Intron 16 I/D association with postoperative bleeding volume [12, 37, 66]. However, our study did not confirm significant differences in 24-hour postoperative blood loss between the three genotype groups under study.

Considerable study is published by *Welsby* et al. [12] analyzed 19 genetic polymorphisms of coagulation proteins and their associations with bleeding after cardiac surgery with CPB. ACE Intron 16 I/D polymorphism demonstrated significant association with bleeding. The deletion (D) allele of the ACE I/D polymorphism was associated with decreased bleeding. This could be explained by higher plasma levels of ACE, PAI-1 and t-PA/PAI-1 complex in combination with an increased vasoconstrictor tone.

A tendency towards increased bleeding was associated with ACE Intron 16 I/I genotype, which was found by *Irribarren* et al. [37], who investigated a possible role of several genetic polymorphisms associated with coagulation, fibrinolysis and inflammation in patients with excessive bleeding after elective CPB. These investigators found that significantly greater bleeding 24-hours postoperatively was associated with ACE Intron 16 I/I genotype. Unfortunately they did not determine PAI-1 and t-PA/PAI-1 plasma levels together with the ACE Intron 16 I/D polymorphism.

In a non-cardiac surgical population is confirmed that homozygosity of the deletion genotype (D/D) has been associated with higher ACE concentrations and the decreased bleeding tendency [66]. In contrary, some investigators analyzing ACE polymorphism and its influence on the risk of bleeding during total hip replacement showed that patients carrying D/D homozygous and I/D heterozygous genotypes of the ACE gene had higher total blood loss [68]. Surprisingly the results suggest that the D allele is a risk factor for increased blood loss, although I/I patients had higher D-dimers suggesting that a more efficient activation of coagulation occurred in this group. In our thesis a tendency towards enhanced fibrinolysis in I/I genotype group also was confirmed by higher D-dimer levels after the surgery. Contrary to the report by Pola et al. [68] we and other investigators support the idea that a greater bleeding tendency could occur in the presence of ACE Intron 16 I/I genotype.

4.3. Changes of coagulation state after CPB detected by thromboelastography and standard coagulation tests

In this comparison of TEG® and standard coagulation tests, we demonstrated that kTEG and hep.kTEG might be reliable parameters for evaluation of the coagulation

state after CPB affecting by hemodilution. There was a trend showing that hemodilution also can influence coagulation as assessed by the kTEG and the hep.kTEG parameters and standard coagulation tests and their potential associations to 24-hour postoperative blood loss in patients after cardiac surgery with CPB.

There is still an ongoing discussion as to whether hemodilution causes hypo- or hypercoagulability. *Bolliger et al.* [214] showed that a 40% dilution with saline solution significantly decreases thrombin generation therefore causing hypocoagulability. On the other hand, some authors assure that thrombin generation is most likely to be increased because concentrations of polymerized fibrin and ATIII are reduced due to hemodilution [215, 216].

Investigators recently reported a study including 26 patients undergoing cardiac surgery with CPB [217]. In that investigation the whole blood coagulation was assessed with ROTEM® and coagulation tests. They found that decreasing levels of fibrinogen could be determined quickly by using FIBTEB and that ROTEM® variables were significantly affected by hemodilution. Hypocoagulation was confirmed by thrombin generation (TG) measurements which was decreased by 13% from baseline after CPB and coagulation factor levels were reduced by 40%. In our study we analyzed TEG® and standard coagulation tests. We are agree with the findings of *Ogawa et al.* [217] that TEG® are progressively affected during CPB by hemodilution showing that hypocoagulation, as well as reduction of fibrinogen levels can be detected by kTEG and hep.kTEG K and A-angle parameters.

Some authors have tried to demonstrate the individual plasma coagulation factor activity after CABG in relation to hemodilution and postoperative bleeding [196]. Plasma concentrations of fibrinogen and the activity in plasma of FII, FV, FVII, FVIII, FIX, FXI, FXI and FXIII were measured. They concluded that there is a marked dissociation in the plasma activity of individual coagulation factors after CPB: the fibrinogen level, and plasma activity of FII, FV, FX and FXIII were reduced two hours after surgery. Simultaneously, FVII and FXI were unchanged, while FVIII and FIX increased. We did not investigate plasma activity of individual coagulation factors after CPB. Performing TEG® we could estimate coagulation factors qualitatively together with the quantitative function. R time is mainly affect by thrombin generation. If thrombin generation is decreased it is presented as a prolonged R time and K clot time

and decreased A-angle, which signifies the deficiency of fibrinogen, reduction of coagulation factors and/or presence of anticoagulants. MA mainly denotes deficiency and/or dysfunction of platelets. In our study, the infusion of higher saline volumes were strongly associated with a drop in kTEG and hep.kTEG parameters that might reflect reduction of plasma activity and concentration of various coagulation proteins, fibrinogen plasma level and impaired platelet function due to dilution coagulopathy.

Standard coagulation tests usually performed in practice represent the fibrinogen level and the PLT count. PI reflects the extrinsic and APTT the intrinsic coagulation pathways. Additionally, these coagulation tests are frequently inadequate for the purpose of monitoring coagulation and hemodilution [84, 108]. As mentioned in our results, we found no significant intergroup differences in standard coagulation tests except for the fibrinogen level, but we detected significant changes in all the kTEG and hep.kTEG parameters with different priming saline volume.

In a large comparative study of 613 adult patients undergoing CABG surgery the investigators found no significant correlations between pre- and postoperative PLT count, INR, APTT and hemodilution [84]. The investigators evaluated hemodilution by analyzing the lowest Ht level during CPB. However, they did not find any differences in the first 12-hour of blood loss, but the mean number of units transfused differed significantly between more and less diluted patients. Thus, apparently, the level of hemodilution can affect postoperative bleeding.

Ruttman et al. [218] studied *in vitro* the influence of hemodilution with saline or hemaccel on TEG® parameters. The R, K time and R+K time were prolonged and the A angle was increased relative to control in both diluents groups. These authors concluded that hemodilution per se increases the initiation of coagulation and speed of clot formation of whole blood *in vitro*, but saline hemodilution has a more marked effect on the final clot strength. In our study, the amount of CPB prime was constant 1400 ml with different additional saline volume of deltajonin, which was selected as the main indicator of patient division into more and less diluted groups. We noticed in kTEG significantly prolonged R, K time and decreased A-angle and MA time in the group with more diluted patients and the same tendency was found in hep.kTEG. We speculate, that most likely, patients with greater hemodilution might have hypocoagulability with prolonged speed of initial clot formation and decreased initial clot cross linking

reactions with activated platelets and clots that have less strength and firmness. It could be caused by a greater decrease in circulating procoagulant activity than anticoagulant activity.

In our study the highest difference between I and II group showed kTEG parameter A-angle, presenting the role of fibrin polymerization making fibrin net for platelet plug stabilization. Our finding is supported by *Mittermayr* et al. [219] who demonstrated that disturbed fibrin polymerization is the main coagulation problem arising from hemodilution that could be reversed by fibrinogen concentrate administration.

Conversely to our results, a few investigators describe the hemodilution-associated hypercoagulability as a result of a change in the procoagulant/anticoagulant proteins balance [215, 216, 220]. *Nielsen et al.* [216] demonstrated that hemodilution causes hypercoagulation due to decreased ATIII activity, which is responsible for the accelerated clot initiation. They found a linear relationship between the degree of dilution with 0%, 10%, 20% and 30% saline solution and TEG® variables. On the other hand, the same authors describes hypocoagulability in the presence of hemodilution with lactated Ringer's solution [221] supporting our finding either.

We believe that several factors influence blood loss during and after cardiac surgery employing CPB. Therefore, an important issue is to find the most appropriate bleeding marker, and many studies are made in this field. There is no single bleeding marker, which by itself has the necessary sensitivity and specificity to predict excessive bleeding. Evidently, blood loss can be influenced by many perioperative factors described in this thesis, moreover we must balance between bleeding and thrombotic complications in cardiac surgery patients. In our study we tried to find associations between standard coagulation tests, two fibrinolysis markers and three genetic polymorphisms affecting the fibrinolytic activity. In addition, we evaluated the TEG® parameters in our attempts to predict bleeding and optimize the hemostatic management of patients undergoing cardiac surgery. Nevertheless, there are still questions to answer and additional studies are warranted to find more precise bleeding markers or their combination as well as coagulation changes affecting by CPB.

5. CONCLUSIONS

Taking into account the complexity of enhanced bleeding after cardiac surgery, it might be difficult to isolate one factor as the denominator of bleeding. The following conclusions could be made:

1. APTT and PLT count constitute the most significant changes from standard coagulation tests after CPB. Decreasing in PLT count can suggest of increased bleeding tendency after cardiac surgery.

2. Preoperative plasma fibrinogen concentration documented the highest predictive value for a greater 24-hour postoperative blood loss, which could be as a possible predictor of increased bleeding risk in the postoperative period.

3. Our investigation indicates that low plasma levels of PAI-1 preoperatively and of t- PA/PAI- 1 complex postoperatively, in parallel with increased plasma concentration of D-dimer can be useful predictors of fibrinolysis, and thus, of increased postoperative blood loss.

4. Tendency to enhanced fibrinolysis affecting postoperative blood volume showed PAI-1 gene -675 5G/5G, PAI-1 gene -844 G/G and ACE Intron 16 I/I genotypes.

5. Our results indicate that kTEG and hep.kTEG can reflect hypocoagulability after CPB that could not be detected by standard coagulation tests.

6. Postoperative 24-hour blood loss can be affected by the volume of saline solution used in the extracorporeal circuit.

6. PRACTICAL RECOMMENDATIONS

1.We recommend to use standard coagulation tests before and after cardiac surgery with CPB for all patients and to focus more attention on preoperative plasma fibrinogen measurements and on changes in PLT count after surgery.

Preoperative fibrinogen level < 3.6 g/L should be observed as a marker for increased bleeding risk after cardiac surgery with CPB.

If PLT count is lower then 150 x 10^9/L transfusion of platelets should be considered for bleeding patient in ICU after cardiac surgery.

2.The established two fibrinolytic system markers PAI-1 and t-PA/PAI-1 should be introduced for practical diagnostic use for bleeding risk stratification before cardiac surgery employing by CPB. Therefore, by including screening of fibrinolytic markers pre – and postoperatively, we might be able to identify patients with low fibrinolytic inhibitory potential, who might benefit the most from antifibrinolytic therapy prior to cardiac surgery.

We recommend for patients with preoperative PAI-1 levels < 25 ng/mL administration of tranexamic acid 20-25 mg/kg followed by 1mg/kg/h infusion instead of 10 mg/kg followed by 1 mg/kg/h infusion during surgery.

We propose for bleeding patient with postoperative t-PA/PAI-1 complex plasma concentration < 5 ng/mL to consider additional administration of tranexamic acid in a dose 10-20 mg/kg in ICU after cardiac surgery.

3.Current knowledge of the effect of gene variability to fibrinolytic activity and bleeding is still limited. Gene analysis could be an additional criterion for diagnosing increased bleeding risk in most complicated clinical cases.

4.Taking into account the results of TEG performed after cardiac surgery with CPB, it is possible to evaluate the compromised coagulation state more precisely with TEG as with standard coagulation tests.

We recommend to perform TEG for bleeding patients after cardiac surgery to evaluate influence of hemodilution to coagulation state.

We propose to perform TEG for bleeding patient to provide more precise initial hemostatic therapy guided by TEG parameters: R > 11 min, 2-4 units of FFP or 10-15 ml/kg, alpha angle < 45^0 , CRIO 1-2 bags/10 kg, maximum amplitude < 54 mm, Desmopressin 3 μg/kg following by platelet transfusion if necessary.

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