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Die Plasma - Sensibilität auf Heparin und Enoxaparin

´´The Plasma Sensibility to Heparin and Enoxaparin``

Inaugural-Dissertation
zur
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Summary

Unfractionated heparin (UFH) and low-molecular-weight-heparin (LMWH) are the most common used clinical anticoagulants for treatment of patients with venous thromboembolism (VTE) or acute coronary syndrome (ACS) and for antithrombotic prophylaxis within hospitals. The clinical tendency increasingly favours LMWH, even intravenously, e.g. in ST elevation myocardial infarction (STEMI).

To test the anticoagulant action of the heparins in the major part of the work, the extrinsic coagulation activity assay (EXCA) was performed with 51 normal citrated plasmas or 213 patient plasmas (with normal PT and APTT in absence of LMWH) after 50 µl sample supplementation with 0-1 IU/ml UFH or LMWH.

To test the procoagulant action of the heparins in the minor part of the work, the recalcified coagulation activity assay (RECA) was performed with 10 normal citrated plasmas or 32 patient plasmas, after 50 µl sample supplementation with 0-10 mIU/ml UFH or LMWH.

1 IU/ml enoxaparin reduced the normal thrombin generation in EXCA to about 13 % of normal, whereas at 1 IU/ml heparin the normal thrombin generation was completely suppressed. This means that 1 IU/ml enoxaparin is a strongly therapeutic anticoagulant, whereas 1 IU/ml UFH is in the toxic range. 0.01 IU/ml heparin or enoxaparin reduced the normal thrombin generation in EXCA only by about 10 % or 20 %, respectively.

Heparin at 0.1 mIU/ml does not reduce thrombin generation in RECA; instead it even increased the thrombin generation in RECA by about 20 %, whereas 0.1 mIU/ml enoxaparin significantly decreased the thrombin generation by about 30%-60%. This means that in ultra-low concentrations, as e.g. occurring in the end of heparin infusion, heparin might be dangerous especially for patients with susceptible intrinsic hemostasis. It is suggested to add enoxaparin at the end of heparin infusion or to replace heparin by enoxaparin.

These results support the concept that the LMWH dosage of each individual patient should be adjusted with an ultra-specific thrombin generation assay as EXCA or INCA (intrinsic coagulation activity assay). The dosing of LMWH-enoxaparin must be individualized according to the patient´s need (prophylactic or therapeutic) and anticoagulant response as reflected by trigger-induced thrombin generation. Target for therapeutic or prophylactic anticoagulation is 10-20 % or 20-40 % of normal trigger-induced thrombin generation, respectively. The adequate dosage of enoxaparin in the individual patient should be monitored by a specific thrombin generation assay.

Zusammenfassung

Unfraktioniertes Heparin (UFH) und niedermolekulares Heparin (NMH) sind die am häufigsten verwendeten klinischen Antikoagulantien zur Therapie von Patienten mit venösen Thromboembolien (VTE), akutem Koronarsyndrom (ACS) und für Thromboseprophylaxe in Krankenhäusern. Die klinische Tendenz favorisiert zunehmend NMH, auch intravenös, z.B. beim transmuralen Myokardinfarkt (STEMI).

Der extrinsische Gerinnungs-Aktivitätstest (EXCA) wurde im Hauptteil der Arbeit bei 51 normalen Citratplasmen oder 213 Citratplasmen der Patienten (bei normaler PT und APTT in Abwesenheit von NMH) nach 50 µl Supplementierung mit 0-1 IU/ml UFH oder NMH durchgeführt.

Der recalcifizierte Gerinnungsaktivitätstest (RECA) wurde im Nebenteil der Arbeit mit 10 normalen Citratplasmen und in 32 Citratplasmen der Patienten nach 50 µl Supplementierung mit 0-10 mIU/ml UFH oder NMH durchgeführt.

1 IU/ml Enoxaparin reduzierte die normale Thrombin-Generierung in EXCA auf etwa 13% der Norm, während bei 1 IU/ml Heparin die normale Thrombin-Generierung komplett supprimiert wurde. Dies bedeutet, dass 1 IU/ml Enoxaparin ein stark therapeutisches Antikoagulans ist, während 1 IU/ml UFH bereits im toxischen Bereich ist. 0.01 IU/ml Heparin oder Enoxaparin reduzierte die normale Thrombin-Generierung in EXCA nur um etwa 10% oder 20%.

UFH bei 0.1 mIU/ml reduzierte die Thrombin-Generierung im RECA nicht, sondern erhöhte sogar die Thrombin-Generierung um etwa 20%, während 0.1 mIU/ml NMH signifikant die Thrombingenerierung um etwa 30% -60% hemmte. Dies bedeutet, dass Heparin in ultra-niedrigen Konzentrationen, wie z. B. am Ende der Heparin-Infusion, besonders für Patienten mit sensibler intrinsischer Hämostase gefährlich werden könnte. Es wird vorgeschlagen, mit NMH das Ende der Heparin-Infusion zu überbrücken oder UFH auf NMH umzustellen.

Diese Ergebnisse unterstützen das Konzept, dass die Dosierung der NMHs für jeden Patienten individuell durch einen ultra-spezifischen Thrombin-Generierungstest (EXCA oder INCA (intrinsischer Gerinnungs-Aktivitätstest)) angepasst werden sollte. Die Dosierung der NMH soll individuell entsprechend dem Ziel der Antikoagulation (prophylaktisch oder therapeutisch) und dem plasmatischen Ansprechen auf das Heparin angepaßt werden. Zielaktivität für therapeutische oder prophylaktische Antikoagulation sind 10-20% oder 20-40% der normalen Trigger-induzierten Thrombin-Generierung respektiv. Die adäquate Dosierung des NMH beim individuellen Patienten sollte durch einen spezifischen Thrombin-Generierungstest kontrolliert werden.

Abbreviations

ΔA/t	Absorption increase per time
ACS	Acute coronary syndrome
ACT	Activated clotting time
APLA	Antiphospholipid antibodies
APTT	Activated partial thromboplastin time
AT-3	Antithrombin-3
BK	Bradykinin
Ca^{2+}	Calcium-Ions
CRP	C - reactive protein
CRT	Coagulation reaction time
CTS	Chromogenic test system
DIC	Disseminated intravascular coagulation
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
EXCA	Extrinsic coagulation activity assay
FSP	Fibrin split products
GAG	Glycosaminoglycan
HC-2	Heparin cofactor-2
HIO	Heparin induced osteopenia
HISN	Heparin induced skin necrosis
HIT	Heparin induced thrombocytopenia
HMWK	High-molecular-weight kininogen
HRG	Histidine-rich glycoprotein
IC50	50 % inhibitory concentration
INCA	intrinsic coagulation activity assay
INR	International normalized ratio
ISI	International sensitivity index
IU	International units
IU/m^2	International units per squared meter
IU/mg	International units per milligram
IV	Intravenous

IVBT-CT	in vitro bleeding test closure time
KCT	Kaolin clotting time
kDa	Kilodalton
LMWH	Low-molecular-weight heparin
mIU	Milli-international unit
mmol/l	Millimol per liter
MV	Mean value
NaCl	Sodium chloride
OAT	Oral anticoagulant therapy
PAR1	Protease-activated receptor 1
PCa	Activated protein C
PdCA	Phospholipid-dependent coagulation assay
PF4	platelet factor 4
PK	Prekallikrein
PL	Phospolipid
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PT	Prothrombin time
PTr	Prothrombin time ratio
RECA	Recalcified coagulation activity assay
SC	Subcutaneous
SD	Standard deviation
SGAG	Sulphated glycosaminoglycans
SHBG	Sex hormone-binding globulin
TAFI	Thrombin-activated fibrinolysis inhibitor
TF	Tissue factor
TGT	Thrombin generation test
TM	Thrombomodulin
TSR	Thrombin-sensitive region
TT	Thrombin time
TVT	Taipan venom test
UFH	Unfractionated heparin
VKD	Vitamin K-dependent
VTE	venous thromboembolism

vWF	von Willebrand factor
WBA	Whole blood aggregometry
WBCT	Whole blood clotting time

Contents

1. Introduction

1.1. Physiology and Pathophysiology of Hemostasis

1.1.1. Definition

Hemostasis is the system of generation and destruction of micro- and macro-thrombi. Primary hemostasis is the thrombocytes system; secondary hemostasis the generation of plasmatic thrombin, tertiary hemostasis is cellular and plasmatic fibrinolysis.

Hemostasis protects life by

1. preventing critical minor bleeding or preventing major blood loss by thrombin-mediated sealing of injury sites in the vascular system,

2. allowing vessel patency by inhibition of excess thrombin and plasmin-mediated lysis of vessel lumen occluding thrombi.

Hemostasis must be strictly controlled so that blood does not coagulate within the vasculature which would prevent normal blood flow. Blood flow may be lost because of pump failure, blood leakage, or blood vessel obstruction. According to the Virchow´s Law physiological hemostasis requires physiological
a) blood flow,
b) blood itself,
c) blood vessels.

Hemostasis consists of multiple processes that center on the generation of α-thrombin, the enzyme that converts soluble fibrinogen into insoluble fibrin. Physiologically, unnecessary fibrin is removed by plasmin (Jenny NS, Mann KG. 1998).

There are two main triggers to initiate mammalian blood coagulation: the intrinsic trigger is an unphysiologic surface (e.g. cell fragments or a xenobiotic), the extrinsic trigger is tissue factor (TF, thromboplastin, CD142, coagulation factor III).

Pathological amounts of TF in blood occur seldomly; only if an organ rich in TF such as brain or placenta is injured, the extrinsic pathway with its main protease F7a is activated.

Most clinical situations are associated with an activation of the intrinsic pathway of blood coagulation, consisting of F12a, kallikrein, HMWK, F11a, F9a, F8a. The common pathway consists of F10a, F5a (accelerin), F2a (thrombin), fibrin. Phospholipids catalyze both intrinsic and extrinsic pathway, they are cofactors for F10a generation and F2a generation. Calcium ions are required to fold factors 2, 7, 9, and 10 into an activatable form. The most important immediate regulator protein of blood coagulation is the polysulphated glycosaminoglycan (SGAG)-dependent antithrombin-3 (AT-3). The Ca^{2+} dependent factors protein C and protein S act by subacute inactivation of F5a and F8a (Davidson CJ et al. 2003; Krem MM, Cera ED. 2002).

1.1.2. History of Hemostasis (Blood Coagulation and Fibrinolysis)

The transformation of fluid blood to a gel-like substance (clot) has been a topic of great interest to scientists, physicians, and philosophers since the days of Plato and Aristotle (Jewett B (ed.). 1892). However, it was not until the beginning of the 18th century that blood clotting (coagulation) was appreciated as a means to prevent blood loss from wounds (hemostasis) (Petit JL. 1731). As with other areas of science, the microscope played a pivotal role in the understanding of coagulation. In the mid-17th century, Marcello Malpighi separated the individual components of a blood clot into fibers, cells, and serum. The fibers were later found to be derived from a plasma precursor (fibrinogen) and given the name fibrin (Babington BG. 1830). Further developments in the mid-19th century included the recognition of an enzyme (later called thrombin) that was capable of coagulating fibrinogen (Buchanan A. 1845) (Figure 1). In the latter half of the 19th century, the scientific community began to appreciate that clotting activities of thrombin could not be a constituent of normal plasma (otherwise clotting would occur continuously and at random) (Schmidt A. Zur Blutlehre. Leipzig, Vogel, 1892). This concept was vital to our understanding of the complex “checks and balances” system of coagulation, wherein inactive precursors are activated precisely where and when they are needed. It also fostered the belief that blood contained many, if not all, of the necessary elements for intravascular coagulation (circulating predominantly in an inactive form).

This hypothesis served as the basis for the theory of intrinsic coagulation (Schmidt A. Zur Blutlehre. Leipzig, Vogel, 1892).

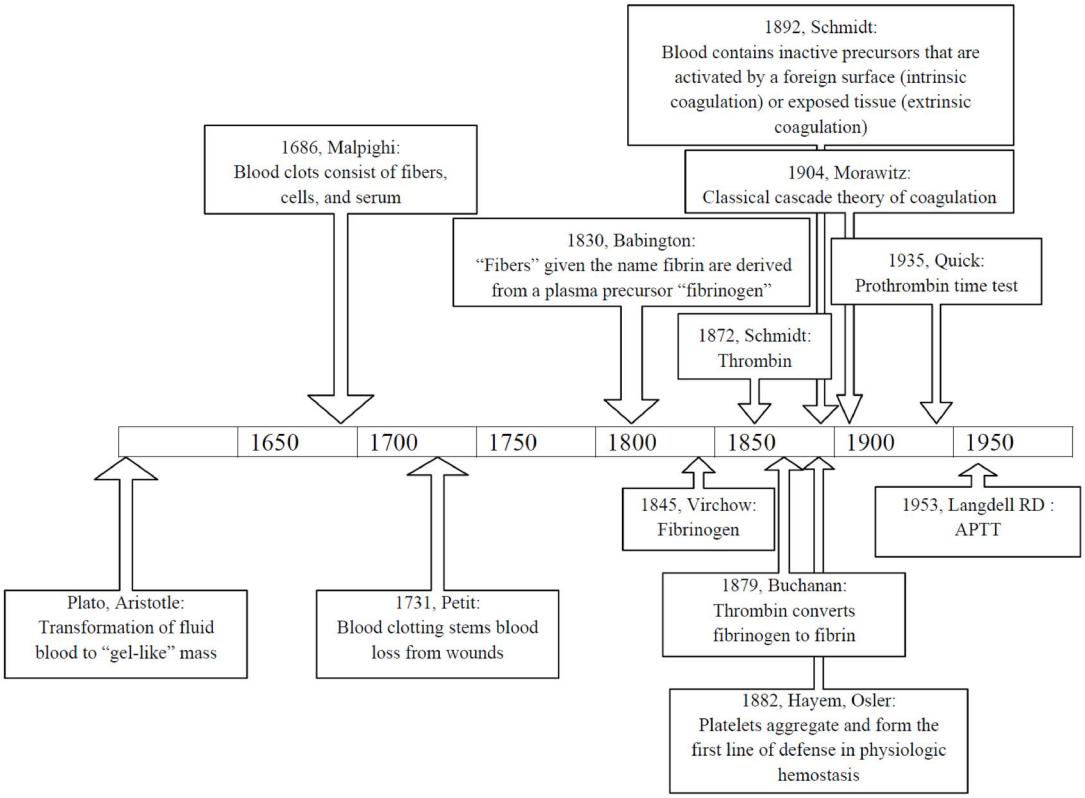

Figure 1. History of Hemostaseology.

Researchers showed that blood coagulated when it came into contact with a foreign surface and that some surfaces were more "thrombogenic" than others. This concept paved the way for an expanding knowledge of hereditary disorders of coagulation (Hay J. 1813). Developments in defining extrinsic coagulation followed the pioneering work of several investigators (De Blainville HMD. 1834; Howell WH. 1912; Mills CA. 1921; Thackrah CT. 1819), all of whom described blood coagulation following the infusion of tissue suspensions (later called tissue thromboplastin or tissue factor). A revised theory of extrinsic coagulation suggested that an exposed tissue surface (from a damaged blood vessel wall) was capable of stimulating blood clotting. Later discoveries included the direct contribution of calcium ions (Bordet J. 1921), phospholipids (Chargaff E, 1944), and other essential components of the prothrombinase complex (factors 5a, 10a) (Hougie C, 1957) to blood coagulation (Figure 2).

Stenflo isolated bovine vitamin K-dependent protein C, which was named protein C because it was the third peak to elute from the DEAE column (Stenflo J. 1976).

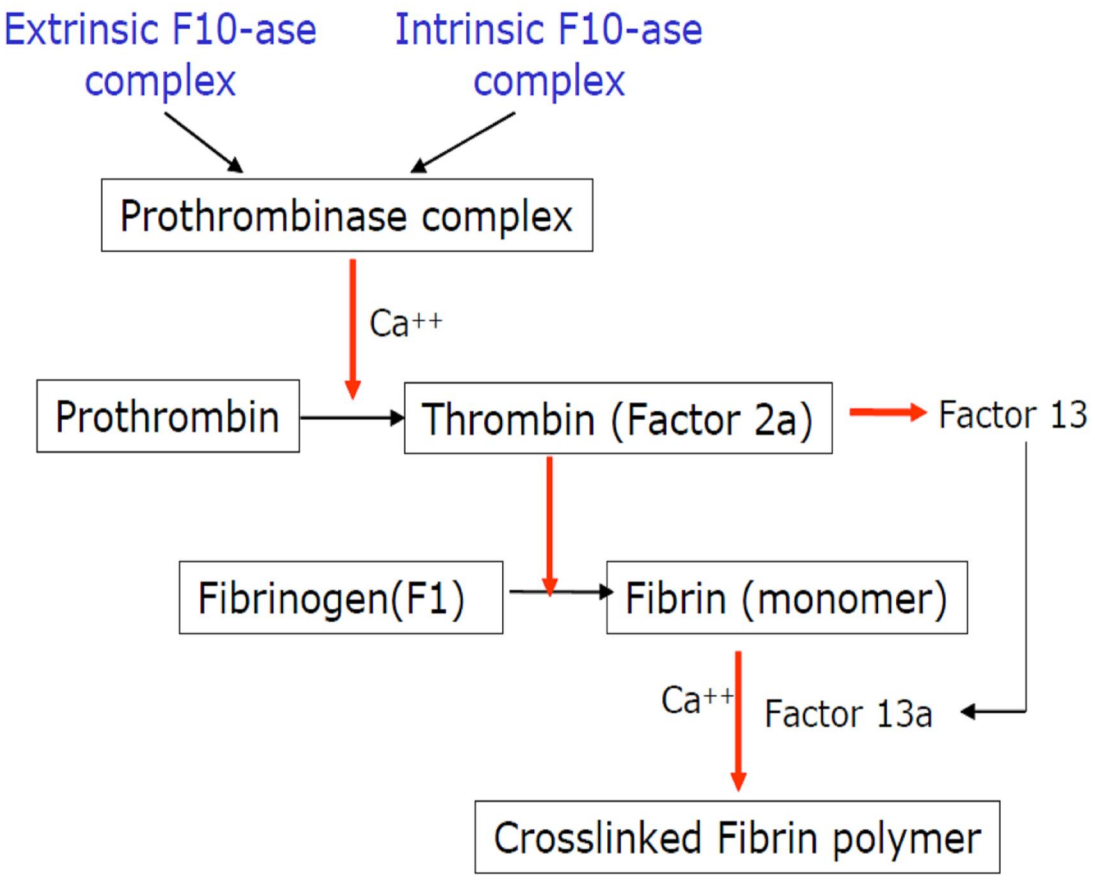

Figure 2. Waterfall or cascade model of hemostasis

The "waterfall" or "cascade" model of coagulation, proposed almost simultaneously by MacFarlane (1964) and Davie and Ratnoff (1964), expanded the former theory of Morawitz (1904) and provided both a structural and biochemical framework for understanding coagulation. The contribution of platelets to the coagulation process can be traced back to the mid-19th century and the original work of Alfred Donne, who discovered platelets with the help of a newly developed microscope lens (achromatic lens) (Donne A. 1842). However, the clinical importance of platelets in normal hemostasis was not appreciated until the end of the 19th century, when Sir William Osler (1874) described platelet aggregation and Hayem (1882) cited the importance of platelet plugs in preventing blood loss after tissue injury. The development of electron microscopy made it clear that platelets adhered to damaged blood vessels (Marcus AJ. 1969) and subsequently were "activated" through a variety of pharmacologic (e.g.,

adenosine diphosphate, epinephrine, thrombin) or mechanical (e.g., shear stress) stimuli (Grette K. 1962; Spaet TH, 1964; Willis AL, 1974). The surface of the activated platelet accelerates coagulation.

The inability of blood to fully coagulate following death was observed centuries ago, possibly as early as the days of Hippocrates. Pioneering work near the end of the 18th century described the process of fibrinolysis and a mechanism whereby a circulating precursor (plasminogen) generated (with the appropriate stimulus) an active enzyme (plasmin) capable of degrading clotted blood (Christensen LR, 1945; Hedin SG. 1904). The potential clinical ramifications of fibrinolysis and its application in treating thrombotic disorders began with the work of Gratia in 1921, who observed that clots could be dissolved by staphylococcal extracts. Tillet and Garner (Tillett WS, Garner RL. 1933) later reported that bacteria-free filtrates of β-hemolytic streptococci contained a substance (streptokinase) that was capable of dissolving blood clots. Soon thereafter, the work of Sherry et al. (1959) highlighted the potential use of fibrinolytics in humans. Plasminogen activators were found in many tissues of the body, including blood vessels themselves (MacFarlane RG, Pilling J. 1947; Williams JRB. 1951). Their physiology and pharmacology is important in human medicine (Figure 3).

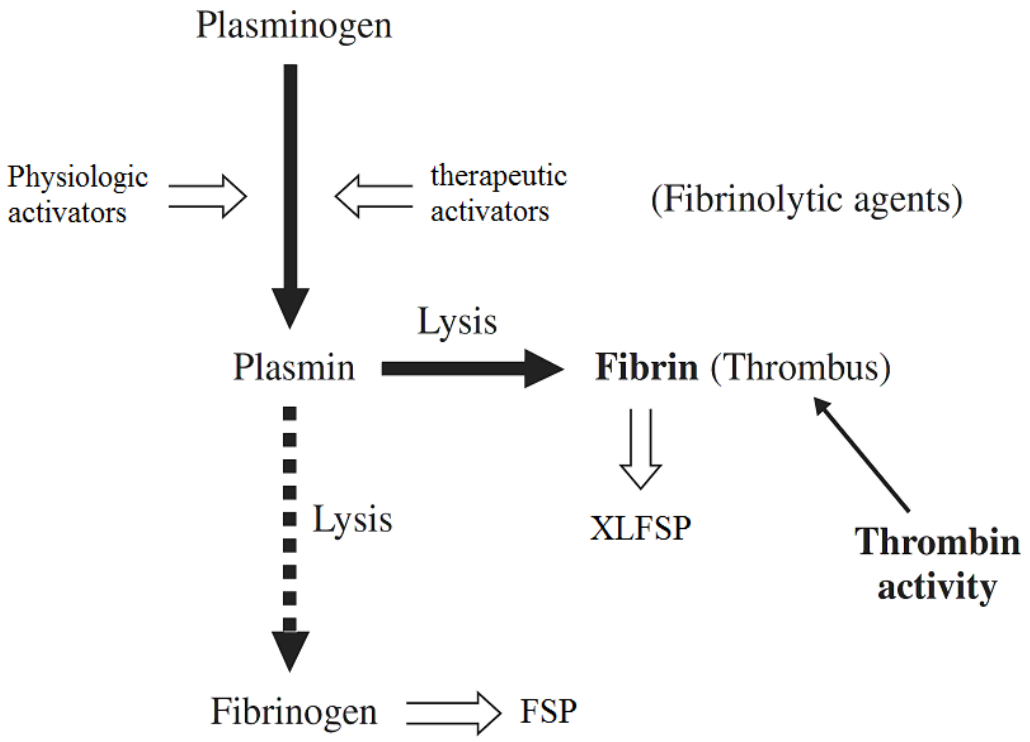

Figure 3. Fibrinolysis. The fibrinolytic system consists of an inactive precursor (plasminogen) that is converted (by the physiologic activators urokinase or t-PA or by therapeutic activators) to the aggressive protease plasmin that preferably splits fibrin but also fibrinogen to the respective split products (FSP).

1.1.3. Vitamin K-Dependent Proteins

Vitamin K-dependent (VKD) proteins, synthesized in the liver, play a central role in blood coagulation through either procoagulant or anticoagulant mechanisms. The vitamin K-dependent protein family includes the zymogen procoagulant factors F7, F9, F10, and prothrombin (F2) and the anticoagulants protein C, protein S (Figure 4). Except protein S, these proteins in their active form are serine proteases related to the trypsin and chymotrypsin superfamily. Limited cleavage of peptides (only at specific sites) converts the vitamin K-dependent zymogens into their active serine protease forms. (Girolami A,2008; Stafford DW 2005; Nelsestuen GL, 2000). Vitamin K is essential for the biosynthesis of these clotting factors by participating in the cyclic oxidation and reduction of the enzyme that converts 9 to 13 amino-terminal glutamic acid residues to γ-carboxyglutamic acid residues (Gla) (Berkner KL: 2008; Oldenburg J,2008). This post-translational multi-Gla linkage adds 9-13 negative

charges to the F 2, 7, 9, 10, PC, PS molecules that enable them to interact with Ca^{2+} and a membrane surface (Schwalbe RA, 1989). Blocking formation of the Gla residues addition is the basis for "blood-thinning" anticoagulant therapy with Phenprocoumon (Marcumar), Coumadin (Warfarin) and Acenocoumarol (Sintrom) derivatives, which are chemically similar in structure to vitamin K. This Ca^{2+} biochemistry is also the basis for the anticoagulant activity of sodium citrate, a reversible Ca^{2+} chelator, used for clinical laboratory testing of clotting activity. VKD-proteins are composed of 10-13 NH_2-terminal Gla residues followed by either a kringle domain (K) in prothrombin or an epidermal growth factor (EGF) like-domain in F7, F9, F10, protein C, and protein S (Figure 4).

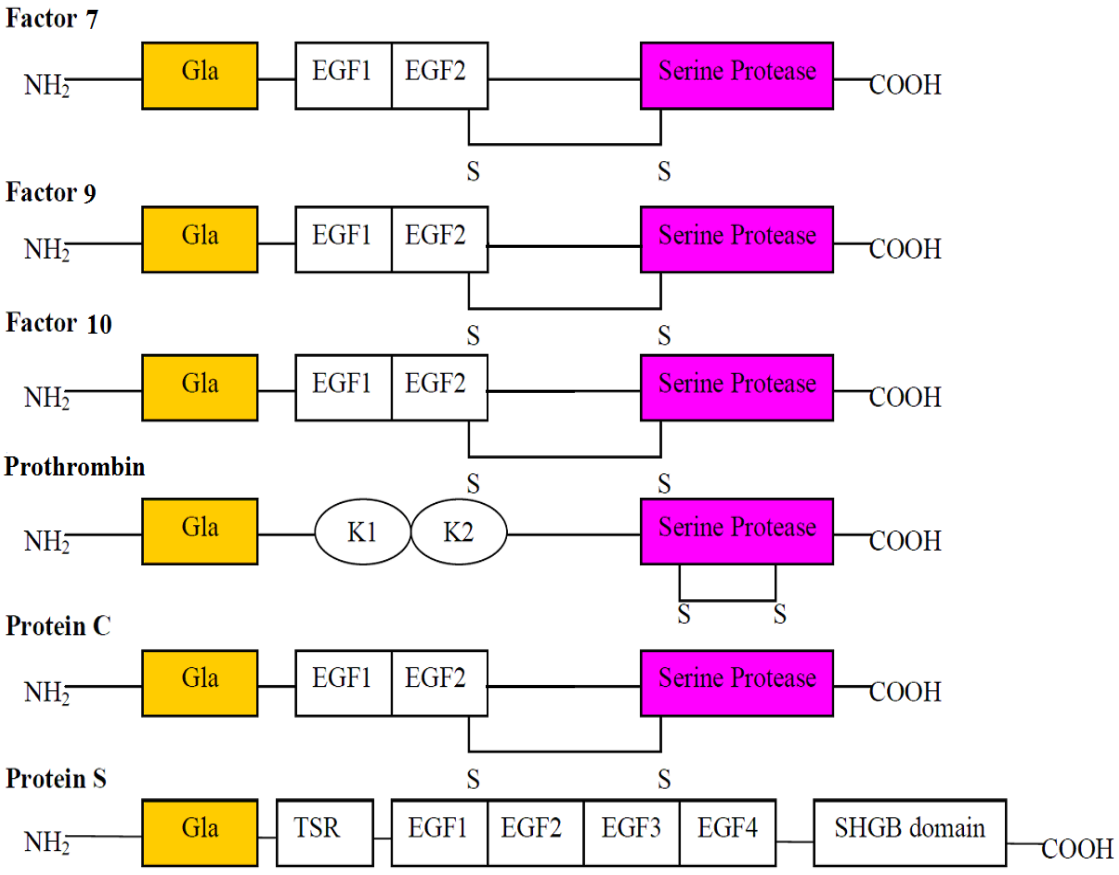

Figure 4. Schematic presentation of the vitamin K-dependent proteins.

TSR: thrombin-sensitive region; SHGB: sex hormone-binding globulin; EGF: epidermal growth factor; K: kringle domain; Gla: γ-carboxyglutamic acid residues.

Thrombin (F2a) is the key serine protease in hemostasis. It is the enzyme that creates the fibrin clot. With regard to procoagulation, F2a activates many of the proteins in the clotting cascade including fibrinogen, F5, F8, F11, F13 (Crawley, J.T.B., 2007). F2a also activates platelets and vascular endothelial cells (Bahou, W.F, 2002) and initiates cellular responses including production and secretion of cytokines, growth factors and cellular adhesion molecules (Davie, E.W, 2006**;** Crawley, J.T.B., 2007). With regard to its anticoagulant function, thrombin activates anti-fibrinolytic components such as protein C and a fibrinolysis inhibitor (TAFI) (Wolberg, A.S. 2007). Thrombin's precursor, prepro-prothrombin (Figure 5), is synthesized in hepatocytes where it undergoes posttranslational modifications. After entering the endoplasmic reticulum (ER), the signal peptide is removed and 10 glutamate residues adjacent to the propeptide are converted to γ-carboxyglutamate (Gla) residues (Stenflo, J., 1974**;** Nelsestuen, G.L., 1974**;** Wu, S.M., 1991). This Gla domain interacts with calcium ions and is critical for creating the conformation required to bind to negatively-charged phospholipid surfaces of injured vascular tissue and activated platelets which concentrates F9, F7, F10, and F2 at the injury site.

After addition of the Gla residues, the propeptide is removed and 3 asparagine N-linked oligosaccharide chains are attached in the mature molecule, 2 in the first kringle domain and one in the B-domain. Thrombin's zymogen, prothrombin (F2; 70 kDa), is then secreted into blood at a concentration of 1.2 μM.

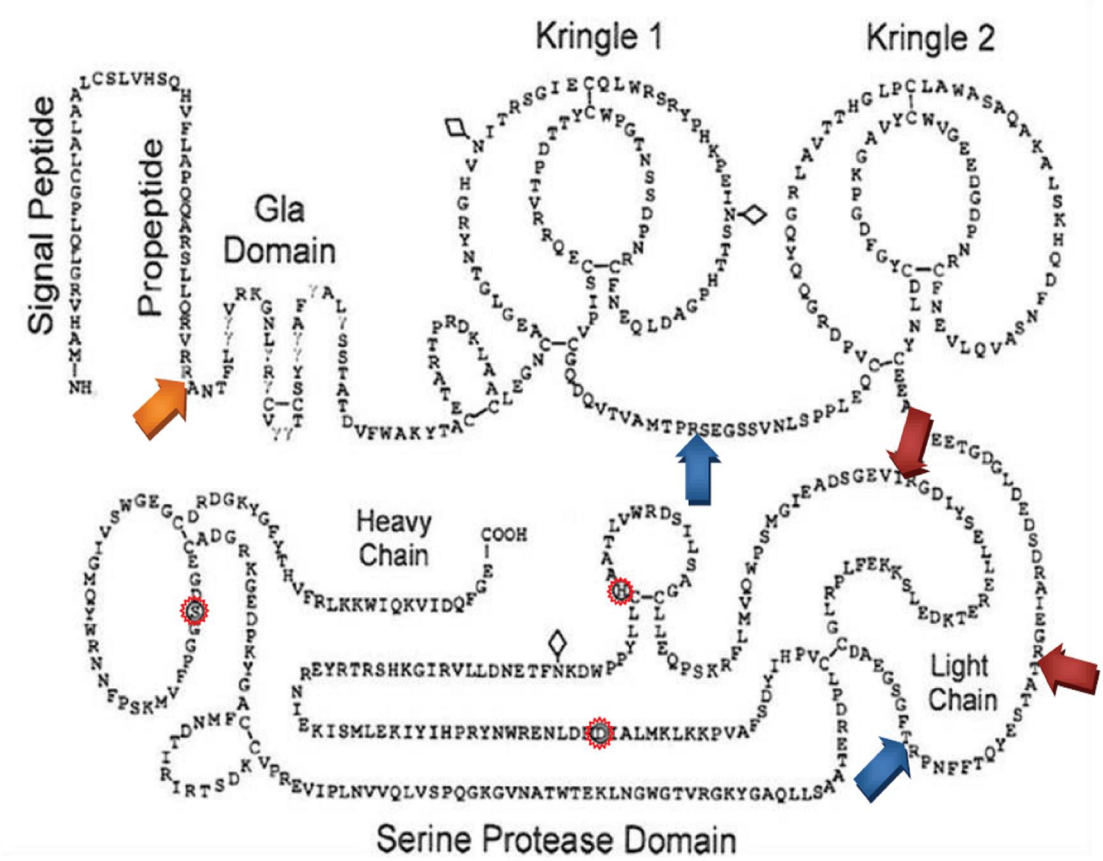

Figure 5. Amino acid sequence of prepro-prothrombin. Prepro-prothrombin contains a signal peptide, propeptide, Gla domain, two Kringle domains, a light (A-chain) and heavy (B-chain) chain. The N-terminal sequence of prothrombin is indicated by an orange arrow. Gla residues are in the sequence as γ and the three ◊ indicate amide-linked carbohydrates at asparagines (N). F10a cleaves at red arrows and F2a at blue arrows. The serine protease catalytic center amino acids (His (H), Asp (D), and Ser (S)) are circled in red. F1.2 is the fragment between the orange arrow and the first (N-terminal) red arrow (modified figure according to Davie and Kulman).

Prothrombin is converted to F2a by prothrombinase complex which consists of F10a, activated Factor 5 (F5a), and calcium ions complexed on the phospholipid surface of activated platelets (Figure 6). F10a activates prothrombin by cleaving Arg273-Thr274 creating the intermediate prethrombin-2 consisting of combined A- and B-chains after releasing fragment 1.2 (Krishnaswamy, S., 1987). This intermediate is subsequently converted to F2a by F10a cleavage at Arg322-Ile323, separating the A- and B-chains that remain linked by a disulfide bridge (Bishop, P.D., 2006). The prothrombinase

complex with the coagulation accelerator F5a activates prothrombin to F2a much faster than F10a alone (Nesheim, M.E., 1979, Bishop, P.D., 2006, Nesheim, M.E, 1981).

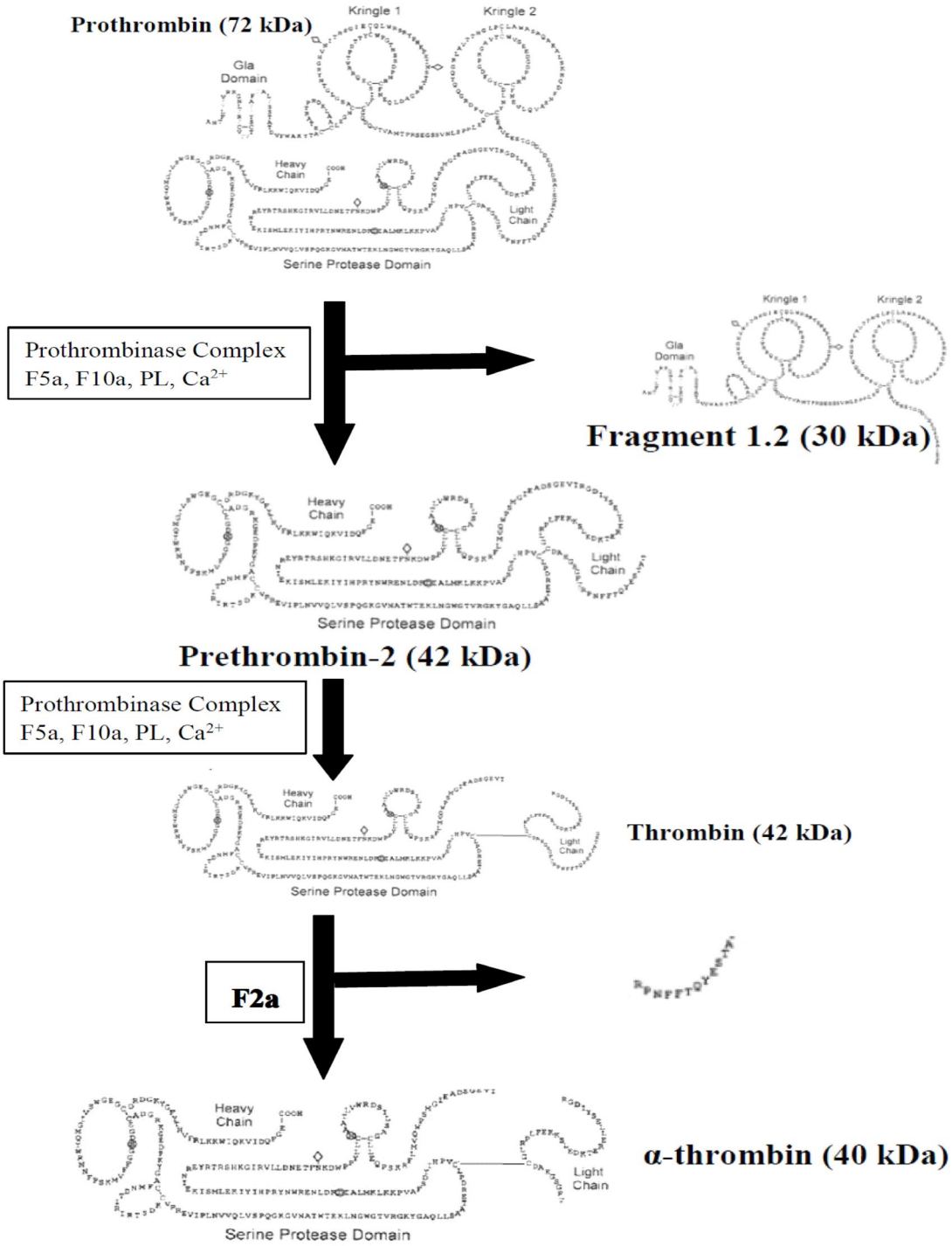

Figure 6. Activation of prothrombin. Prothrombin is activated by the prothrombinase complex to 42 KDa prethrombin-2a. Autocatalytic activation results in 40 KDa α-thrombin splitting off the 13 amino acid N-terminal of the A-chain (modified figure according to Davie and Kulman).

The serine protease, F2a, consists of the 49 residue A-chain linked to the 259 residue B-chain by a disulfide bridge (Figure 6). α-thrombin is formed when F2a is cleaved at Arg284 releasing a 13 residue peptide (Figure 7). Both F2a and α- thrombin (α-F2a) are active serine proteases (Davie, E.W, 2006). F2a is homologous to other serine proteases. The His···Asp···Ser catalytic center is in a cleft within the B-domain (Fehlhammer, H., 1977) and upon activation are brought into close proximity by salt bridges (Bode, W., 1992). The active site is surrounded by three surface loops (Bode, W., 1992**;** Di Cera, 1995; Dang, Q.D., 1997) and two charged surface areas (exosites) that create specificity for the respective substrates. F2a is removed quickly from the blood stream in vivo; however, in vitro, F2a can be degraded into less active products: β- and γ-thrombin (Figure 1.7). These degraded species result from proteolysis of the B-chain by plasmin, trypsin, (Boissel, J.P, 1984) F10a (Soslau, G., 2004) or F2a itself (Boissel, J.P., 1984). β-thrombin is formed when F2a cleaves the B-chain after Arg380 and Arg391 (prothrombin numbering, Arg62 and Arg73 in F2a numbering) releasing a small 11 residue peptide important for recognizing fibrinogen thereby creating the B1 and B2 fragments (Boissel, J.P, 1984). γ-thrombin is subsequently formed with the cleavage after Arg442 and Lys472 (prothrombin numbering, Arg123 and Arg154 in F2a numbering) resulting in the creation of the B3 and B4 fragments (Boissel, J.P. 1984). The B-chain in β- and γ-thrombin are held together by noncovalent interactions and because they still contain the catalytic triad they retain some activity (Soslau, G., 2004).

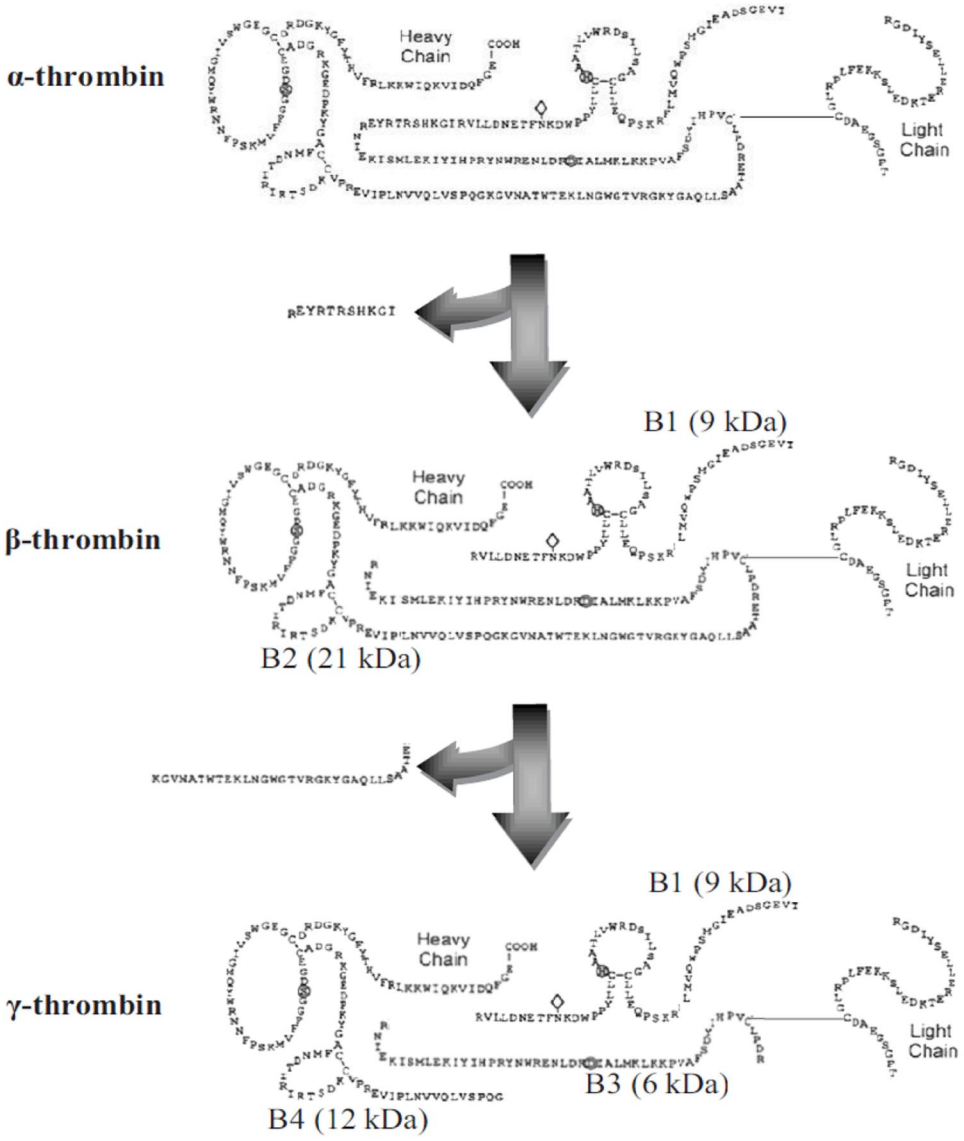

Figure 7. Autodegradation of thrombin. α-thrombin can be autolytically degraded to β- and γ-thrombin. This figure modifies the prothrombin schema by Davie and Kulman.

Analytically, prothrombin is frequently activated to F2a by snake venom such as ecarin (Kornalik, F, 1975**;** Morita, T., 1976; Speijer, H., 1986).

Thrombin as multifunctional molecule in hemostasis cleaves fibrinogen into fibrin, activates cofactors F5 and F8, activates protein C, activates factors F11 and F13, and activates thrombocytes. Thrombin is a promoter of angiogenesis by activating PAR1 (protease-activated receptor-1) in platelet and endothelial cells. PAR1 has been shown to mediate extensively thrombin-induced neurodegeneration and neuroprotection in the

brain. Therefore, thrombin and thrombin receptors represent novel therapeutic targets for treating neurodegenerative diseases (Weibo Luo, 2009). Thrombin and thrombin peptides are important in wound healing and tissue regeneration (Barbara Olszewska-Pazdrak, 2009).
Protein S is not a serine protease precursor but instead contains a thrombin-sensitive region (TSR) before the epidermal growth factor (EGF) domain and a sex hormone-binding globulin (SHBG)-like domain in the COOH-terminus. (Joseph DR, Baker ME. 1992).

Vitamin K-dependent protein complexes are the essential components for establishing hemostatic balance. Each complex is composed of a serine protease enzyme [factor 7a, factor 9a, factor 10a, or thrombin (factor 2a)], a cofactor that functions as a surface receptor/enhancer for the enzyme [factor 8a (heavy- and light-chain), factor 5a (heavy- and light-chain), tissue factor (TF) or thrombomodulin (TM)], Ca^{2+}, and a negatively charged membrane surface of cells (e.g., endothelial cells, monocytes, and platelets).
There are 3 vitally important vitamin K-dependent complexes:
extrinsic ten-ase (factor 7a-TF-PL-Ca^{2+}),
intrinsic ten-ase (factor 9a-cofactor 8a-PL-Ca^{2+}),
prothrombinase (factor 10a-cofactor 5a-PL-Ca^{2+}).

Another vitamin K-dependent complex is the anticoagulant protein C-ase complex (thrombin-TM).

When the serine protease enzyme is associated with its respective cofactor on an appropriate membrane surface with Ca^{2+}, the specific reactions occur at an enhanced rate 10^4 to 10^9 -fold greater than the enzyme-substrate combination alone (Mann KG, 1990).

1.1.4. Cofactor Proteins

Two categories of procoagulant cofactor proteins are recognized: the soluble plasma derived procoagulant procofactors (cofactor 5 and cofactor 8) and the cell-bound cofactors (TF and TM).

Factor 5 (F5) is a large single-chain glycoprotein that circulates in human plasma and 20% of the total factor F5 is also in the alpha granules of human platelets (Tracy PB, 1982). Factor 5a functions as both a factor 10a receptor and a positive modulator of factor 10a catalytic proteolytic potential in the prothrombinase complex (Mann KG, Kalafatis M. 2003).

Factor 5a is proteolytically inactivated by activated protein C (PCa) (Mann KG et al. 1997; Nicolaes GA, Dahlback B. 2002). A common genetic mutation (R506Q) that introduces a glutamine (Gln) for an Arginin (R) at PCa-cleavage position 506 has been termed $F5^{LEIDEN}$ (PCa resistance syndrome) (Dahlback B, 1993; Bertina RM, 1994). Individuals with homozygous carriers $F5^{LEIDEN}$ defect have an 80-fold increase in risk of hypercoagulability with venous thrombosis compared to similar individuals with normal F5 (Kalafatis M, 1995).

The mature antihemophilic factor (F8) is synthesized in hepatocytes and in the hepatic sinusoidal endothelial or Kupffer cells as a single-chain polypeptide. Insufficient expression of F8 or expression of nonfunctional F8 results in hemophilia A, one of the most common severe sex-linked hereditary bleeding disorders. F8 circulates in plasma as noncovalent complex with the higher molecular weight (HMW) multimeric protein von Willebrand factor (vWF) (Leyte A, 1989). vWF regulates the synthesis and cofactor activity of F8 (Weiss HJ, 1977) and concentrates the F8 at the site of vascular injury (Sakariassen KS, 1979). vWF prevents premature interaction of F8 with phospholipid (PL) prior to F8 activation (Andersson LO, 1981) and protects F8 from various PL dependent proteases, such as factor F9a, F10a, and PCa (O'Brien DP, 1992; Nogami K, 1999; Fay PJ, 1991).

The quantitative deficiency (Type 1) and complete deficiency (Type 3) of vWF or the qualitative deficiency (Type 2) of vWF results in a secondary decrease of F8 activity and a bleeding tendency known as von Willebrand disease (Hoyer LW. 1981).

α-thrombin and activated serine protease factor 10 (F10a) cleave Factor 8 to generate the heterotrimeric activated cofactor 8. Activated protein C (PCa) inactivates activated F8 by proteolytic cleavage in the presence of negatively charged PL (Eaton D et al. 1986).

Tissue Factor (TF) (thromboplastin, CD142, coagulation factor III) is a glycosylated membrane single polypeptide chain protein consisting of extracellular protein composed of two fibronectin type III domains, hydrophobic membrane-spanning domain and short cytoplasmic domain (Morrissey JH et al. 1987; Paborsky LR, Harris RJ. 1990). TF is a type I integral membrane protein, which means that the amino-terminus of the protein is located outside the cell, whereas the carboxy terminus is located inside the cell. The majority of the TF is in functionally inactive (encrypted) state. Upon cell lysis or stimulation, TF is activated (de-encrypted) and supports F7a binding and activation of F10 (Chen VM, 2006). Protein disulfide isomerase (PDI), glutathione and nitric oxide have all been implicated in mediating of de-encryption process. TF is abundant on the surface of cell including adventitial cells surrounding all blood vessels larger than capillaries, differentiating keratinocytes in the skin, and a number of epithelial-mesenchymal cell types, including those present in mucous membranes and many organs (brain, placenta, lung and prostate). TF antigen is present in the acellular core of atheromas, most likely derived from cells that have undergone necrosis (Marmur JD, 1996). TF is not normally expressed on vascular endothelial cells or monocytes. TF expression occurs during activation of monocytes/macrophages and endothelium, e.g. by tumor necrosis factor (TNFα), interleukin-1β (IL1β), or endotoxin (Geczy CL. 1994; Camerer E, 1996). TF is expressed during severe sepsis by endothelial cells in only a few, highly restricted areas, such as in the splenic microvasculature (Drake TA, 1993). Endothelial expression of TF has been observed *in vivo* in other conditions, including placental villitis (Faulk WP, 1990) and graft rejection (Blakely ML, 1994**;** Salom RN, 1998). TF may also circulate in the blood, associated with cell-derived membrane microvesicles as well as in a soluble, alternatively-spliced form (Giesen PL, 1999**;** Bogdanov VY, 2003). These microvesicles derive from lipid rafts on the surface of stimulated monocytes/macrophages, and might be capable of fusing with platelets, initiating coagulation (Del Conde, 2005). TF binds factor 7 or 7a with high affinity, resulting in a 1:1 complex of the two proteins on the cell surface. Once bound to TF, factor F7 is rapidly converted to factor F7a by limited proteolysis (Nemerson Y, 1985). The TF:F7a complex is formed through direct capture of circulating factor 7a by TF or through capture of factor 7 by TF followed by conversion of the bound factor 7 to 7a. Once formed, the TF:F7a complex directly activates factor F10.

1.1.5. Intrinsic Pathway and contact system proteins

The 3 proteins Factor 12 (F12; Hageman factor), prekallikrein (PK; Fletcher factor), high-molecular-weight kininogen (HMWK; Williams-Fitzgerald-Flaujeac factor), are the initial phase of the contact system (intrinsic hemostasis); the serpin C1 esterase inactivator (C1-ina) controls the proteolytic activity of F12a and kallikrein (K) (Figure 8).

The intrinsic pathway of coagulation is initiated by factor F12a, often in a reaction involving high molecular weight kininogen and plasma kallikrein. Contact with negatively charged surfaces induces a conformational change in zymogen F12 resulting in a small amount of activated F12 (F12a). F12a cleaves PK into active kallikrein, which in turn reciprocally activates the F12 zymogen (Figure 8). F12a or K initiate fibrin formation through F11 activation, K releases the inflammatory mediator bradykinin (BK). Binding of BK to the kinin B2 receptor (B2R) activates various proinflammatory signalling pathways that dilate vessels, induce chemotaxis of neutrophils, and increase vascular permeability and fluid efflux (”leakiness”).

Individuals with factor F11 deficiency (hemophilia C) express variable bleeding disorders with surgical challenge, (Sidi A, 1978) thus are establishing an essential role for factor F11 in hemostasis. Formation of factor 11a appears to be catalyzed also by α-thrombin as part of a positive feedback loop (Lawson JH, 1994).

Factor F12 and prekallikrein are zymogens that are activated to generate serine proteases, and HMWK is a nonenzymatic procofactor. Activation of this pathway *in vitro* is accomplished when factor F12 autoactivates to factor 12a with exposure to foreign surfaces, including kaolin, dextran sulfate, and sulfatides (Schmaier AH, 2008; Wiggins RC, 1979; Silverberg M, 1980). Two main trigger types activate the intrinsic enzyme system: (delta)-negatively charged molecules and lipophilic molecules. The stimulation of thrombin generation by (delta)-negatively charged molecules depends on their maximal plasma concentration prior to plasma dilution (Stief TW, 2012a), and contact activation of coagulation depends on the maximal lipophilic trigger concentration in plasma prior to dilution (Stief TW, 2012b). The substrates for factor F12a, prekallikrein and factor F11, exist in a noncovalent complex with HMWK and become activated to kallikrein and factor F11a, respectively (Mandle RJ, 1976). The intrinsic pathway is positively or negatively regulated via cleavage of HMWK e.g. by factor 11a (Scott CF, 1984; Scott CF, 1985). These proteins are associated with the systemic inflammatory response syndrome, (Bone R: 1992; Schousboe I: 2008).

Biologic activation of the contact pathway system may be accomplished through assembly of these proteins on endothelial cell membranes; also prekallikrein is activated by an endothelial cell membrane cysteine protease (Schmaier AH, 1998; Rojkjaer R, 1998). The accessory pathway is especially important in cardiopulmonary bypass because of contact between blood components and synthetic surfaces.

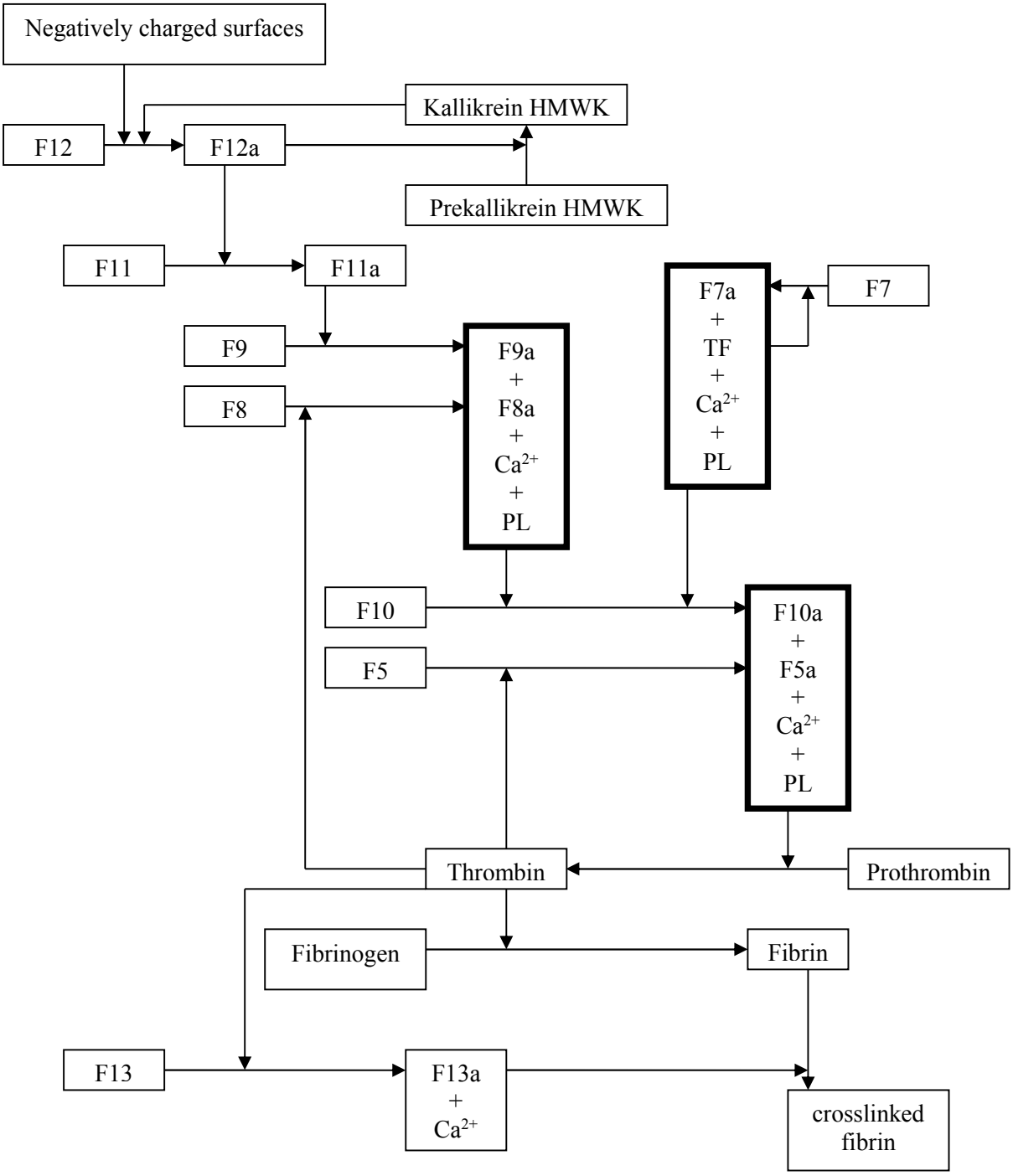

Figure 8. Coagulation System.

PL: phospholipids; TF: tissue factor; a: activated coagulation factors**.**

Hageman-Factor, F12, synthesized in the hepatocytes as single polypeptide chain, is the serine protease precursor of a β-globulin fraction of plasma (Griffin JH 1976). Six distinct domains can be identified in a putative signal peptide sequence of Hageman factor: a type II region homologue to collagen-binding site in fibronectin, epidermal growth factor (EGF)-like domain, a type I homology or the fibrin finger in fibronectin, second EGF-like domain, a kringle (k) structure and a proline-rich region, and the large catalytic domain (Cool DE, 1987). F12a activates PK and F11 of coagulation (Kurachi K, 1980), and C1 of complement (Ghebrehiwet B, 1983). F12a downregulates the Fc receptor on monocytes (Chien P, 1988), releases interleukin 1 (IL-1) and IL-6 from monocytes and macrophages (Toossi Z, et al. 1992), and stimulates neutrophils (Wachtfogel YT, 1986). Two pathways for activation of factor 12 can be identified: Plasma proteinases-dependent activation and surface/contact-dependent activation. Plasma proteinases, including plasma kallikrein, trypsin or plasmin activate F12 to F12a, cleaving at Arg353-Val354 and generating a two-chain molecule composed of a heavy chain and a light chain, held together by a single disulfide bridge (Cool DE, 1985). Trypsin, plasmin, and kallikrein can proteolytically activate the zymogen F12 to F12a or to β-F12a (Revak SD, 1977**;** Cochrane CG, 1973). The 80kDa form of activated F12 has the ability to bind to negatively charged surfaces and activate F11. Because the 28-30kDa enzymatic form of F12 (β-F12a) has no heavy chain, it has no surface-binding properties or ability to activate F11 but retains its ability to activate PK and C1 (Revak SD, Cochrane CG, et al. 1978**;** Revak SD, et al. 1976). Components, which include fatty acids, high concentrations of dextran sulfate or phosphatidyl inositol phosphate, cerebroside sulfates (Tans G, 1983), glycosaminoglycans (Hojima Y, 1984), are very potent activators of F12 via surface-dependent activation pathway. The major plasma protease inhibitor of F12a and K is C1-ina, which binds both proteins in 1:1 stoichiometry with concomitant loss of activity of both, protease and inactivator.

Prekallikrein (PK; Fletcher factor) and High-molecular-weight kininogen (HK; Williams-Fitzgerald-Flaujeac factor)

PK, synthesized at high levels in pancreas, kidney, testis, spleen, and prostate, but at highest levels in the liver (Neth P, 2001), is a fast γ-globulin with an isoelectric point of 8.7 (McConnell DJ, 1970) and with a plasma concentration of about 42 μg per ml (0.49 μM) (Fisher CA, 1982). Approximately 75% of plasma PK circulates bound to HK (Mandle R Jr, 1976**;** Scott CF, 1980), and only 25% circulates as free PK. HK is a 120 kDa, β-globulin glycoprotein that circulates in plasma at a concentration of 670 nM (80 μg/ml) and is a member of the cystatin family of cysteine protease inhibitors. The two plasma kininogens, HK and low-molecular-weight kininogen (LK) are known. In humans and rodents, HK and LK are predominantly expressed in the liver but can also be found in endothelial cells, granulocytes, renal tubular cells, and α-granules of platelets. The conversion of PK to kallikrein, its active form, is catalyzed by F12a on a surface to which HK is bound or by F12 fragment in the fluid phase (Wuepper KD, 1972). Kallikrein catalyzes an autolytic cleavage at Lys140-Ala141, resulting in β-kallikrein (Colman RW, 1985). This enzyme exhibits decreased coagulation activity, a diminished rate of cleavage of HK, and decreased ability to stimulate neutrophils. Plasma kallikrein activates pro-urokinase (Ichinose A, 1986), pro-renin (Sealey JE, 1978), and F12 (Fujikawa K, 1980), and cleaves HK, thereby releasing bradykinin (BK) (Jacobsen S, 1967). HK inhibits adhesion of neutrophils to blood-compatible surfaces under flow conditions (Yung LL, 1996), enhances cellular fibrinolysis (Lin Y, 1997), and inhibits thrombin-induced platelet activation. In plasma, kallikrein is inactivated by the serpin C1-ina, forming a 1:1 stoichiometric complex with kallikrein (Gigli I, 1970), resulting in loss of both, the proteolytic activity of the enzyme and the inhibitory function of C1-ina (Schapira M, 1981). Kallikrein bound in α2-macroglobulin inhibits the release of BK from kininogens, but it does not inhibit its amidolytic activity. Antithrombin 3 (AT-3), in the presence or absence of heparin, is an inefficient inhibitor of kallikrein.

1.2. Hemostasis Screening Assays: Use and Interpretation

Routine assays of usual coagulation testing are the prothrombin time (PT), partial thromboplastin time (APTT) and Thrombin Time (TT) (Figure 9).

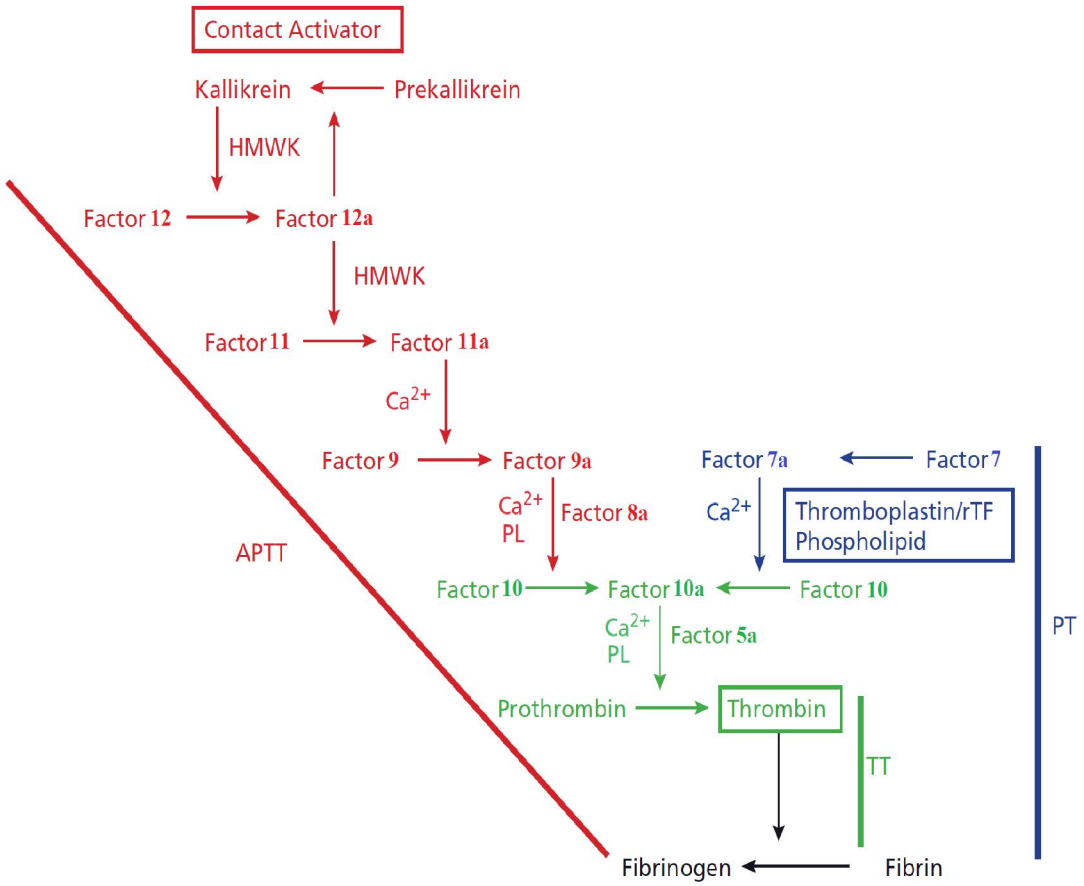

Figure 9. The coagulation cascade. Blood coagulation with separate intrinsic (red) and extrinsic (blue) pathways converging on the common pathway (green) with the generation of F10a.

1.2.1. PT (prothrombin time, Thromboplastin time, Quick-Test)

The prothrombin time (PT) test is used mainly to control oral anticoagulant therapy (OAT). PT is the most widely used coagulation test in clinical laboratories. The number of tests is increasing about 10 % a year. OAT is based on the ability of Phenprocoumon (Marcumar), Coumadin (Warfarin) and Acenocoumarol (Sintrom) as a vitamin K antagonists, to slow down the synthesis of active coagulation factors in the liver (F2, F7, F9, and F10). Marcumar, Warfarin and Sintrom medications require continuous monitoring to prevent the serious consequences of thrombosis or bleeding (Odén A, 2002). Mortality has been seen to be strongly related to the level of the international normalized ratio (INR), and accuracy in patient care is very important.

The prothrombin time is commonly measured by either the "Quick PT", which is based on the technique described by Quick and co-workers in 1935 (Quick AJ, Stanley-Brown M, Bancroft FW. 1935**;** Quick AJ. 1935) or by the "Owren PT" (Owren PA. 1959) (combined thromboplastin reagent).

The PT measures the time required for the following reaction:

$$\textbf{Plasma+Thromboplastin+}Ca^{2+}\rightarrow\textbf{Fibrin Clot}$$

Thromboplastins are reagent preparations rich in tissue factor and phospholipids. The prothrombin time (PT) is used to assess the extrinsic pathway of clotting, which consists of tissue factor and factor F7, and coagulation factors in the common pathway (factors F2 [prothrombin], F5, F10, and fibrinogen). In this test, clotting is initiated by recalcifying citrated patient plasma in the presence of thromboplastin (tissue factor). The endpoint for the PT (and also for the partial thromboplastin and thrombin times) is the time (in seconds) for the formation of a fibrin clot, which is detected by visual, optical, or electromechanical means. The sensitivity of the PT to reduced activity of the vitamin K-dependent factors within this pathway (ie, factors F7, F10, and F2; especially factor F7) comprises the rationale for the use of the PT to monitor Marcumar, Warfarin and Sintrom medications therapy.

Results of the prothrombin time can be expressed in one of four different ways:

- **PT with control value**: The patient's PT (in seconds) is reported along with the PT obtained from control (normal) plasma. The control value is needed since there can be significant inter-laboratory variability in the PT with different reagent/instrument combinations.
- **PT expressed as INR**: In order to promote standardization of the PT for monitoring oral anticoagulant therapy, the World Health Organization (WHO)

developed an international reference thromboplastin, currently recombinant tissue factor, and recommended that the PT ratio be expressed as the INR (Hirsh J, Poller L. 1994). This allows values of the PT from various locations to be directly compared, as may happen when a patient taking Marcumar, Warfarin and Sintrom has blood sampled at different laboratories.

Measurement of INR: The INR, which compensates for differences in sensitivity of various PT reagents to the effects of warfarin, is used to monitor warfarin therapy (Hirsh J, Poller L. 1994)**.** The INR is calculated from the following formula: **INR = [Patient PT / Normal PT]^{ISI}**

The ISI (international sensitivity index) should be determined for each PT reagent and instrument combination. Although the ISI is traceable to an international reference thromboplastin reagent, it is useful to have the ISI value confirmed within each laboratory, since this may be affected by differences in handling of the reagents and the type of equipment used (Becker DM, 1993**;** van den Besselaar AM, 1999). The control value for the PT is the mean normal prothrombin time for the laboratory, and should be determined from ≥20 fresh normal plasmas handled identically to patient material. An apparatus that allows the patient to monitor warfarin therapy at home is now available.

- **Prothrombin time ratio (PTr)**: The patient's PT is expressed as a ratio, where PTr = (patient PT ÷ control PT). As an example, a PTr >1.2 was associated with a significantly increased risk of acute traumatic coagulopathy in a large multicenter retrospective study (Frith D, 2010). In this study, reagents used had similar sensitivities (ISI range 1.03-1.09). A limitation of this method is that reagent/instrument variability may affect the results.

The PT has several disadvantages (Stief TW, 2008a). First, anticoagulants not always induce the respective test inhibition. Second, the normal range of extrinsic haemostasis is too blunted and not truly represented. Third, patients prone to hyper-activated extrinsic pathway are not detected. Fourth, fibrinogen/fibrin concentration influences the test result. Fifth, plasma matrix is changed significantly in the assay, and sixth, assay results are not IU/ml thrombin but coagulation seconds, which requires special hemostasis machines.

1.2.2. Activated partial thromboplastin time (APTT)

The APTT was first described in 1953 by Langdell et al. Kaolin cephalin clotting time is a historic name for the activated partial thromboplastin time.

The activated partial thromboplastin time (APTT or PTT) is used to test the intrinsic coagulation pathway (prekallikrein, high molecular weight kininogen, factors F12, F11, F9, and F8) and final common pathway (factors F2, F5, F10, and fibrinogen), and to monitor heparin therapy.

The test is performed by recalcifying citrated plasma in the presence of a thromboplastic material that does not have tissue factor activity (therefore: partial thromboplastin) and a negatively charged substance (eg, ellagic acid, celite, kaolin, silica), which results in contact factor activation, thereby initiating coagulation via the intrinsic clotting pathway (Schmaier AH. 1997).

The APTT measures the time required for the following reaction:

$$\textbf{Plasma+Phospholipid+Activator+}Ca^{2+}\textbf{→Fibrin Clot}$$

Clot formation occurs via the intrinsic and common pathway, involving high molecular weight kininogen, prekallikrein, factors F12, F11, F10, F9, F8, F5, and F2, and fibrinogen (Van Cott EM, 2001). There are four etiologies to consider when the APTT is elevated: First, factor deficiency. Second, lupus inhibitors. Third, factor inhibitors, and Fourth, heparin.

The importance of the APTT for monitoring UFH therapy is based on:

1. Tests should measure the physiologic anticoagulant effect rather than simply providing a heparin concentration.
2. The APTT is widely available with relatively short turnaround time, has good reproducibility, and is inexpensive.
3. Clinicians have decades of experience with the APTT and have achieved a comfort level with its use for UFH monitoring.
4. Until recently there have not been good alternatives.

The APTT also possesses severe limitations (Hirsh J, 2004; Olson JD, 1998):

1. The APTT does not measure the clinically most important heparin type that is low-molecular weight heparin.
2. The APTT is a non-standard assay so therapeutic ranges must be established in each laboratory. Different types of heparin may produce different PTT response curves.
3. The APTT is useful only for monitoring therapeutic intravenous doses or high-dose subcutaneous UFH therapy. The APTT is not useful for standard-dose subcutaneous

therapy, due to limited APTT response, or for the high-dose iv UFH required for cardiac catheterization or other procedures, due to an excessive APTT response.

4. The APTT is affected by variables other than UFH, including increased concentrations of factor F8 and fibrinogen, decreased concentrations of AT or intrinsic and common pathway proteins, lupus anticoagulant, and thrombolytic agents. When lupus anticoagulants are present, the APTT becomes unpredictable to test the response to UFH and should not be used.

5. The routine APTT only detects 30% of patients with APLA and are inadequate as a single test for APLA (Antiphospholipid antibodies) screening. One can increase sensitivity by using different APTT reagents.

6. The APTT does not adequately respond to the new oral anticoagulants rivaroxaban or dabigatran, neither to hirudin.

1.2.3. Thrombin Time (TT): The TT measures the time required for the following reaction:

Plasma+Thrombin→Fibrin Clot

The Thrombin time test is performed by adding thrombin to plasma. The added thrombin directly clots fibrinogen. The TT is only affected that interfere with thrombin or fibrinogen. The TT is elevated in DIC (FSPs interfere with polymerization), low fibrinogen levels, dysfibrinogenemia, uremia and in the presence of Heparin (very sensitive).

1.2.4. Activated clotting time (ACT)

The activated clotting time (ACT) is a whole-blood clotting test commonly used to monitor high-dose UFH therapy, particularly cardiac catheterization and cardiopulmonary bypass procedures. (Olson JD, Arkin CF, et al. 1998)

The ACT consists of the following reaction:

Whole Blood+Particulate Activator→Fibrin Clot

Celite and kaolin are commonly used as activators. Typically, the ACT is a point-of care test because non-anticoagulated blood specimens cannot be transported to a central laboratory quickly enough to avoid pre-test activation of clotting.

The ACT offers several advantages for UFH monitoring:

1. The ACT has a wide dose-response range so it can be used for assessing high-dose UFH therapy.

2. The ACT is simple to perform and results are available with rapid turnaround time. The rapid turnaround facilitates its use in assessing both induction and reversal of UFH anticoagulation in near-real-time during procedures.

3. Extensive clinical experience over several decades provides a high level of familiarity and comfort with its use.

4. Suitable alternatives are not readily available. Consequently, the ACT is widely used for high-dose UFH management.

The ACT also has several important limitations and drawbacks.

1. The ACT is non-standard and imprecise. (Bosch YP, 2006)

2. Since non-anticoagulated blood is used, results are affected by deficiencies in specimen collection and application technique, including delayed application of specimens.

3. The ACT reaction follows the same pathways as the PTT reaction, so the ACT is affected by the same biological variables. In addition, because whole blood is used, the ACT is also affected by variations in platelet count inasmuch as platelets provide phospholipid surfaces for reactions of the intrinsic and common pathways.

By contrast, in the PTT assay phospholipid is provided as a reagent in a controlled amount.

4. Although the dose-response of the ACT extends into the standard-dose UFH range, it is less precise and offers no advantages over the PTT for this use.

5. Many ACT analyzers have only basic functionality and cannot be interfaced with information systems for data management.

1.2.5. Anti-F10a Assay

The heparin anti-F10a assay, or factor F10a inhibition test, is increasingly used to supplement or replace the PTT for monitoring heparin therapy (Olson JD, 1998). The principle of the anti-F10a assay is the inhibition of factor F10a by AT-3-heparin complexes, as illustrated in the following reactions:

$$\mathbf{AT{-}Heparin + F10a \rightarrow AT{-}Heparin{-}F10a + Residual\ F10a}$$

$$\mathbf{Residual\ F10a + Substrate \rightarrow Signal}$$

The anti-F10a assay has several favourable characteristics:

1. The anti-F10a assay is simple to perform and automatable on many coagulation analyzers.
2. Citrated specimens used for other coagulation tests may be used without additional special handling.
3. The test is not affected by coagulation factor concentrations, lupus anticoagulants, or other biological variables.
4. The anti-F10a assay is sensitive to the anticoagulant effect of LMWH and fondaparinux.
5. The anti-F10a assay can theoretically be standardized to provide consistency between laboratories and facilitate the adoption of common therapeutic ranges.

Anti-F10a assays have limitations:

1. The anti-F10a assay is not an F10a generation assay (see APTT), it just measures the antigenic concentration of an anticoagulant.
2. The anti-F10a assay is much more expensive than the APTT.
3. It may not be financially or technically feasible to offer the anti-F10a assay in small laboratories, depending on test volume and instrumentation.
4. Different UFH preparations may yield different standard curves. It is generally not feasible to establish a unique standard curve for every UFH preparation, so the potential exists for the standard curve of an anti-F10a assay to not be representative of the anticoagulant response for some UFH preparations. The same limitation applies to LWMH preparations.

1.2.6. Phospholipid-dependent coagulation assays (PdCAs):

PdCAs are used to optimize identification of the LA (lupus anticoagulant). Test options include the PTT, dilute PT, dilute Russell's viper venom time (dRVVT), kaolin clotting time (KCT), Taipan venom test (TVT) and Textarin time (Triplett DA, 2002 ; Wisloff F, 2003).

- **Dilute Russel viper venom time (dRVVT):** This test is very sensitive to any interference with phospholipids and is very sensitive to Russel Viper venom which directly activates factor F10 and is very sensitive to phospholipids.

- **Kaolin cloting time (KCT):** This test uses no added phospholipids and is a sensitive test to detect APLA. However, it is technically demanding to do properly.
- **Platelet neutralization test:** This test takes a coagulation reaction that is prolonged by plasma and does not correct with a 50:50 mix. Extracts of platelet phospholipids are added to the plasma and an APTT is performed. The platetet phopholipid is very avid for APLA and ``soaks up`` the antiphospholipid antibody and corrects the APTT. If the APTT corrects with addition of platelets this is diagnostic for APLA.
- **Hexagonal phospholipids neutralization:** This test is based on the same principle as the platelet neutralization test but it uses hexagonal phospholipids which is more specific for APLA. Current test kits use hexagonal PL also have added plasma and inhibitors of heparin. The additional reagents allow this assay for lupus inhibitors to be performed on anticoagulated patients.

1.3. Anticoagulants: classification, pharmacology

Antithrombotic drugs are used for prevention and treatment of thrombosis. Targeting the components of thrombi, these agents include

1. Antiplatelet drugs
2. Anticoagulants
3. Fibrinolytic agents

Anticoagulants had been divided into parenteral and oral anticoagulants. Currently available parenteral anticoagulants include

1. Unfractionated Heparin (UFH)
2. Low-molecular-weight Heparins (LMWHs)
3. Fondaparinux, a synthetic pentasaccharide.
4. Heparin-related sulphated glycosaminoglycans (SGAG; heparinoids) include heparin derivatives such as danaparoid, dermatan sulfate, pentosan polysulfate, apolate, suleparoid, and sulodexide.

The usual oral anticoagulants have been the vitamin K antagonists (Phenprocoumon (Marcumar), Coumadin (Warfarin) and Acenocoumarol (Sintrom) derivatives).

Dabigatran etexilate, an oral thrombin inhibitor, and rivaroxaban, an oral Factor F10a inhibitor, are now licensed in the US and in Europe. (Haines ST, 1995; Nutescu EA, 2005; 2006) (Table 1).

Drug	Method of Preparation	Mean Molecular Weight (Daltons)	Plasma Half-Life	Anti-F10a:Anti-F2a Activity	Bioavailability
Unfractionated heparin (UFH)	Extracted from porcine gut mucosa or beef lung	≈15,000	30–90 min (dose dependent)	1:1	SC: 30–70% (dose dependent)
Low molecular weight heparins (LMWHs)					
Ardeparin	Peroxidative depolymerization	≈6,000	200 min	1.9:1	SC: 90%
Dalteparin	Nitrous acid depolymerization	≈6,000	119–139 min	2.7:1	SC: 87%
Enoxaparin	Benzoylation and alkaline depolymerization	≈4,200	129–180 min	3.8:1	SC: 92%
Nadroparin	Nitrous acid depolymerization	≈4,500	132–162 min	3.6:1	SC: 99%
Tinzaparin	Heparinase digestion	≈4,500	111–234 min	2.8:1	SC: 90%
Heparinoid					
Danaparoid	Extracted from porcine gut mucosa	≈6,500	22–24 h	20:1	SC: 95%
Anti–factor F10a inhibitors					
Fondaparinux	Synthetic	1,728	15–18 h	100% anti-F10a	SC: 100%
Idraparinux	Synthetic	≈1,700	≈80 h	100% anti-F10a	SC: 100%
Direct thrombin F2a inhibitors					
Argatroban	Synthetic	509	30–50 min	100% anti-F2a	
Bivalirudin	Semisynthetic	2,180	25 min	100% anti-F2a	
Desirudin	Recombinant DNA technology	6,964	120 min	100% anti-F2a	SC: >90%
Lepirudin	Recombinant DNA technology	6,980	80 min	100% anti-F2a	SC: 70%
Dabigatran	Synthetic	471	14 h	100% anti-F2a	Oral: 7%
Vitamin K antagonists					
Warfarin	Synthetic	330	40 h	1:1	Oral: 90–100%

Table 1. anticoagulants: systematic classification and pharmacology

1.3.1. Unfractionated Heparin

1.3.1.1. History of Heparin discovery and biochemical structure

The heparin story (Bigelow, 1990) began in 1916 at Johns Hopkins University in Baltimore. Jay McLean isolated an extract of dog liver, which inhibited blood coagulation (Bick RL. 2002). Howell determined that heparin was not phosphorylated and was a carbohydrate. The student Sune Bergstrom identified glucosamine (GlcN) as a sugar component in heparin while working with Eric Jorpes in Sweden. Jorpes established that heparin contained a high proportion of sulfo groups and determined that the GlcN residue in heparin was primarily N-sulfonated. By the 1920s, several groups were manufacturing heparin.

In the 1930s, Gordon Murry in Toronto and Clarence Crafoord in Stockholm successfully began using heparin in surgery patients. Since the 1930s, clinicians have used unfractionated heparin (UFH) for the prevention and treatment of thrombosis (Hirsh J, 2004). Like all other natural polysaccharides, heparin is a polydisperse mixture containing a large number of chains having different molecular weights (Ahsan A, 1995; Mulloy B, 1997). The polydispersity (the ratio of overnormal weight in relation to averaged molecular weight) of pharmaceutical heparin is 1.1–1.6 (Ahsan A, 1995). The chains making up polydisperse pharmaceutical grade heparin range from 5,000 to over 40,000 Da (Linhardt RJ, 1997) and contain a significant level of sequence heterogeneity. Heparin is composed of a major (75–95%) trisulfated disaccharide repeating unit (Figure 10, A), a 2-O-sulfo-α-L-iduronic acid 1 → 4 linked to 6-O-sulfo-N-sulfo-α-D-glucosamine IdoA2S(1 → 4)GlcNS6S, as well as a number of additional minor disaccharides structures corresponding to its variable sequences (Figure 10, B) (Linhardt RJ, 1988; Loganathan D, 1990; Pervin A, 1995). There are some fully sulfated heparin chains that are simply composed of uniform repeating sequences of trisulfated disaccharide. Most heparin chains, however, have an intermediate level of sulfation (2.5 sulfo groups/disaccharide) and are composed of long segments of fully sulfated sequences with intervening undersulfated domains. Some chains, primarily composed undersulfated sequences, are classified as heparan sulfate, a closely related sulphated glycosaminoglycan (SGAG).

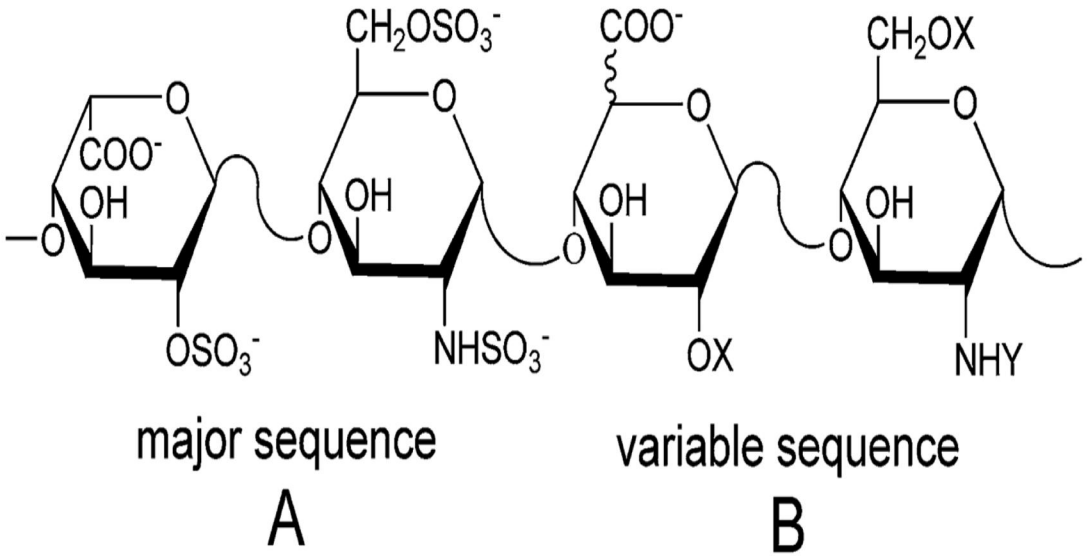

Figure 10. The structures of the major (A) and minor (B) repeating disaccharides comprising heparin: X =SO_3^- or H, and Y =SO_3^- or $COCH_3$.

A unique saccharide combination comprises the antithrombin-3 (AT-3) pentasaccharide binding site, GlcNAc/NS6S → GlcA → GlcNS3S,6S → IdoA2S → GlcNS6S, important for heparin's anticoagulant activity (Figure 11) (Lindahl U, 1980; Atha DH, 1985).

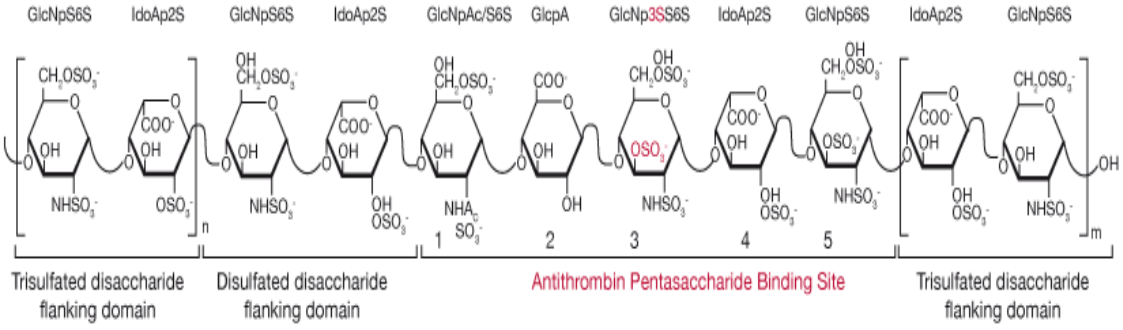

Figure 11. Heparin's antithrombin-3 binding site and its structural variants.

GlcA = β-D-glucuronic acid; **IdoA** = α-L-iduronic acid; **IdoA (2S)** = 2-O-sulfo-α-L-iduronic acid **GlcNAc** = 2-deoxy-2-acetamido-α-D-glucopyranosyl; **GlcNS** = 2-deoxy-2-sulfamido-α-D-glucopyranosyl; **GlcNS(6S)** = 2-deoxy-2-sulfamido-α-D-glucopyranosyl-6-O-sulfate

AT-3 binds to a specific AT-3 pentasaccharide sequence in heparin (Lindahl U, 1980; Atha DH, 1985). When heparin binds to the serine protease inhibitor AT-3, AT-3 undergoes a conformational change resulting in the inhibition of thrombin and other coagulation cascade proteases (Munoz EM, 2004; Linhardt RJ, 2003). Only a third of the chains comprising pharmaceutical-grade heparin contain an AT-3 binding site, and these are called "high affinity heparin" (Edens RE, 1995). In contrast, heparin interacts with low specificity to thrombin based on its high negative charge density. Thus, if a heparin chain containing an AT-3 binding site is sufficiently long to accommodate thrombin, it can form a tertiary complex, inhibiting thrombin's conversion of soluble fibrinogen to an insoluble fibrin clot. Low molecular weight heparins (LMWHs) are prepared through the controlled chemical and enzymatic depolymerization of heparin (Barrowcliffe TW, 1995). LMWH chains are often too small to accommodate thrombin in a ternary complex, and thus inhibit the coagulation cascade primarily through coagulation factor 10a. The clinical value of LMWHs comes primarily from their enhanced subcutaneous bioavailability (Linhardt RJ, 2003).

Heparin and other glycosaminoglycans are generally isolated by extraction from animal tissues, but some simple unsulfated glycosaminoglycans can be obtained from the capsules of bacteria (Casu B, 1989; Hook M, 1984; Chakrabarti B, 1980; Casu B. 1979). Heparins from tissues of various species also differ in structure and activity (Table 2) (Loganathan D, 1990).

Tissue	N-Acetyl AT-3 binding sites	N-Sulfo AT-3 binding sites	Trisulfated disaccharides	Disulfated disaccharides
Porcine intestine	0.5 (0.3–0.7)	0.1	10 (10–15)	1.2 (1–2)
Bovine lung	0.3	0.3	14	1.0
Bovine intestine	0.3	0.3	10	1.7
Ovine intestine	0.7	0.4	11	1.4

Table 2. Structural variability of heparins between different tissues with different average number in a single heparin chain

For example, porcine intestinal heparin has an AT-3 binding site primarily containing an N-acetyl (NAc) group, (Linhardt RJ, 1999) while bovine lung heparin primarily contains an N-sulfo (NS) group, resulting in slight differences in their affinities for AT-3 (Linhardt RJ, 2003). Pharmaceutical heparins are most commonly isolated in tons quantities from porcine intestines (Coyne E, 1981). The disaccharide composition of individual porcine intestinal heparins can also differ (Table 2) (Linhardt RJ, 1988). Some porcine intestinal heparin is prepared from porcine intestinal mucosa, scraped from the intestine, while other preparations use the whole intestine ("hashed pork guts"). These two raw materials contain differing amounts of structurally related heparan sulfate that can carry over into the final pharmaceutical product.

Today, heparin is often described in the literature as standard heparin or unfractionated heparin to distinguish it from low-molecular weight heparins. Unfractionated heparin is an intravenuosly or subcutaneously administered anticoagulant, widely used for the treatment and prevention of thromboembolic events, including deep venous thrombosis, pulmonary embolism, stroke, myocardial infarction, unstable angina, and some cases of disseminated intravascular coagulation (DIC), and for anticoagulation during cardiopulmonary bypass, percutaneous coronary intervention, and extracorporeal membrane oxygenation (ECMO) procedures (Hirsh J, Raschke R. 2004).

1.3.1.2. Mechanism of Action of Unfractionated Heparin

UFH is a heterogeneous group of anionic mucopolysaccharides, called sulphated glycosaminoglycans (SGAGs), with anticoagulant properties. The molecular weight ranges from 3,000 to 30,000 daltons, averaging 15,000-18,000 daltons, around 45-50 saccharides. It carries the name *heparin* because it was originally extracted from liver (Hirsh J, Raschke R. 2004). The term "heparin" generally refers to unfractionated heparin, so named because of its heterogeneity in size and function. Heparin exerts its anticoagulant effect via several mechanisms:

1. Binding to AT-3 and catalyzing inactivation of factors F2a and F10a: major mechanism for anticoagulant effect, produced by only one third of heparin molecules (those containing the unique AT-3-binding pentasaccharide) (Lam LH, 1976; Andersson LO, 1976).
2. Binding to heparin cofactor-2 (HC-2) and catalyzing inactivation of factor F2a: anticoagulant effect requires high concentrations of heparin and independent of the pentasaccharide (Tollefsen DM, 1982)
3. Binding to factor F9a requires very high concentrations of heparin, and is independent of AT-3 or HC-2 (Weitz JI, 1999).
4. Binding to platelets inhibits platelets function and contributes to the hemorrhagic effects of heparin: high-molecular-weight fractions have greater effect than low-molecular-weight fractions (Eika C, 1971; Kelton JG, 1980).
5. Heparin displaces TFPI (tissue factor pathway inhibitor) bound to endogenous SGAGs on the surface of the endothelium (Arieru RA, 1994; Holst J, 1993). Repeated heparin administration releases TFPI without diminishing releasability with TFPI levels reaching 2- to 10-fold over baseline. LMWHs have a variable effect on TFPI release (Vogel GM, 1989). Neutralization of heparin by protamine sulfate results in a dramatic decrease in the plasma TFPI level (Harenberg J, 1993).

But the most important by far is the potentiation of the serine protease inactivator antithrombin 3 (AT-3). By itself, AT-3 is a slow inhibitor of thrombin. Heparin binds to AT-3 through a specific pentasaccharide sequence, inducing a conformational change that increases the rate of binding to thrombin by 1000-fold (Figure 12). Heparin-AT-3 also inhibits factor F10a, and to a much lesser degree factor F9a, factor F7a-tissue factor complex, factor F11a (factor F12a, kallikrein). It indirectly inhibits thrombin-induced activation of platelets and factors F5 and F8. Thrombin is 10-fold more

sensitive to heparin-AT-3 inhibition than is factor 10a, and factor F10a is more sensitive than the other coagulation factors. AT-3 binds covalently to the active serine centers of coagulation factors, then heparin dissociates and can be reutilized. Inhibition of thrombin by heparin-AT-3 requires heparin molecules of 18 or more saccharides. Complexes with small heparin molecules inhibit factor F10a and other factors, but not thrombin.

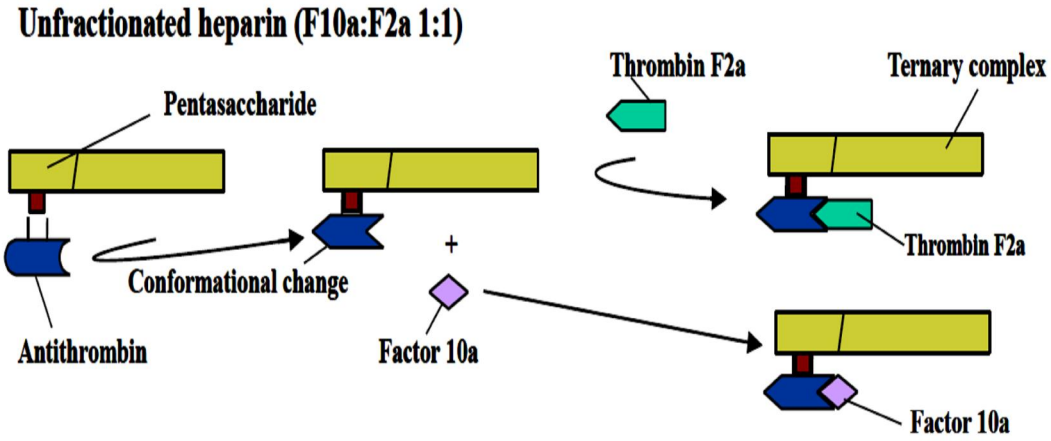

Figure 12. Pharmacologic activity of unfractionated heparin (UFH).

At high concentrations, UFH molecules of 24 or more saccharides, with or without the high-affinity pentasaccharide, bind to heparin cofactor 2 and catalyze the inactivation of thrombin. Heparin is a 10000 to 20000 Dalton sized polynegative molecule that not only activates antithrombin-3 but also pre-kallikrein or F12; kallikrein or F12a initiate the intrinsic phase of hemostasis generating thrombin that activates F5 and F8, the two main accelerators of hemostasis. Small heparin derivatives of about 6000 Dalton, such as the low molecular weight heparin enoxaparin, still activate AT-3 but they do not seem to significantly trigger intrinsic hemostasis (Stief TW. 2007a). Heparin binds to von Willebrand factor (vWF), inhibiting platelet adhesion mediated by vWF (Hirsh J, Raschke R. 2004). Heparin inhibits the secretion of aldosterone and so may cause hyperkalaemia (Oster JR, 1995). Although all patients treated with heparin may develop reduced aldosterone concentrations, most are able to compensate through the reninangiotensin system. Patients on prolonged heparin therapy or those unable to compensate, such as patients with diabetes mellitus or renal impairment or those also receiving potassium-sparing drugs such as ACE inhibitors, may present with K^+ increase symptoms. Long term use of heparin for treatment and prophylaxis of

thromboembolism in pregnant women has been complicated with heparin-induced osteoporosis in pregnancy (Nelson-Piercy C.1998). Symptomatic osteoporosis in pregnants given heparin long term occurs in about 2% (Bates SM, 2008). Subclinical reduction in bone density occurs in up to one-third of patients, (Bates SM, 2008) but it is not possible to predict which of these patients will develop osteoporotic fractures. Use of low-molecular-weight heparins is associated with a lower risk of heparin-induced osteoporosis (Bates SM, 2008). Using of heparin leads to the release of lipoprotein lipase into the plasma. Postprandial lipidaemia is reduced due to increased hydrolysis of triglycerides into free fatty acids and glycerol (Riemersma RA, 1981). With long-term use reserves of lipoprotein lipase may be depleted; severe hypertriglyceridaemia reported in a pregnant woman was attributed to long-term heparin prophylaxis that was thought to have resulted in lipoprotein lipase deficiency (Watts GF, 1991). Increaseing in liver transaminase in patients given heparin, usually reversible on discontinuing of heparin admenstration, have been reported with therapeutic (Sonnenblick M, 1975**;** Dukes GE, 1984) or prophylactic (Monreal M, 1989) doses of heparin. Dermal necrosis is a rare dermatological complication of heparin use (Ulrick PJ, 1984**;** Fowlie J, 1990). It may be a localised reaction at the site of subcutaneous injection or possibly be related to heparin-induced thrombocytopenia. Eczematous plaque reactions have developed several days after starting subcutaneous heparin. An intravenous hypersensitivity reaction has been implicated (Bircher AJ, 1990). Low-molecular-weight heparins may be an alternative but cross-reactivity can occur (O'Donnell BF, 1993). Heparin has been associated with the development of thrombocytopenia (HIT). Thrombocytopenia induced by heparin may be of two types. HIT-1 is an acute, but usually mild, fall in platelet count occurring within 1 to 4 days of starting therapy and which often resolves without stopping treatment. A direct effect of heparin on platelet aggregation appears to be responsible. HIT-2 has an immunological basis and is more serious. It usually occurs after 5 to 11 days although its onset may be more rapid in patients previously exposed to heparin; (Warkentin TE, 2001) it is often associated with thromboembolic complications due to platelet-rich thrombi (the 'white clot syndrome') or, more rarely, bleeding. This type of thrombocytopenia appears to occur more often with bovine heparin than with heparin from other species, (Bell WR, 1980) and least frequently with lowmolecular-weight-heparins; (Warkentin T, 2008) the mechanism appears to be development of antibodies to a complex formed between heparin and platelet factor-4 (a cationic protein released from the α granules during

platelet activation, neutralizes heparin and prevents it from interacting with AT-3), which then cause platelet activation and thrombin generation (Menajovsky LB, 2005).

Heparin has been postulated to have an antineoplastic effect due to a decrease in neoangiogenesis, possibly mediated by binding to vascular endothelial growth factors, cytokines, and adhesion molecules. Heparin inhibits the release of P-selectin from platelets and endothelial cells and also binds to P-selectin and L-selectin and CD11b/CD18 expressed on leukocytes (anti-inflammatory effect).
Heparin (sodium or calcium salt) is administered either by intravenous bolus ingection followed by continuous infusion or subcutaneous injection. Upon entering the blood stream, heparin binds to a variety of plasma proteins, thereby lowering its bioavailability and producing a variable anticoagulant response. Major binding proteins include histidine-rich glycoprotein (HRG), PF4, fibronectin, vitronectin, and vWF (Lijnen HR, 1983). Heparin chains of varying MW exhibit different binding capacities reflected in varying pharmacokinetic behaviour, with shorter heparin chains exhibiting a higher and more reliable bioavailability and slower clearance than larger chains. Heparin exhibits complex pharmacokinetics, and its anticoagulant effect is not linearly related to the dose. The half-life of heparin anticoagulant activity is approximately 1 hour at therapeutic doses; it increases from 30 minutes following an intravenous bolus dose of 25U/kg to 150 minutes following a dose of 400U/kg. Heparin is cleared by two mechanisms. The rapid, saturable phase of elimination is due to receptor-mediated internalization of heparin by endothelial cells and macrophages of reticuloendothelial system (de Swart CA, 1982), while a slower nonsaturable renal mechanism is also operative. Heparin is excreted in the urine, mainly as metabolites, although after large doses up to 50% may be excreted unchanged (Estes JW. 1980**;** Kandrotas RJ.1992). Accumulation of UFH is modest in severe renal deficiency in contrast to LMWH.

1.3.1.3. Limitations of Unfractionated heparin therapy

Unfractionated heparin has several limitations:

1. Nonhemorrhagic limitations are caused by the AT-3-independent binding of UFH to (plasma proteins, thrombocytes proteins, endothelium, and macrophages) and charge-dependent binding properties of UFH to proteins and surfaces.

- Pharmacokinetic limitations are caused by the AT-3-independent binding of heparin to plasma proteins, (Young E, 1994) to proteins released from platelets, (Hirsh J. 1991) and endothelial cells, which result in the variable anticoagulant response to heparin and to the phenomenon of heparin resistance (Granger CB, 1996); AT-3-independent binding to macrophages and endothelial cells also result in the dose-dependent mechanism of clearance.
- The biophysical limitations occur because the heparin-AT-3-complex is unable to inactivate factor F10a in the prothrombinase complex and thrombin bound to fibrin or to subendothelial surfaces.
- The biological limitations of heparin include HIO (heparin induced osteopenia), HIT (heparin induced thrombocytopenia) and heparin induced skin necrosis (HISN). Osteopenia is caused by the binding of heparin to osteoblasts, (Shaughnessy SG, 1995) which then release factors that activate osteoclasts (HIO via interleukin 11-dependent pathway osteocalsts activation) (Rajgopal R , 2006), whereas HIT is caused by IgG subclass, heparin-dependent antibodies. These antibodies bind to a conformationally modified epitope on platelet factor 4 (PF4) (Visentin GP, 1994; Greinacher A, 1994). Simultaneous binding of these antibodies to Fc receptors on the platelet surface causes platelet activation. Activated platelets release highly prothrombotic microparticles and are then removed from the circulation causing thrombocytopenia. In addition, these activated platelets and microparticles provide a surface onto which coagulation factor complexes can assemble to promote thrombin generation. This phenomenon can then trigger venous or arterial thrombosis with venous thrombosis being more common (Warkentin TE, 1996).

2. Hemorrhagic limitations

The non-specific binding of heparin to platelets inhibits platelet function (usually) and contributes to the hemorrhagic effects of heparin therapy. Platelet binding is more pronounced with large heparin molecules than with small heparin molecules.

Finaly, the pharmacokinetic and non-anticoagulant biological limitations of unfractionated heparin are less evident with LMWH, (Hirsh J, 1992) while the limited affinity of the heparin-AT-3 complex to fibrin-bound thrombin and factor F10a has been overcome by several new classes of AT-3-independent thrombin inhibitors and factor F10a inhibitors (Hirsh J, 1999).

1.3.2. Low-Molecular-Weight Heparin

1.3.2.1. History of LMWHs discovery and biochemical structure

In 1976 it was confirmed (Johnson EA, 1976; Andersson LO, 1976) that LMWH fractions prepared from standard heparin have been shown to have progressively less effect on the APTT, as they were reduced in molecular size, while still inhibiting activated factor F10 (factor F10a). The chemically enzymatic depolymerization of UFH changed the anticoagulant activity of UFH, resulting to dissociation of AT-3 dependent anti-factor F10a activity of UFH from its effect on thrombin (F2a) activity.

LMWHs have reduced inhibitory activity against thrombin relative to factor F10a, (Harenberg J, 1990 ; Holmer E , 1981) have a more favorable benefit-to-risk ratio than heparin when used to treat VTE, (van Dongen CJ, 2004) and have superior pharmacokinetic properties (Bara L, 1988).

LMWHs are polysulfated glycosaminoglycans that have a mean molecular weight of 4 to 5 kDa (about 15 monosaccharide units per molecule) ranging from 2 to 9 KDa. Increasing anti-F10a/anti-F2a ratio of polysulfated glycosaminoglycans is directly proportional to the increasing in number of monosaccharide units per molecule (Table 3) (Lane DA, 1984).

		Anticoagulant Activity	
Heparin Oligosaccharides	Molecular Weight	Anti-10a	Anti-2a
8	2,400	1.3	-
12	3,600	2.58	-
16	4,800	1.60	-
18	5,400	0.95	0.51
24	7,200	1.30	1.21

Table 3. Relationship between Molecular Weight and Anticoagulant Activity of Heparin Fractions.

All heparin molecules contain at least 18 saccharide units; therefore unfractionated heparin has an anti-factor F10a/anti-factor F2a ratio of 1:1. In contrast, LMWHs have anti-factor F10a/anti-F2a ratios between 2:1 and 4:1, depending on their molecular size distribution. LMWHs are differing in their pharmacokinetic properties (binding to proteins and cells) and anticoagulant profiles (anti-F2a/anti-F10a ratio) and in their recommended dosing regimens. LMWHs have several advantages over UFH, including:

1. Predictable anticoagulation dose response
2. Improved subcutaneous bioavailability
3. Dose-independent clearance
4. Longer biologic half-life
5. Lower incidence of thrombocytopenia
6. Reduced need for routine laboratory monitoring

LMWH such as enoxaparin are the most common used anticoagulants within hospitals for prophylaxis of venous thromboembolism (VTE) during surgical procedures and treatment patients with VTE and ACSs (Hirsh J, 2004; Laposata M, 1998; Montvale, NJ. 2006, Stief TW. 2007b). Enoxaparin is the sodium salt of a depolymerised heparin obtained by alkaline depolymerisation of the benzyl ester derivative of heparin from porcine intestinal mucosa. The majority of the enoxaparin components have a 4-enopyranose uronate structure at the nonreducing end of their chain. About 20% of the components contain a 1,6 anhydro derivative on the reducing end of the chain. The molecular mass ranges between 3.8 and 5 Kda, characteristically about 4.5 Kda, the sulfation degree is about 2 per disaccharide unit. The chemical structure of enoxaparin $(C_{26}H_{40}N_2O_{36}S_5)n$ is illustrated in (Figure 13)

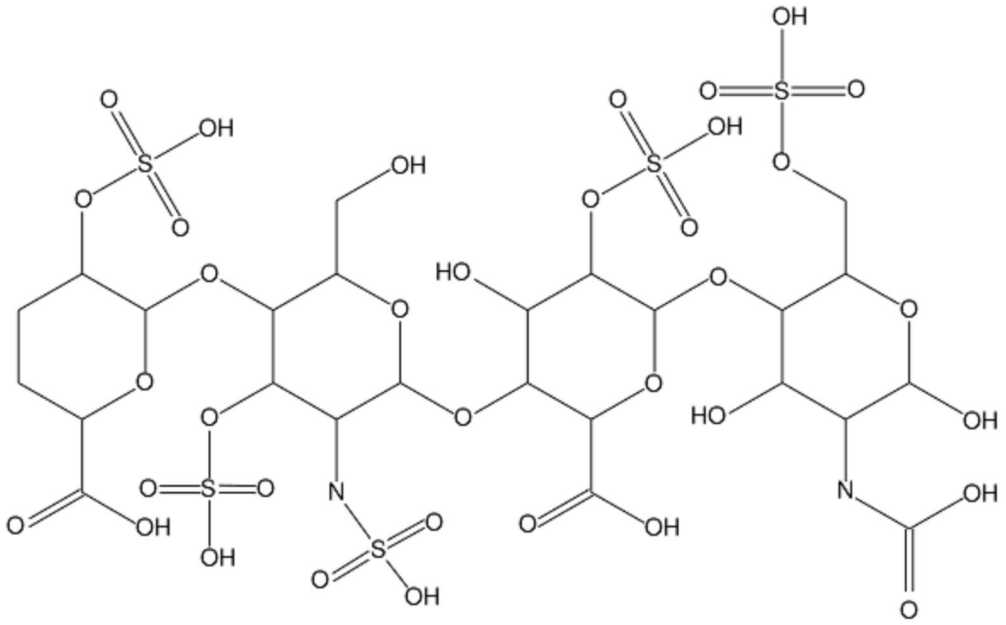

Figure 13. The chemical structure of enoxaparin ($C_{26}H_{40}N_2O_{36}S_5$)n

Pharmacokinetically, enoxaparin is rapidly and almost completely absorbed after subcutaneous injection with a bioavailability of about 100%. Peak plasma activity is reached within 1 to 5 hours. The elimination half-life is about 4 to 5 hours but anti-factor F10a activity persists for up to 24 hours after a 40-mg dose. Elimination is prolonged in patients with renal impairment. Enoxaparin is metabolised in the liver and excreted in the urine, as unchanged drug and metabolites.

1.3.2.2. Mechanism of Action of Low-Molecular-Weight Heparin

LMWHs produce their major anticoagulant effect by the potentiation of AT-3 (Figure 14). The interaction with AT-3 is mediated by a unique specific pentasaccharide sequence (Choay J, 1983) which is found on less than one third of LMWH molecules.

All LMWH chains containing the high-affinity pentasaccharide catalyze the inactivation of factor F10a. Because only pentasaccharide-containing heparin chains composed of at least 18 saccharide units are of sufficient length to bind AT-3 to thrombin, 50% to 75% of LMWH chains are too short to catalyze thrombin inhibition. AT-3-dependent F10a inactivation via high-affinity pentasaccharide of LMWHs does not require bridging between AT-3 and F10a compared to AT-3 dependent F2a

inactivation. Anti-factor F10a/anti-F2a ratio of LMWH is between 2:1 and 4:1, depending on their molecular size.

LMWHs have a reduced ability to inactivate thrombin because the smaller fragments cannot bind simultaneously to AT-3 and thrombin. Reduced binding of LMWHs to plasma proteins is responsible for the more predictable dose-response relationship of LMWHs (Hirsh J, 1992). A lower incidence of binding to macrophages and endothelial cells increases the plasma half-life of LMWH, allowing once- or twice-daily dosing (Weitz JI, 1997), whereas reducing binding to PF4 and to osteoblasts may explain the lower incidence of HIT (heparin indused thrombocytopenia) (Warkentin TE, 1995) and a lower incidence HIO (heparin indused osteopenia) (Shaughnessy SG, 1995; Bhandari M, 1998) respectively. A comparison of LMWH with UFH is given in Table 4.

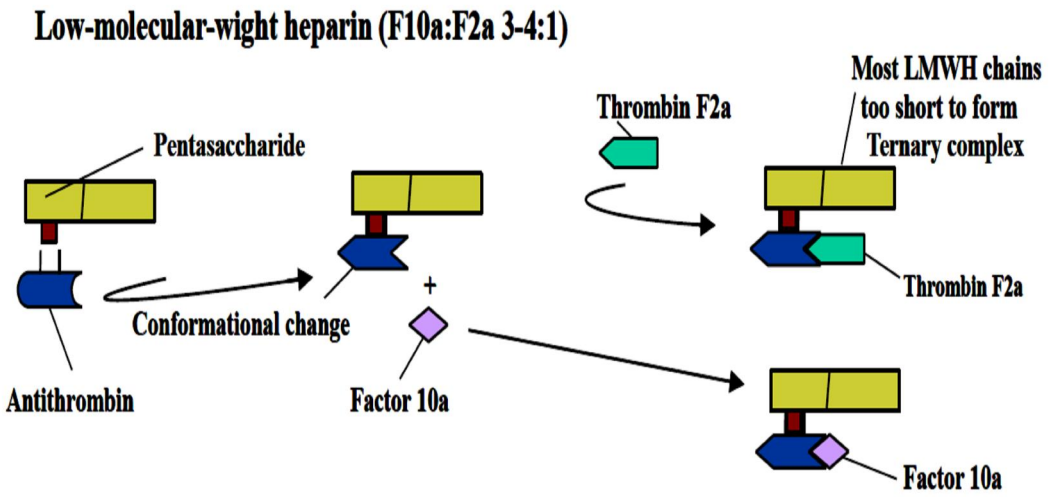

Figure 14. Pharmacologic activity of Low-Molecular-Weight Heparins (LMWHs).

Property	UFH	LMWH	Fondaparinux
Mode of action	Activation of AT-3	Activation of AT-3	Activation of AT-3
Molecular weight, daltons	3000–30,000	1500–10,000	1728
Molecules containing high-affinity pentasaccharide, %	33	33	100
Administration	IV or SC	SC	SC
Plasma half-life, h	0.5–2.5	3–4	17–21
Binding to plasma proteins, endothelial cells, platelets, and macrophages	Strong	Moderate	No
Dose-response	Unpredictable	Predictable	Predictable
Dosage	Titrated to lab value	Fixed dose or weight-based	Fixed dose or weight-based
Anti-F10a/anti-F2a ratio	1.2	2–4	No anti-F2a activity
APTT at therapeutic levels	Prolonged	Not prolonged or only slightly prolonged	Not prolonged or only slightly prolonged
Thrombocytopenia <50,000–100,000/ μL, incidence, %	1.0–1.5	1.0–1.5	0
Thrombocytopenia (<50,000/ μL, incidence, %)	0.2	0.1	0
HIT-2 (immune-mediated thrombocytopenia), %	0.1–5	< 0.1–1	None
Bleeding complications, %	3–5	Same or lower	Same or lower
Protamine reversal	Effective for IV, less effective for SC	Less effective	No effective

Table 4. Comparison of Unfractionated Heparin, Low-Molecular-Weight Heparin, and Fondaparinux. AT-3 = antithrombin; UFH = unfractionated heparin; LMWH = low molecular- weight heparin; IV = intravenous; SC = subcutaneous; APTT = activated partial thromboplastin time; HIT = heparin-induced thrombocytopenia.

1.4. Thrombin generation assays with use of synthetic Substrates.

1.4.1. Chromogenic test system (CTS)

As early s 1954 Sherry & Troll discovered the esterolytic activity of proteases and found that amino acid esters are very sensitive substrates for proteolytic enzymes. This was followed by the development of simple amino acid ester substrates (e.g. Tosyl-Arginin-Methyl-Ester) and chromogenic amide substrates (e.g. Benzoyl-Arginin-p-Nitro-Anilide) (BAPNA). Assuming that the cleavage of oligopeptide substrates reflects much more the biological activity of a protease than the cleavage of ester substrates or of substrates containing a single amino acid, e. g. BAPNA, Blombäck & Svendsen were the first to synthesize chromogenic peptide substrates, e.g. Phe-Val-Arg-pNA (S-2160), in which the chromogenic group, 4-nitroaniline, is amide-linked to the carboxyl group of arginine. Serine proteases hydrolyze the molecule and release 4-Nitroaniline from this peptide. Chromogenic peptide substrates are generally composed of 3, sometimes also of 4 or 5 amino acids (Becker et al., 1984, Gallimore and Friberger, 1991). The C-terminal amino acid is often arginine, with a chromogenic group amide - coupled to its free carboxyl group. Today, the most currently used peptide substrates employ 4-nitroaniline as the chromogenic group, the release of which is measured at 405 nm, during the reaction in a photometer cuvette. The photometric measuring signal, the change in absorbance per min (ΔA/min), is directly proportional to the enzyme activity. The CTS consists of the following reaction:

Amino acid residues-pNA (chromogen) + Protease →Amino acid residues + pNA (chromophore) + Protease

The CTS has several disadvantages (Mosesson et Kaminski 1990, Stief TW, 2006).

• Nonspecific protease-substrate (thrombin-substrate), i.e. cleavage of the protease-substrate (thrombin-substrate) by thrombin-like enzymes such as kallikrein

• Turbidity during of clot formation with generation of antithrombin-1 = fibrin.

1.4.2. Fluorogenic test system (thrombin generation test; TGT)

To avoid the disadvantages resulting from chromogenic test systems, thrombin activity was measured by a fluorogenic thrombin substrate (Hemker HC. et al. 2002; Hemker HC et al. 2003). Using a "slow" fluorogenic thrombin substrate and continuous comparison to a simultaneously run calibrator, thrombin generation can be monitored automatically, online, in clotting platelet-rich plasma or platelet-poor plasma, (PRP or PPP).

The resulting "Calibrated Automated Thrombogram (CAT)" in PPP tries to measure hypocoagulability (haemophilias, oral anticoagulants, heparins, heparinoids, direct inhibitors) and hypercoagulabilities (AT deficiency, F2 hyperexpression, PC and PS deficiency, $F5^{Leiden}$, oral contraceptives) (Hemker H C. 2002).

In PRP it is diminished in thrombopathies, in von Willebrand disease, by antibodies blocking GPIIb-IIIa or GPIb, or by antiplatelet drugs like aspirin and clopidogrel. Lupus anticoagulant increases -but unfortunately also retards- thrombin generation in the CAT. The thrombogram thus appears to be a broad function test of the haemostatic-thrombotic mechanism of blood (Hemker H C. 2002).

The CAT has several disadvantages

1. Unspecificity due to contact-phase activation of coagulation (multi-talented kallikrein splits all kind of chromogenic substrates)
2. Dependence on antithrombin-1 (fibrin), an in vitro test phenomenon that is of minor in vivo importance
3. Generated fibrin leads to renewed activation of coagulation
4. Long processing time
5. Calibration in nM
6. Internal filter effect
7. Necessity of special equipment
8. Instability of thrombin-alpha2macroglobulin complexes
9. Overestimation of thrombin activities > 1 IU/ml, underestimation of clinically relevant thrombin activities < 0.1 IU/ml.
10. Expensive

2. Objective

Low-molecular-weight heparin (LMWH) is the most important anticoagulant inside hospitals. There might be great inter-individual variation of the plasma response to a certain LMWH dosage. The present work quantifies this inter-individual variation using the best thrombin generation tests internationally available.

3. Material and Methods

3.1. Arginine

Important for the performance of Recalcified Coagulation Activity Assay (RECA) and Extrinsic Coagulation Activity Assay (EXCA) was the discovery of the serine protease inhibitory properties of arginine (Dano and Reich, 1979). Most of the coagulation factors, except protein S, in their active form are serine proteases (Girolami A et al 2008; Stafford DW 2005; Nelsestuen GL et al. 2000) that interact with their target-substrats often involves arginine residue in the P1 position. Arginine is an amino acid of the P1-P1´ reactive center of many serpins (Stief T W. 2007c; Gettins PG. 2002). Arginine or guanidine inhibits serine proteases (Stief et.al. 2001). The proteinolytic activity of thrombin, plasmin, and C1-esterase is inhibited by arginine (Stief TW. 2007c). Arginine inhibited the Whole blood clotting time (WBCT), PT, APTT, in vitro bleeding test closure time (IVBT-CT), and whole blood aggregometry (WBA) (Stief T W. 2007c). Arginine resulted in a two-fold prolongation of WBCT, PT, or IVBT-CT (the anti-epinephrine action is superior to the anti-ADP action), a four-fold prolongation of APTT or a 60% inhibition of WBA (Stief T W. 2007c).

3.2. Recalcified Coagulation Activity Assay (RECA)

3.2.1. Reagents

•Polypropylene monovettes containing 0.5 ml 106 mM sodium citrate, (Sarstedt, Nümbrecht, Germany)

•Untreated F-well polystyrene plates (NUNC, Wiesbaden, Germany, article nr. 446140)

•Bovine thrombin (Siemens Healthcare, Munich, Germany; article no. B 4233-25, 130 IU/vial) 1 IU/ml in 6.7% of human albumin (Kabi, Stockholm, Sweden)

•Siliconized glass bottles (Siemens Healthcare, Munich, Germany; article nr. OVKE49)

•Multipette (Eppendorf AG, Hamburg, Germany)

•Unfractionated Heparin (ratiopharm, Ulm, Germany) in siliconized glass bottles

•LMWH enoxaparin (Aventis, Frankfurt, Germany) in siliconized glass bottles

•$CaCl_2$ (Sigma, Deisenhofen, Germany) 250 mM in siliconized glass bottles

• Digitally controlled water bath (37°C) (Memmert, Schwabach, Germany)

•Arginine, 2.5 M, pH 8.6, 0.16% Triton X 100® (Sigma)

•Chromogenic thrombin substrate cyclohexylglycyl-alanyl-arginyl-para-nitroanilide (CHG-Ala-Arg-pNA) (Pentapharm, Basel, Switzerland; article no. 081-20), 1 mM in 1.25 M arginine, pH 8.7

•Microtiter plate photometer (Milenia- DPC, Los Angeles, USA)

3.2.2. Test performance of Recalcified Coagulation Activity Assay (RECA)

Citrated blood (0.5 ml 106 mM sodium citrate + 4.5 ml venous blood) of healthy donors after thankful written informed consent was centrifuged within 2 h (23ºC) at 2800g for 10 min (23ºC). In the recalcified coagulation activity assay (RECA) 50 µl normal plasma or patient plasma (for routine quality assurance of plasmatic F2a activity determinations taken out of the routine waste) were pipetted in duplicate by an Eppendorf-Multipette® with 0.9 % NaCl-rinsed and completely emptied new polypropylene tips into polystyrene F-well microtiterplates. 2 µl 0-260 mlU/ml unfractionated heparin in 0.9 % NaCl or 2 µl 0-260 mlU/ml LMWH enoxaparin in 0.9 % NaCl were added to generate final anticoagulant concentrations of 0, 0.1, 1, and 10 mIU/ml, starting with the lowest concentration. The plates were intensely shaken for 10s. After 10 min (23ºC) 5 µl 250 mM $CaCl_2$ were added. After intense shaking for 10s, the plates were brought into a 37ºC digitally controlled water-bath. After 40 min

(main value) or 45 min (control value) at 37ºC, 100 µl 2.5 M arginine, pH 8.6, 0.16 % Triton X 100® were added. After 3 min 25 µl 1 mM chromogenic thrombin substrate CHG-Ala-Arg-pNA in 1.25 M arginine, pH 8.7, were added, and the linear increase of absorbance with time at 23ºC (ΔA/t) was measured (linear range: up to 40 % of maximal ΔA (900 mA). The results were standardized against 1 IU/ml bovine thrombin in 6.7% human albumin that replaced the plasma sample and resulted in about 160 mA/min (23ºC). The basal thrombin activity in citrated plasma prior to any CRT was also determined; this about 10 mIU/ml basal thrombin activity was substracted from all thrombin activities measured in RECA. The plasmas were analyzed in the RECA with a coagulation reaction time (CRT) of 40 min (37ºC) (RECA-40). 40 min reaction time was chosen for these lots of polyproplene blood sampling tubes and of microwells because at 40 min CRT the thrombin generation was still ascending, i.e. the ratio between thrombin activity at 40 min CRT and thrombin activity at 45 min CRT in unsupplemented plasmas was less than 1.

3.3. Extrinsic Coagulation Activity Assay (EXCA)

3.3.1. Reagents

•Untreated polystyrene microtiterplates with round bottom U-wells (Greiner, Frickenhausen, Germany; article nr. 650161, lot nr. 05281161)

•TF-reagent; 1 ml aliquots of once frozen/thawed 1 ng/ml tissue factor without polybrene® (Thromborel S®, Behringwerke, Marburg, Germany), 250 mM $CaCl_2$, 5% human albumin (Kabi, Stockholm, Sweden) in siliconized 15 ml glass vials (Stief TW et al. 2008a)

3.3.2. APTT and EXCA in pooled normal plasma

500 µl samples of unfrozen pooled normal plasma were supplemented with 20 µl 0-26 IU/ml unfractionated heparin in 0.9 % NaCl or with 20 µl 0-26 IU/ml of the LMWH enoxaparin in 0.9 % NaCl. After 90 min at 23°C the samples were assayed for APTT (Pathromtin SL, Behring Coagulation Timer (BCT), DadeBehring, Marburg, Germany). The control value was that of 0 IU/ml drug. At the same time point, these samples were also analyzed in the EXCA: 50 µl plasma in polystyrene U-wells were activated after 90 min preincubation at 23°C with 5 µl TF-reagent that was added by an Eppendorf-Multipette® with 0.9% NaCl-rinsed polypropylene-tips. After intense shaking for 5s, the plates were brought into a 37°C digitally controlled water-bath. After 1 min or 2 min at 37°C, the plates were taken out of the water bath and 100 µl 2.5 M arginine, pH 8.6, 0.16% Triton X 100® were added. The coagulation reaction time (CRT) of 1 min (37°C) is the main value =EXCA-1, the CRT of 2 min is the control value =EXCA-2 (EXCA-1/EXCA-2 ration was always < 1, i.e. thrombin generation at 1 min CRT was always in the increasing part of the thrombin generation curve). After 3 min at 23°C, 25 µl 1 mM chromogenic thrombin substrate cyclohexylglycyl-alanyl-arginyl-para-nitroanilid (CHG-Ala-Arg-pNA) in 1.25 M arginine, pH 8.7, were added, and the linear increase of absorbance with time at 23°C (ΔA/t) was measured (linear range: up to 40 % of maximal ΔA, which is about 1050 mA) by a microtiter plate photometer with a 1 mA resolution. The results were standardized with 1 IU/ml bovine thrombin (DadeBehring) in 6.7 % human albumin that replaced the plasma sample, and resulted in about 16 mA/min (23°C). The basal thrombin activity in citrated plasma prior to any CRT was also determined by first addition of the arginine-reagent and then addition of the TF-reagent. This basal thrombin activity was substracted from all thrombin activities measured in EXCA, transforming the thrombin activities into thrombin generations. The basal thrombin activity in citrated patient plasmas was 8.0±2.4 mIU/ml (100±30%). The basal thrombin activity in citrated plasmas of healthy donors was 7.3±2.0 mIU/ml (100±27%). Since the basal thrombin activity in pooled normal EDTA-plasma is 5.5 mIU/ml (Stief TW, 2006), hemostasis in the citrated samples was only minimally pre-activated.

3.3.3. Extrinsic Coagulation Activity Assay (EXCA) in normal plasma and patient plasma

Citrated blood (0.5 ml 106 mM sodium citrate + 4.5 ml venous blood in polypropylene monovettes) of healthy donors after thankful written informed consent was centrifuged at 2800g for 10 min (23ºC). 50 µl normal plasma or patient plasma for routine quality assurance of chromogenic F2a determinations taken out of the routine waste were analyzed in duplicate in the extrinsic coagulation activity assay (EXCA): 50 µl samples were pipetted with an Eppendorf-multipette® with 0.9 % NaCl-rinsed and completely emptied new polypropylene tips into polystyrol microwells with round bottom (U-wells; Greiner) 2 µl 0- 26 IU/ml unfractionated heparin in 0.9 % NaCl or 2 µl 0-26 IU/ml enoxaparin in 0.9% NaCl were added to generate final anticoagulant concentrations of 0, 0.01, 0.1, 0.2, 0.5, and 1 IU/ml, starting with the lowest concentration. After intense shaking for 5s, the plates were brought into a 37ºC digitally controlled water-bath. After 1min or 2 min at 37ºC, the plates were taken out of the water bath and 100 µl 2.5 M arginine, pH 8.6, 0.16 % Triton X 100® were added. The coagulation reaction time (CRT) of 1 min (37ºC) is the main value =EXCA-1, the CRT of 2 min is the control value =EXCA-2 (EXCA-1/EXCA-2 ration was always < 1, i.e. thrombin generation at 1min CRT was always in the increasing part of the thrombin generation curve). After 3 min at 23ºC, 25 µl 1 mM chromogenic thrombin substrate cyclohexylglycyl-alanyl-arginyl-para-nitroanilide (CHG-Ala-Arg-pNA) in 1.25 M arginine, pH 8.7, were added, and the linear increase of absorbance with time at 23ºC ($\Delta A/t$) was measured (linear range up to 40 % of maximal ΔA = about 1050 mA) by a microtiter plate photometer with a 1 mA resolution. The results were standardized with 1 IU/ml bovine thrombin (DadeBehring) in 6.7 % human albumin that replaced the plasma sample, and resulted in about 16 mA/min (23ºC). The basal thrombin activity in citrated plasma prior to any CRT was also determined by first addition of the arginine reagent and then addition of the tissue factor reagent. This basal thrombin activity was substracted from all thrombin activities measured, transforming thrombin activities into thrombin generations.

4. Results

4.1. Individual anticoagulant efficiency of heparin and of the LMWH enoxaparin

4.1.1- LMWH (enoxaprin) and unfractionated heparin in APTT and EXCA.

Unfrozen pooled normal plasma was supplemented with heparin or with the low-molecular-weight-heparin (LMWH) enoxaparin. After preincubation time of 90 min at 23°C the samples were assayed simultaneously for APTT and extrinsic coagulation activity assay (EXCA).

Figure 4.1.1a demonstrates that 0.5 IU/ml enoxaparin prolonged the APTT of pooled normal plasma from 29s to about 40s; the same prolongation of APTT is noted by only 0.1 IU/ml unfractionated heparin.

Normal APTT control (0 IU/ml drug concentration) = 29s. The 1.5fold prolongation of the normal APTT control (43s) is reached at 0.11 IU/ml heparin, 2fold prolongation of the normal APTT control (58s) is reached at 0.15 IU/ml heparin and 3fold prolongation of the normal APTT control (87s) is caused by 0.25 IU/ml heparin.

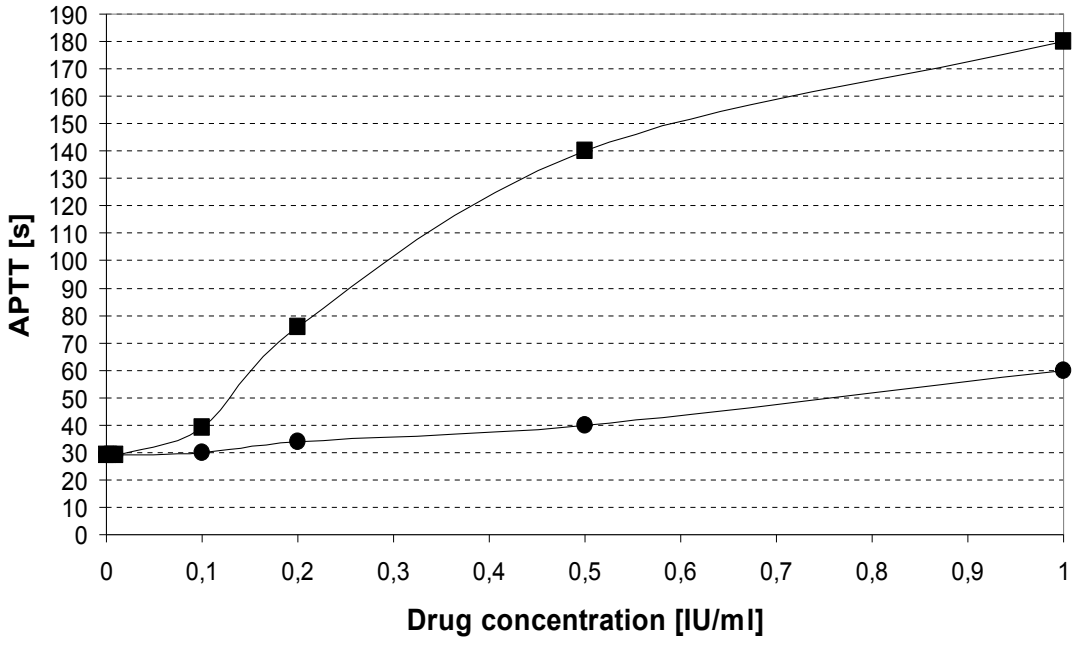

Figure 4.1.1a. APTT in pooled normal plasma. Unfrozen pooled normal plasma was supplemented with heparin (■) or with the low-molecular-weight-heparin (LMWH) enoxaparin (●).

Figure 4.1.1b demonstrates that 0.11 IU/ml, 0.15 IU/ml, or 0.25 IU/ml heparin corresponded to about 40 %, 20 %, or 10 % of normal thrombin generation in the EXCA, respectively.

100 % EXCA = generation of 462±23 mIU/ml (mean value ± 1 standard deviation) thrombin (F2a) within 1 min (37°C) in unsupplemented controls.

1.5 fold (43s) prolongation of the normal APTT corresponded to about 40 % EXCA, 2fold (58s) prolongation of the normal APTT corresponded to about 20 % EXCA and 3fold (87s) prolongation of the normal APTT corresponded to about 10 % EXCA.

The slightest prophylactic anticoagulation, equivalent to about 40 % of normal EXCA, occurred at about 0.3 IU/ml (LMWH) enoxaparin concentration. The therapeutic anticoagulation, equivalent to about 10-20 % of normal EXCA, occurred at about 0.6 IU/ml - 1 IU/ml (LMWH) enoxaparin concentration (Fig. 4.1.1b).

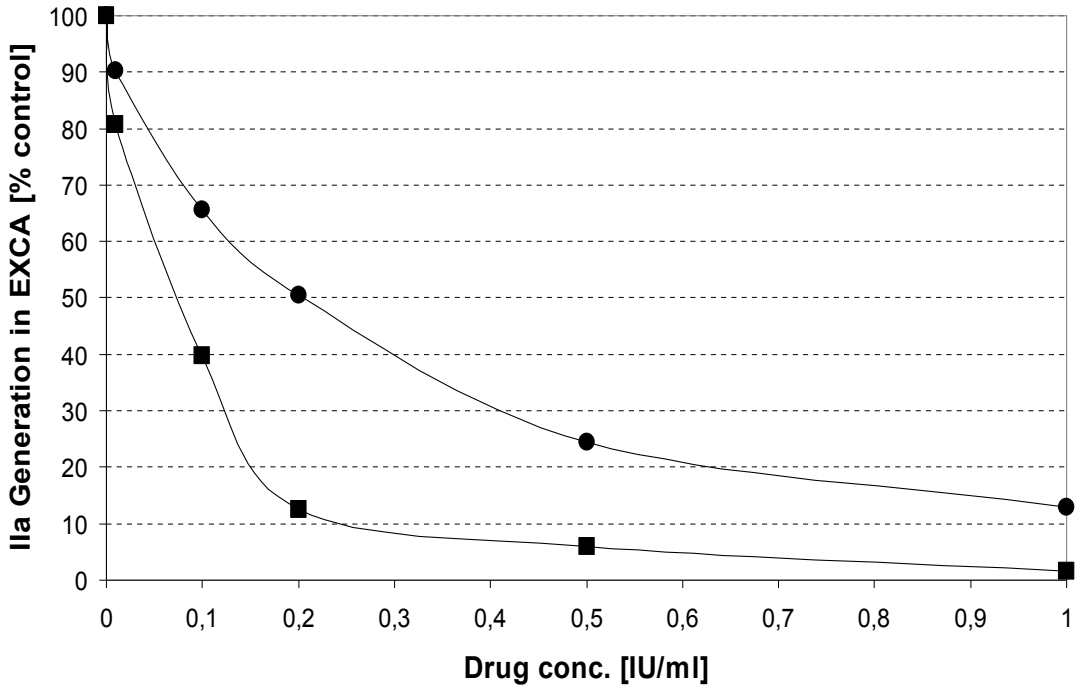

Figure 4.1.1b. EXCA in pooled normal citrated plasma. EXCA values of the same samples of Figure 4.1.1a (heparin (■), enoxaparin (●)).

4.1.2. Thrombin generation in EXCA [mIU/ml] at different supplementations of heparin or enoxaparin in normal citrated plasmas.

Citrated plasma samples of normal healthy donors (n=51) were supplemented with unfractionated heparin or with low-molecular-weight-heparin (LMWH) enoxaparin.

The EXCA was performed (main value = 1 min incubation at 37°C = EXCA-1).

Mean values were defined a percent of thrombin generation in EXCA by unsupplemented citrated plasma control [% of unsupplemented citrated plasma control].

Thrombin generation in EXCA at 0 IU/ml heparin was 423±127 mIU/ml = 100 % F2a generation, at 0 IU/ml LMWH it was 358±96 mIU/ml.

Figure 4.1.2a demonstrated that the mean approximate 50 % inhibitory concentration (IC50) of unfractionated heparin was 0.08 IU/ml; the mean IC50 of low-molecular-weight-heparin (LMWH) enoxaparin was 0.14 IU/ml.

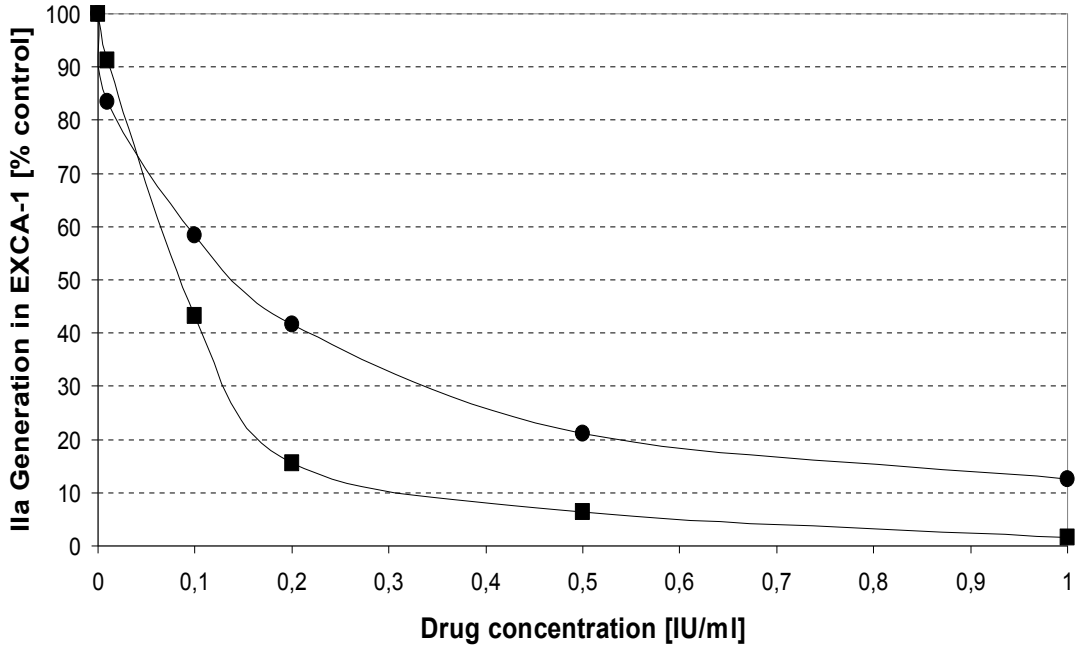

Figure 4.1.2a. EXCA inhibition by heparin and (LMWH) enoxaparin in normal citrated plasmas (of n=51 healthy donors). Supplementation with heparin (■) or with enoxaparin (●).

0.01 IU/ml low-molecular-weight-heparin (LMWH) enoxaparin or 0.01 IU/ml heparin decreased the normal thrombin generation in EXCA by about 17 % and 9 %, respectively.

1 IU/ml (LMWH) enoxaparin concentration reduced the normal thrombin generation in EXCA to about 13 % of normal whereas at 1 IU/ml heparin the normal thrombin generation in EXCA was completely suppressed. This means that 1 IU/ml LMWH is strongly therapeutic, but 1 IU/ml heparin is in the toxic range.

0.1 IU/ml heparin in individual normal citrated plasmas resulted in a thrombin generation of 43±16 % of the unsupplemented plasma control. A thrombin generation of more than 59 % of normal indicated plasmatic resistance against 0.1 IU/ml heparin, a thrombin generation of less than 27 % of normal indicated plasmatic hyper-responsiveness to 0.1 IU/ml heparin (Fig. 4.1.2b). There was nearly no correlation (r=0.227) between normal thrombin generation in EXCA [mIU/ml] at 0 IU/ml heparin and thrombin generation in EXCA [% of unsupplemented citrated plasma control] at 0.1 IU/ml heparin in plasma.

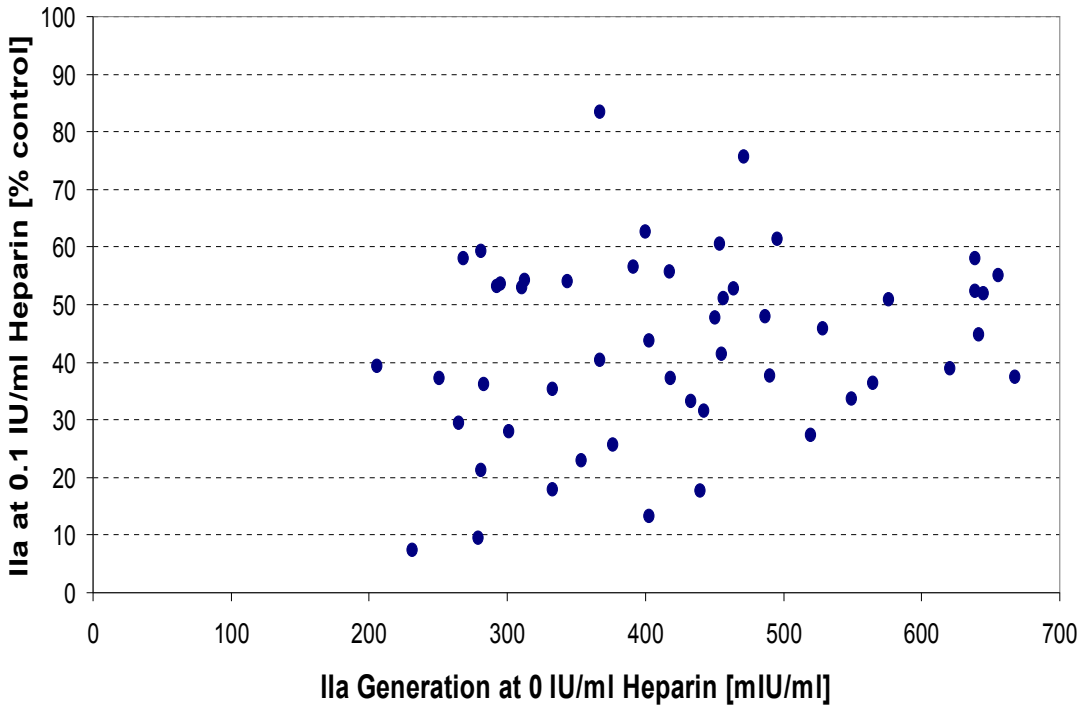

Figure 4.1.2b. Thrombin generation in normal citrated plasmas with 0 IU/ml or with 0.1 IU/ml heparin.

0.1 IU/ml LMWH-enoxaparin in individual normal citrated plasmas resulted in a thrombin generation of 57±12 % of the unsupplemented plasma control. A thrombin generation of more than 69 % of normal indicated plasmatic resistance against 0.1 IU/ml LMWH-enoxaparin, a thrombin generation of less than 45 % of normal

indicated plasmatic hyper-responsiveness to 0.1 IU/ml LMWH-enoxaparin (Fig. 4.1.2c). There is no correlation (r=0.026) between normal thrombin generation in EXCA [mIU/ml] by supplementation with 0 IU/ml LMWH-enoxaparin concentration and thrombin generation in EXCA [% of unsupplemented citrat-plasma control] at 0.1 IU/ml enoxaparin concentration.

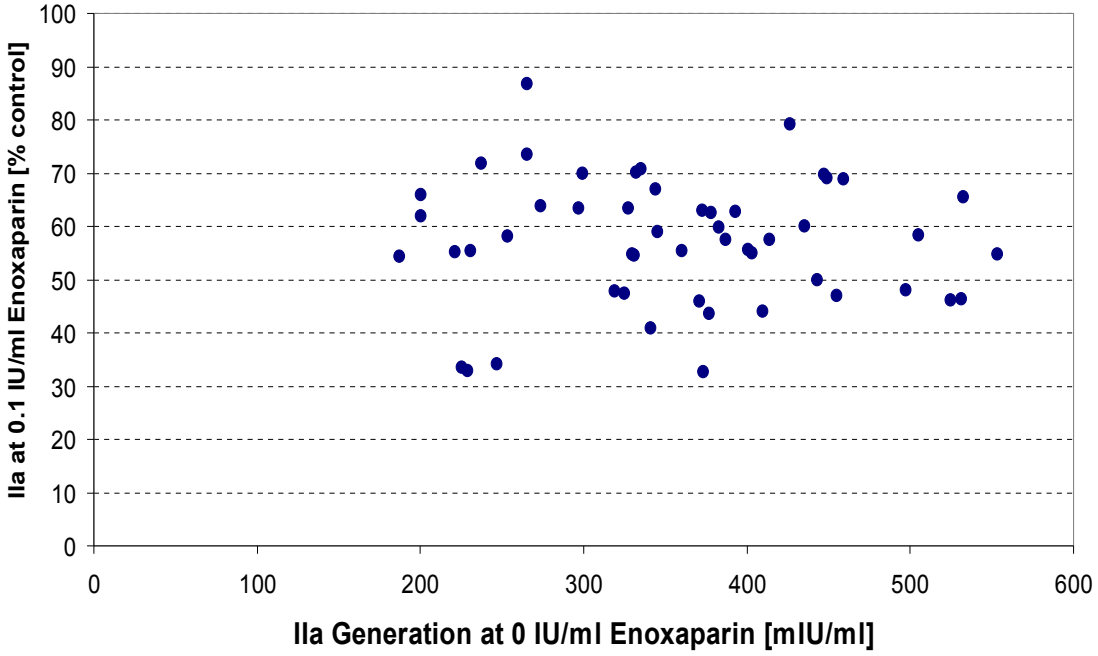

Figure 4.1.2c. Thrombin generation in normal citrated plasmas with 0 IU/ml or with 0.1 IU/ml LMWH-enoxaparin.

A comparison between normal thrombin generation in EXCA at 0.1 IU/ml heparin concentration and normal thrombin generation in EXCA at 0.1 IU/ml LMWH-enoxaparin concentration had only a slight correlation (r=0.334) (Fig. 4.1. 2d), i.e. the response of patients against unfractionated heparin or LMWH-enoxaparin might be completely individually different.

0.2 IU/ml enoxaparin resulted in 42±11 %, 0.5 IU/ml enoxaparin in 21±10 %, and 1 IU/ml in 13±7 % of normal F2a generation. The respective values for heparin were 16±10 %, 6±4 %, and 2±3 %.

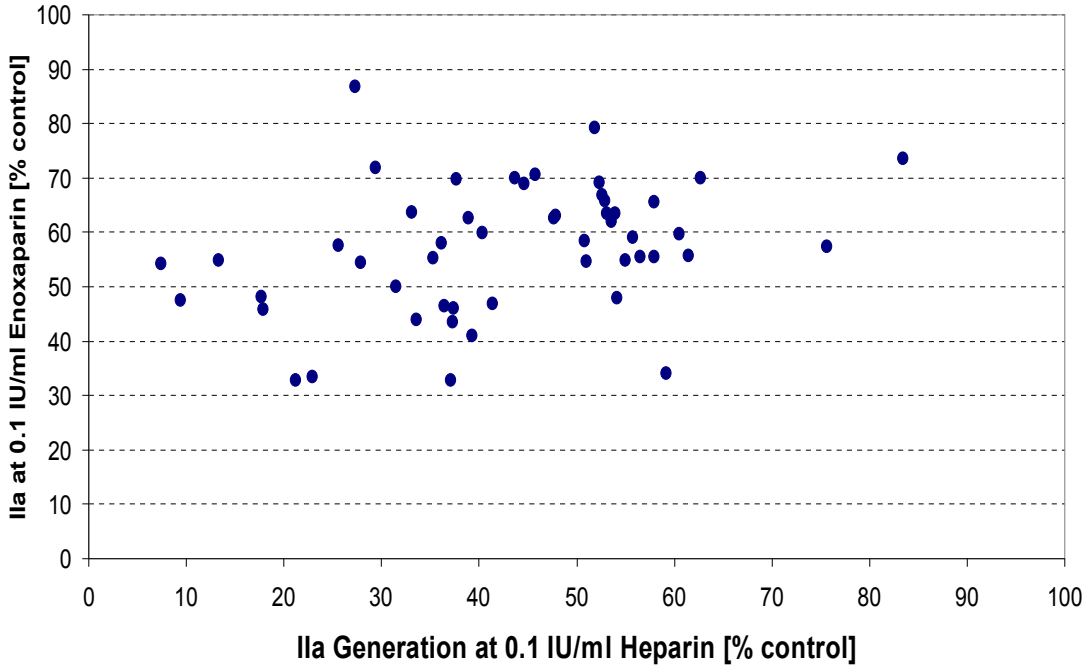

Figure 4.1.2d. Thrombin generation in normal citrated plasmas with 0.1 IU/ml heparin or with 0.1 IU/ml LMWH.

4.1.3. Thrombin generation in EXCA [mIU/ml] at different supplementations of heparin or of enoxaparin in patient plasmas.

Citrated plasma samples of patients (n=213) without anticoagulation therapy and with normal hemostatic parameters (PT, APTT) were supplemented with unfractionated heparin or with low-molecular-weight-heparin (LMWH) enoxaparin.

The EXCA was performed (main value = 1 min incubation at 37°C = EXCA-1).

Thrombin generation in EXCA at 0 IU/ml heparin supplementation and at 0 IU/ml LMWH-enoxaparin were 708±341 mIU/ml = 100 % F2a generation, 643±340 mIU/ml = 100 % F2a generation respectively.

Figure 4.1.3a demonstrated that the mean approximate 50 % inhibitory concentration (IC50) of unfractionated heparin was 0.09 IU/ml; the mean IC50 of low-molecular-weight-heparin (LMWH) enoxaparin was 0.16 IU/ml.

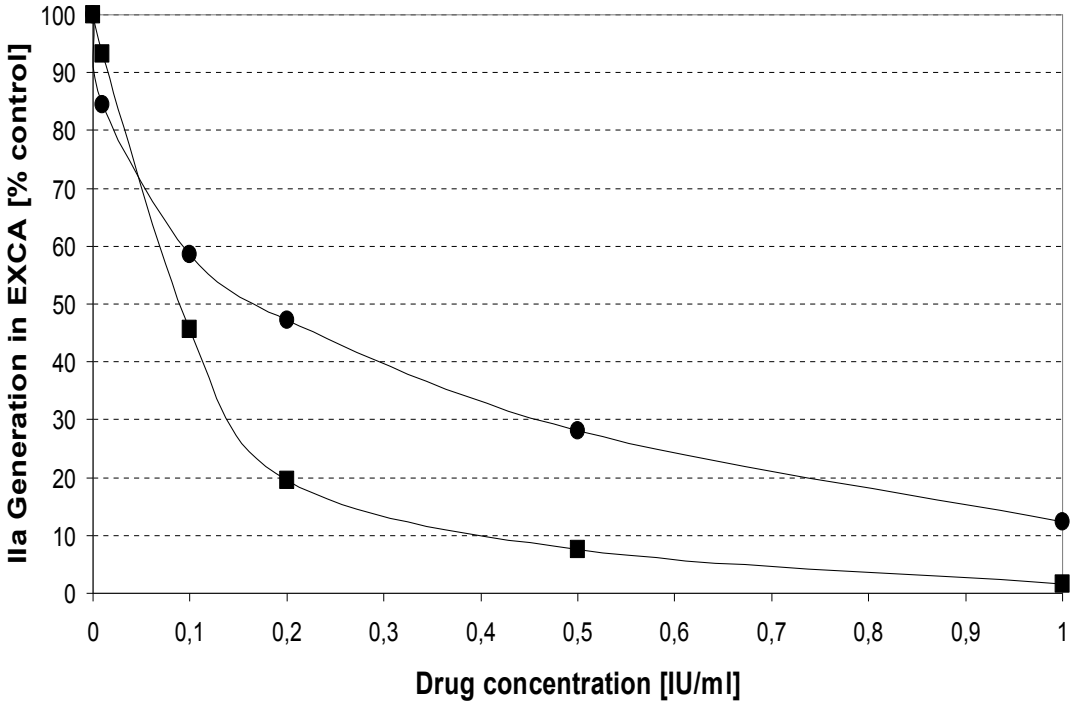

Figure 4.1.3a. EXCA inhibition by heparin and (LMWH) enoxaparin in citrated patient plasmas (of n=213 patients without anicoagulants and with normal hemostasis). Supplementation with heparin (■) or with enoxaparin (●).

Mean values of anticoagulant efficiency of 0.01 IU/ml concentration LMWH-enoxaparin and 0.01 IU/ml concentration unfractionated heparin were 84 % and 93 % respectively. The Comparison of mean values demonstrated that anticoagulat efficiency of 0.01 IU/ml enoxaparin was superior to 0.01 IU/ml heparin with high statistic significance (χ^2corr = 8.58, p< 0.01).

0.1 IU/ml heparin in individual patient citrated plasmas resulted in a thrombin generation of 46±14 % of the unsupplemented plasma control. A thrombin generation more than 60 % of normal indicated plasmatic resistance against 0.1 IU/ml heparin; a thrombin generation less than 32 % of normal indicated plasmatic hyper-responsiveness to 0.1 IU/ml heparin (Fig. 4.1.3b). There was nearly no correlation (r=0.163) between normal thrombin generation in EXCA [mIU/ml] by supplementation with 0 IU/ml heparin concentration and thrombin generation in EXCA [% of unsupplemented citrated plasma control] at 0.1 IU/ml heparin concentration (Fig. 4.1.3b). The higher F2a generation [mIU/ml] in citrated plasma samples of

patients when compared to that in citrated plasma samples of normal healthy donors might be attributed to

1. Pre-analytic differences (longer pre-analytic time interval till analysis, the patient samples were taken out of the routine waste),
2. Some procoagulant tendencies in blood of patients when compared with healthy donors.

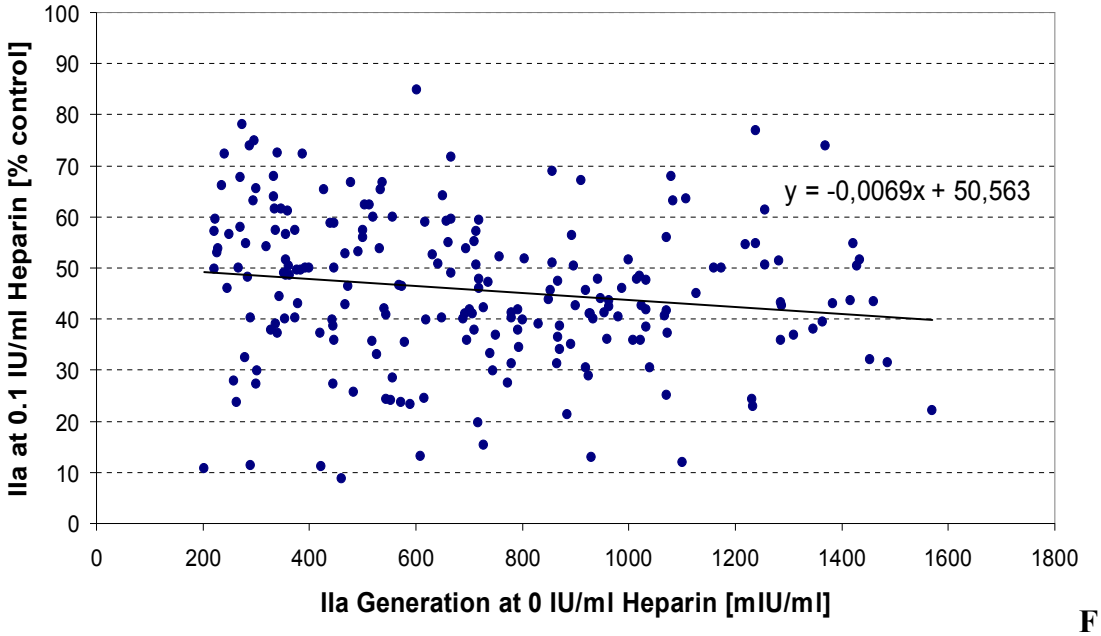

Figure 4.1.3b. Thrombin generation in patient plasmas supplemented with 0 IU/ml or with 0.1 IU/ml heparin.

0.2 IU/ml LMWH-enoxaparin in individual patient citrated plasmas resulted in a thrombin generation of 47±13 % of the unsupplemented plasma control. A thrombin generation more than 60 % of normal control indicated plasmatic resistance against 0.2 IU/ml LMWH-enoxaparin, a thrombin generation less than 34 % of normal control indicated plasmatic hyper-responsiveness to 0.2 IU/ml LMWH-enoxaparin (Fig. 4.1.3c). There was no correlation (r= 0.049) between normal thrombin generation in EXCA [mIU/ml] by supplementation with 0 IU/ml LMWH-enoxaparin concentration and thrombin generation in EXCA [% of unsupplemented citrat-plasma control] at 0.2 IU/ml LMWH-enoxaparin concentration.

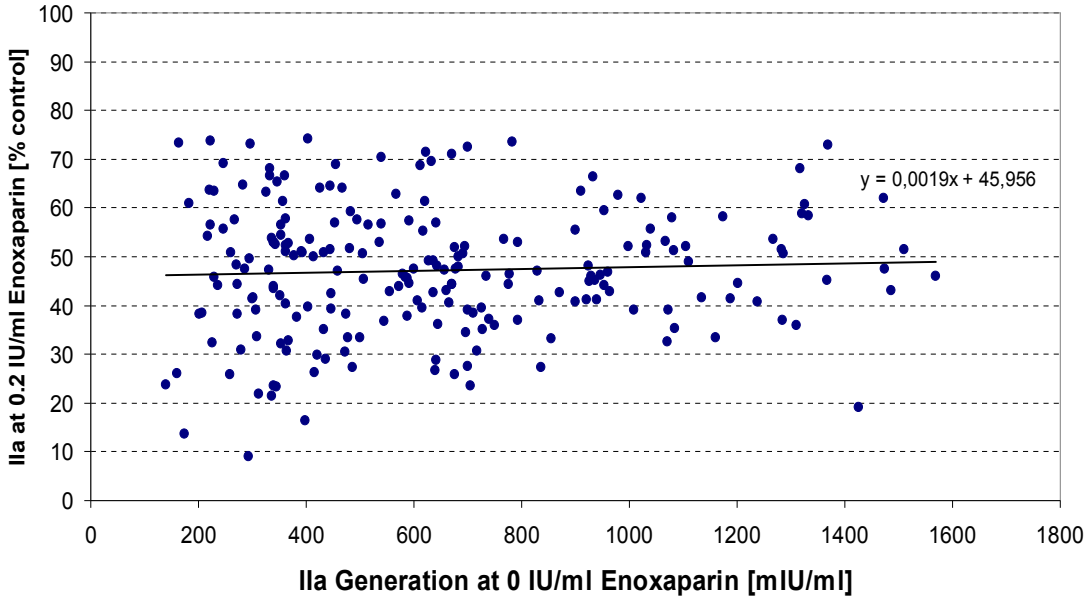

Figure 4.1.3c. Thrombin generation in patient plasmas supplemented with 0 IU/ml or with 0.2 IU/ml LMWH.

But there was slight correlation (r=0.262) between normal thrombin generation in EXCA [mIU/ml] by supplementation with 0.1 IU/ml unfractionated heparin concentration and thrombin generation in EXCA [% of unsupplemented citrat-plasma control] at 0.2 IU/ml LMWH-enoxaparin concentration (Fig. 4.1.3d).

The response of individual patients to enoxaparin when compared to heparin seems to be better predictable.

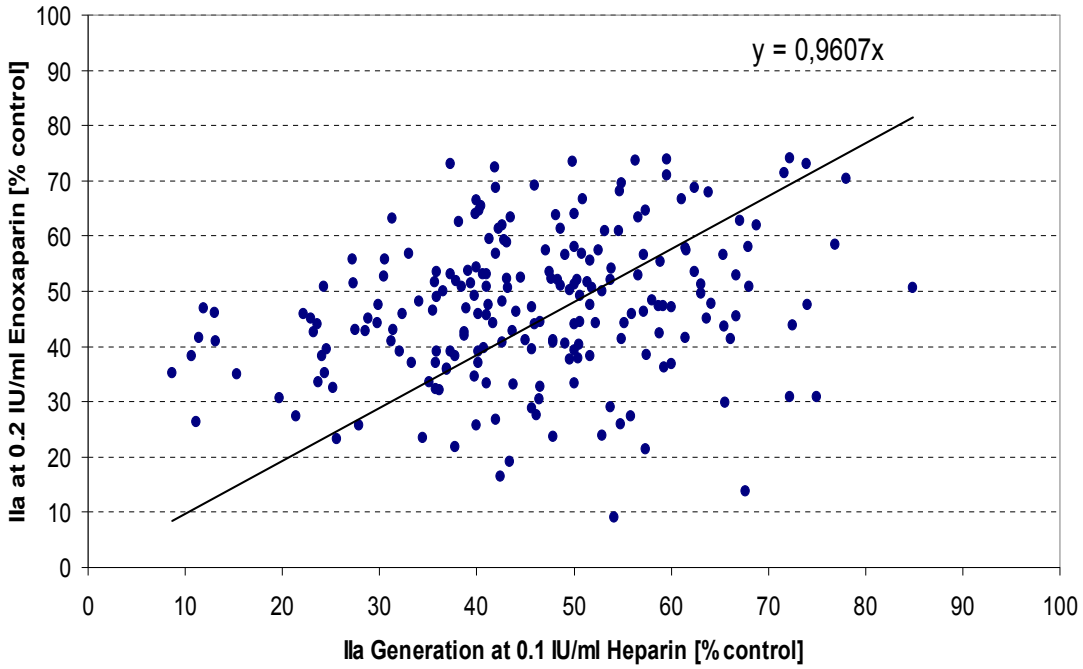

Figure 4.1.3d. Thrombin generation in normal citrated plasmas supplemented with 0.1 IU/ml heparin or with 0.2 IU/ml LMWH-enoxaparin

0.2 IU/ml enoxaparin resulted in 47±13 %, 0.5 IU/ml enoxaparin in 28±11 %, and 1 IU/ml in 12±9 % of normal F2a generation. The respective values for heparin were 19±9 %, 7±5 %, and 2±3 % (greater standard deviation). The results in percent of thrombin generations of unsupplemented patient citrated plasma were similar to the results in precent of the thrombin generations of unsupplemented normal healthy donor citrated plasma.

4.2. Comparison of routine parameters (APTT, INR, CRP, Fibrinogen, Thrombocytes) with EXCA

The EXCA-1 values [% of unsupplemented citrat-plasma control] in citrated plasma samples of patients (n=213) after supplementation with heparins (heparin, enoxaparin) at approximatly 50 % inhibitory concentration (IC50) were compared with the routinely measured hemostatic parameters values APTT, INR, Fibrinogen concentration, platelets count, and C reactive protein (CRP).

4.2.1. Comparison of EXCA-1 with APTT

APTT (Pathromtin SL®; Behring Coagulation Timer (BCT) DadeBehring) (31.4±3.3s) and EXCA-1 results in the n=213 samples of patients of Fig. 4.1.3a after supplementation with 0.1 IU/ml heparin had no correlation, r= -0.072 (Figure 4.2.1.).

APTT (31.4±3.3s) and EXCA-1 values in the n=213 samples of patients of Fig. 4.1.3a after supplementation with 0.2 IU/ml LMWH-enoxaparin did not correlate, too (r= 0.023).

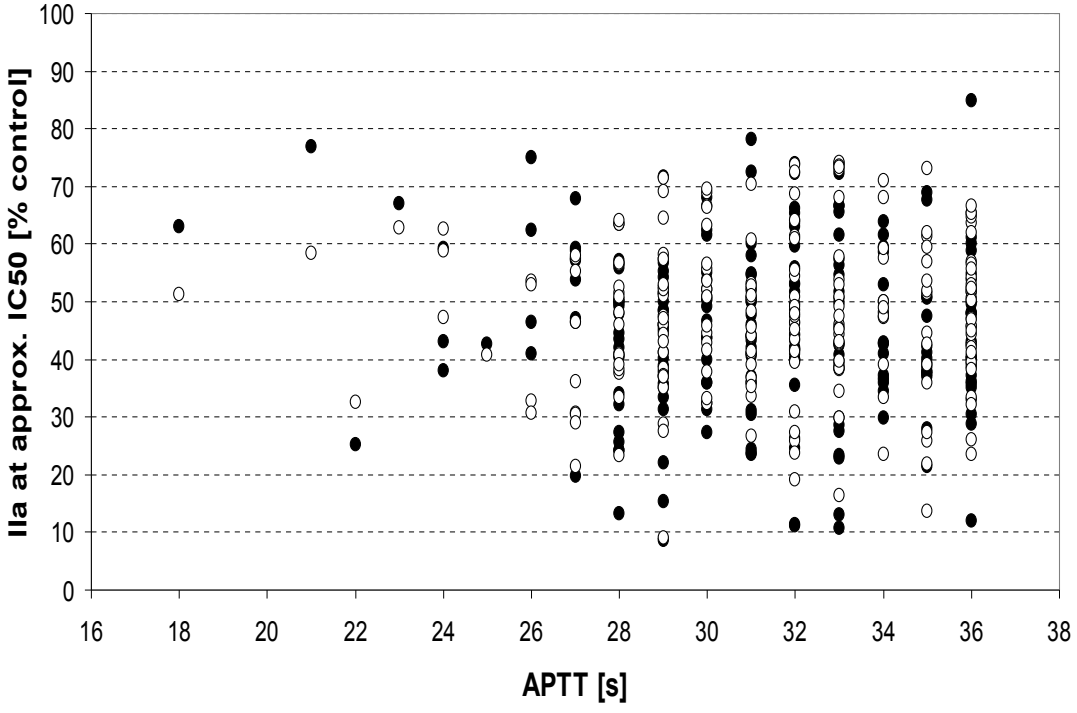

Figure 4.2.1. APTT and thrombin generation in patient plasmas after supplementation with heparin (●) or enoxaparin (o).

4.2.2. Comparison of EXCA-1 with international normalized ratio (INR)

INR (Thromborel S®) (1.02±0.07) and EXCA-1 values in the n=213 samples of patients of Fig. 4.1.3a after supplementation with 0.1 IU/ml heparin had no correlation, r= -0.045 (Fig. 4.2.2.)

INR (1.02±0.07) and EXCA-1 results in the n=213 samples of patients of Fig. 4.1.3a after supplementation with 0.2 IU/ml LMWH-enoxaparin also had no correlation (r=- 0.101).

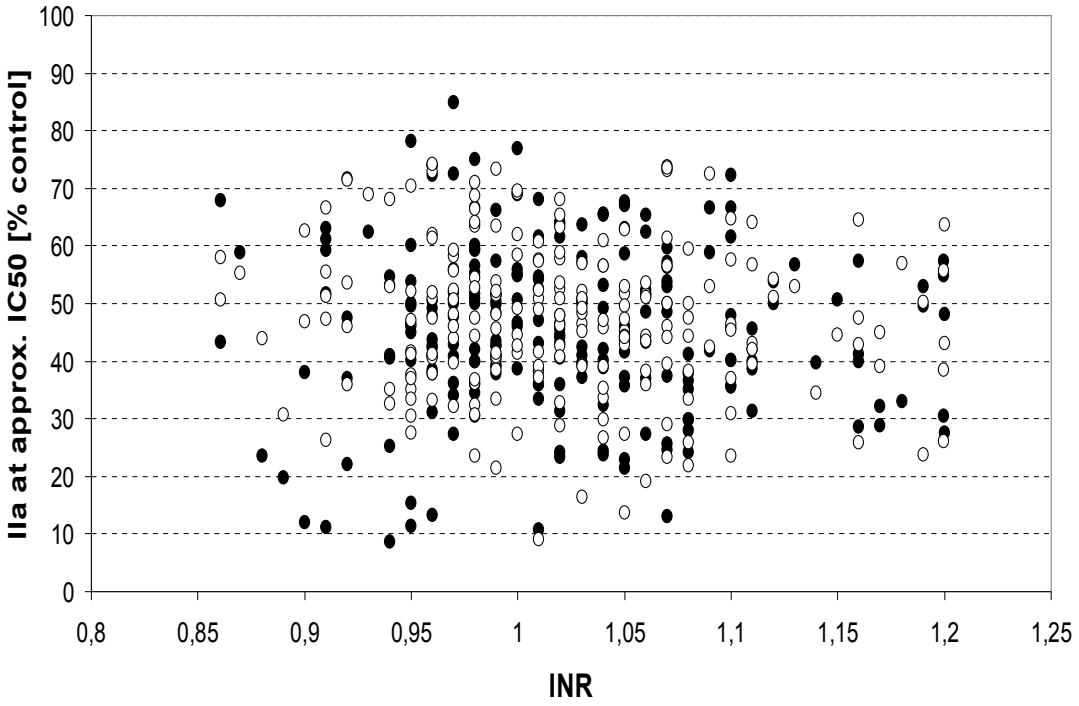

Figure 4.2.2. INR and thrombin generation in patient plasmas after supplementation with heparin (●) or enoxaparin (o).

4.2.3. Comparison EXCA-1 with functional fibrinogen concentration

Fibrinogen concentration (modified Clauss-Method; BCT; DadeBehring) (3.7±1.4 g/l) and EXCA-1 values in the n=213 samples of patients of Fig. 4.1.3a after supplementation with 0.1 IU/ml heparin did not correlate, r= 0.157 (Fig. 4.2.3.)

Fibrinogen concentration (3.7±1.4 g/l) and EXCA-1 values in the n=213 samples of patients of Fig. 4.1.3a after supplementation with 0.2 IU/ml LMWH-enoxaparin also did not correlate (r=- 0.003).

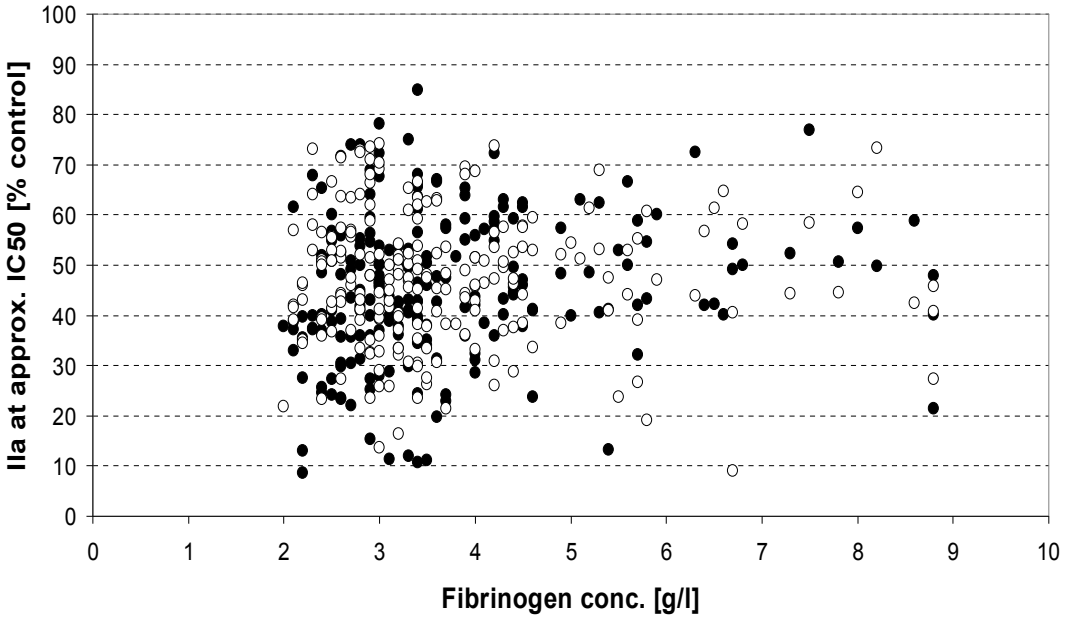

Figure 4.2.3. Fibrinogen concentration and thrombin generation in patient plasmas after supplementation with heparin (●) or enoxaparin (o).

4.2.4. Comparison EXCA-1 with C reactive protein

Samples with CRP values < 5 mg/l were pooled; the pool was analyzed with an ultra-sensitive assay for CRP, resulting in 2 mg/l CRP. All CRP results < 5 mg/l were attributed to 2 mg/l.

C - reactive protein concentration (CRP; Beckman, Krefeld, Germany) (9.7±25.9 mg/l) and EXCA-1 values in the n=213 samples of patients of Fig. 4.1.3a after supplementation with 0.1 IU/ml heparin had no correlation, r= 0.118 (Fig. 4.2.4).

C - reactive protein concentrations (9.7±25.9 mg/l) and EXCA-1 results in the n=213 samples of patients of Fig. 4.1.3a after supplementation with 0.2 IU/ml LMWH-enoxaparin did not correlate, too (r=- 0.130).

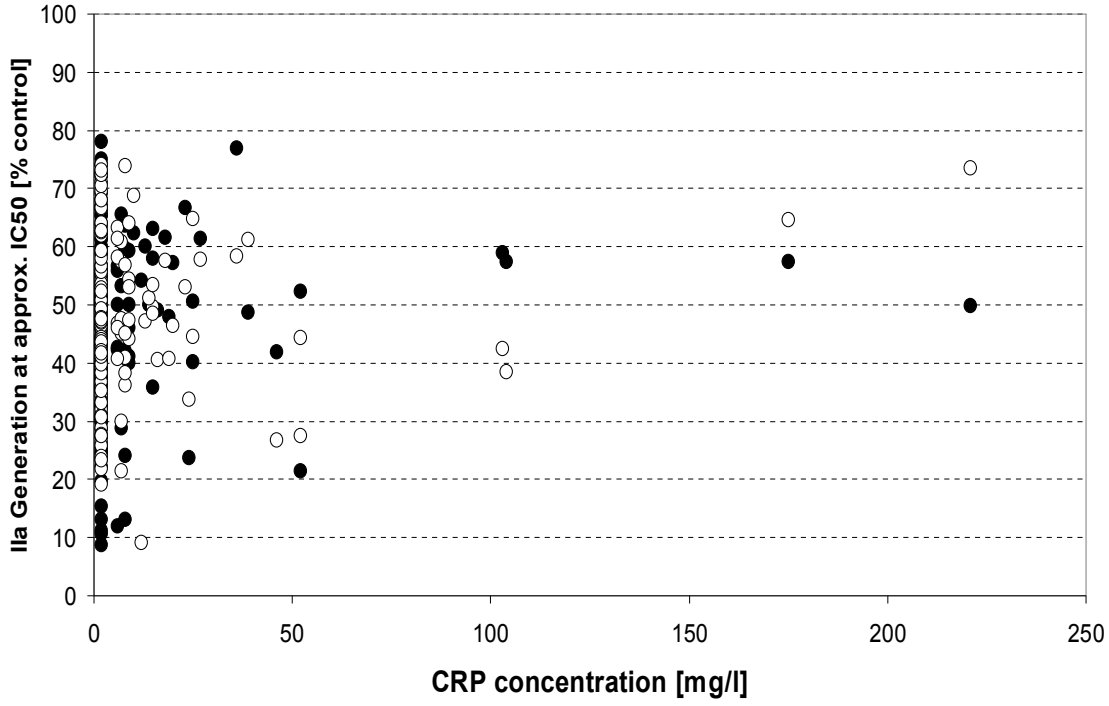

Figure 4.2.4. C - reactive protein (CRP mg/l) concentration and thrombin generation in patient plasmas after supplementation with heparin (●) or enoxaparin (o).

4.2.5. Comparison of EXCA-1 values with platelets count

The platelets count (SysmexXE2100, Hamburg, Germany) (240000±70000/µl) and the EXCA-1 values in the n=167 samples of patients of Fig. 4.1.3a after supplementation with 0.1 IU/ml heparin (46±15%) had no correlation, r= 0.095.

The platelets count (240.000±70.000/µl) and the EXCA-1 results in the n=167 samples of patients of Fig. 4.1.3a after supplementation with 0.2 IU/ml LMWH-enoxaparin (47±13%) did not correlate, r=- 0.299.

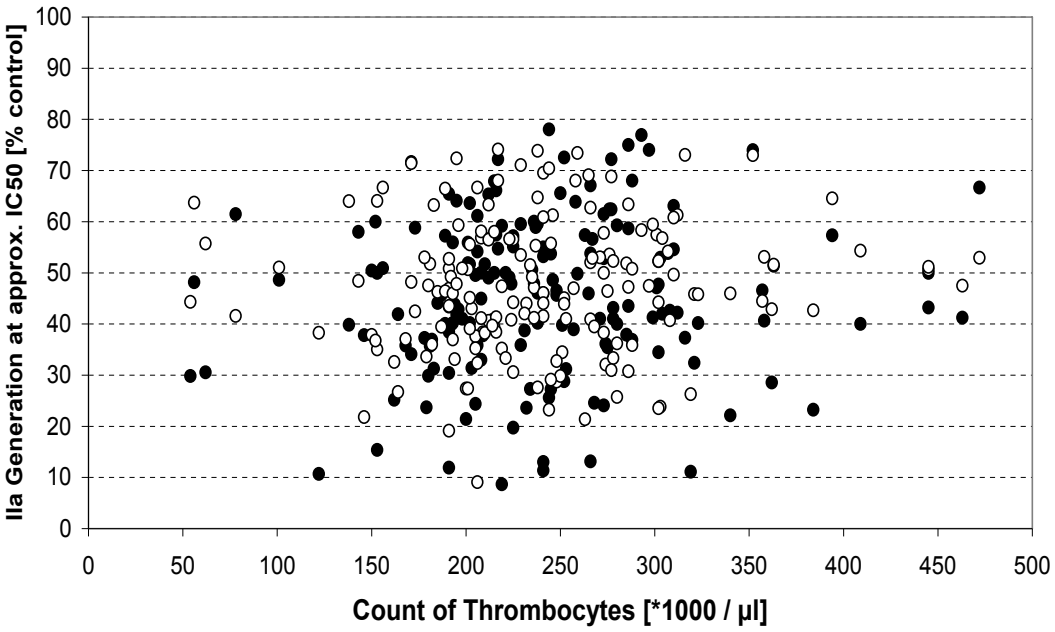

Figure 4.2.5. Count of thrombocytes and thrombin generation in patient plasmas after supplementation with heparin (●) or enoxaparin (o).

4.3. Loss of anticoagulant action by ultra-low dose heparin

4.3.1. No anticoagulant action of ultra-low dose heparin, pronounced anticoagulant action of ultra-low dose enoxaparin in normal plasmas

RECA-40 in citrated plasma (50 µl) samples of healthy donors (n=10) demonstrated that heparin lost anticoagulant action at ultra-low concentrations, whereas at the same concentration enoxaparin did act as an anticoagulant.

Citrated plasma (50 µl) of healthy donors (n=10; each donor has an individual symbol) in flat bottomed polystyrene microtiterplates were supplemented with gradually increasing concentrations of unfractionated heparin (0-10 mIU/ml) or with gradually increasing concentrations low-molecular-weight-heparin (LMWH) enoxaparin (0-10 mIU/ml).
The recalcified coagulation activity assay with a coagulation reaction time (CRT) of 40 min (37°C) (RECA-40) was performed and and ΔA/t was measured.
Thrombin generation in RECA-40 at supplementation with 0 IU/ml heparin concentration and at 0 IU/ml LMWH-enoxaparin are 325±250 mIU/ml = 100 % thrombin generation, 295±180 mIU/ml = 100 % thrombin generation respectively.
Figure 4.3.1a / Figure 4.3.1b demonstrate that unfractionated heparin or LMWH-enoxaparin reduced thrombin generations in RECA-40 more than 50% at final plasma concentration more than 1 mIU/ml in citrated plasma of healthy donors.
At 0.1 mIU/ml heparin does not reduce thrombin generation in RECA-40; instead it even increased the thrombin generation in RECA-40 by some 20 %, whereas 0.1 mIU/ml enoxaparin significantly decreased thrombin generation in RECA-40 by about 30 %.

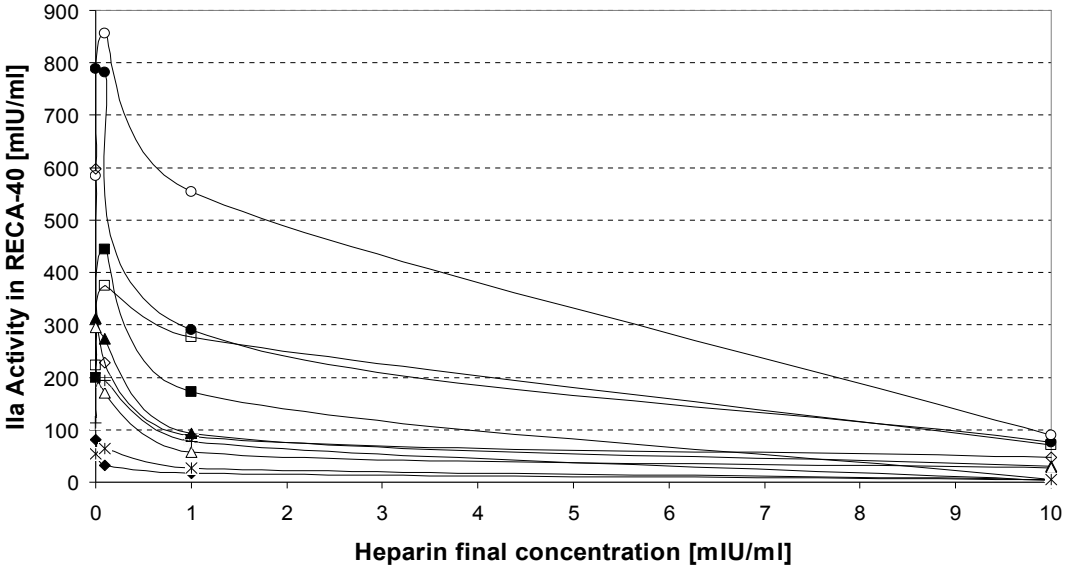

Figure 4.3.1a Inhibition of RECA-40 by increasing concentrations of unfractionated heparin (0-10 mIU/ml) in citrated plasma (50 µl) of healthy donors (n=10; each plasma has an individual symbol)**.**

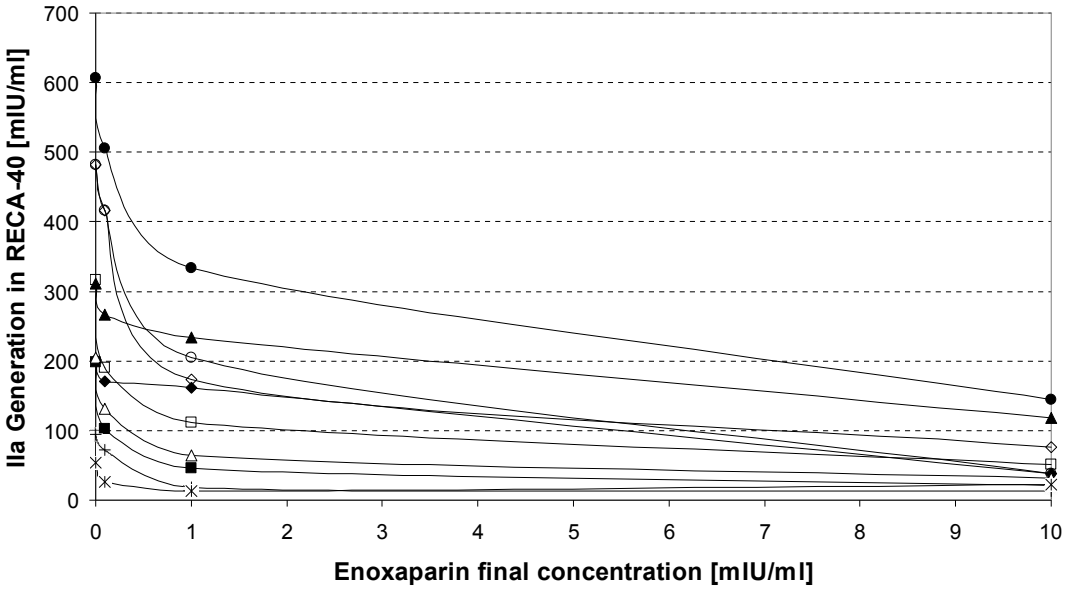

Figure 4.3.1b Inhibition of RECA-40 by increasing concentrations of LMWH-enoxaparin (0-10 mIU/ml) in citrated plasma (50 µl) of healthy donors (n=10; each plasma has an individual symbol).

Decreasing thrombin generation in RECA-40 at final concentrations of unfractionated heparin of 10 mIU/ml when compared to that of LMWH-enoxaparin at same final concentration demonstrated that heparin at this concentration was a stronger anticoagulant than enoxaparin (Figure 4.3.1c)

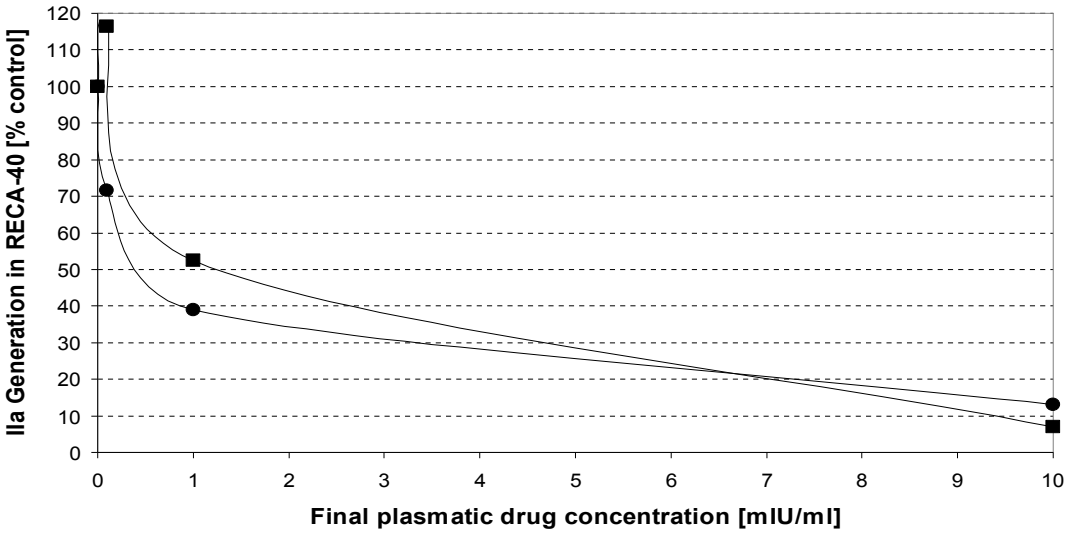

Figure 4.3.1c Inhibition of mean values in RECA-40 by increasing concentrations of heparin (■) (0-10 mIU/ml) or enoxaparin (●) (0-10 mIU/ml) in 50 µl normal citrated plasma.

4.3.2. Slight anticoagulant action of heparin, pronounced anticoagulant action of enoxaparin, both at ultra-low concentrations

RECA-40 in citrated plasma (50 µl) samples of patients (n=32) demonstrated that heparin slightly (10%) reduced thrombin generation at ultra-low concentrations, whereas at the same concentration enoxaparin significantly reduced it (55%).

Citrated plasma (50 µl) samples of patients (n=32; each patient has an individual symbol) without anticoagulation therapy and with normal hemostatic parameters (PT, APTT) were supplemented with gradually increasing concentrations of unfractionated heparin (0-10 mIU/ml) or with gradually increasing concentrations low-molecular-weight-heparin (LMWH) enoxaparin (0-10 mIU/ml).
The recalcified coagulation activity assay with a coagulation reaction time (CRT) of 40 min (37°C) (RECA-40) was performed and ΔA/t was measured.
Thrombin generation in RECA-40 at supplementation with 0 IU/ml heparin concentration and at 0 IU/ml LMWH-enoxaparin were 449±239 mIU/ml = 100 % thrombin generation, 413±227 mIU/ml = 100 % thrombin generation respectively.
Figure 4.3.2a / Figure 4.3.2b showed that 0.1 mIU/ml concentration of unfractionated heparin or 0.1 mIU/ml concentration of LMWH-enoxaparin decreased thrombin generation in RECA-40 by 10%, 55% respectively, whereas at 10 mIU/ml anticoagulant heparin was at least as efficient as enoxaparin.

The slight differences between Fig.4.3.1c and 4.3.2c might be due to
1. different storage time of medical student samples and patient samples (taken out of routine waste), which might result in different generation of procoagulant microparticles (Sinauridze EI. 2007),
2. different lots of polypropylene blood tubes with polypropylene tops, that might contain different amounts of pro-or anti-heparinic plastic material (Stief TW. 2008b),
3. different lots of polystyrene microtiter plates, which might also contain different amounts of pro- or anti-heparinic plastic material, especially if the plates are gamma-irradiated (Stief TW. 2006).

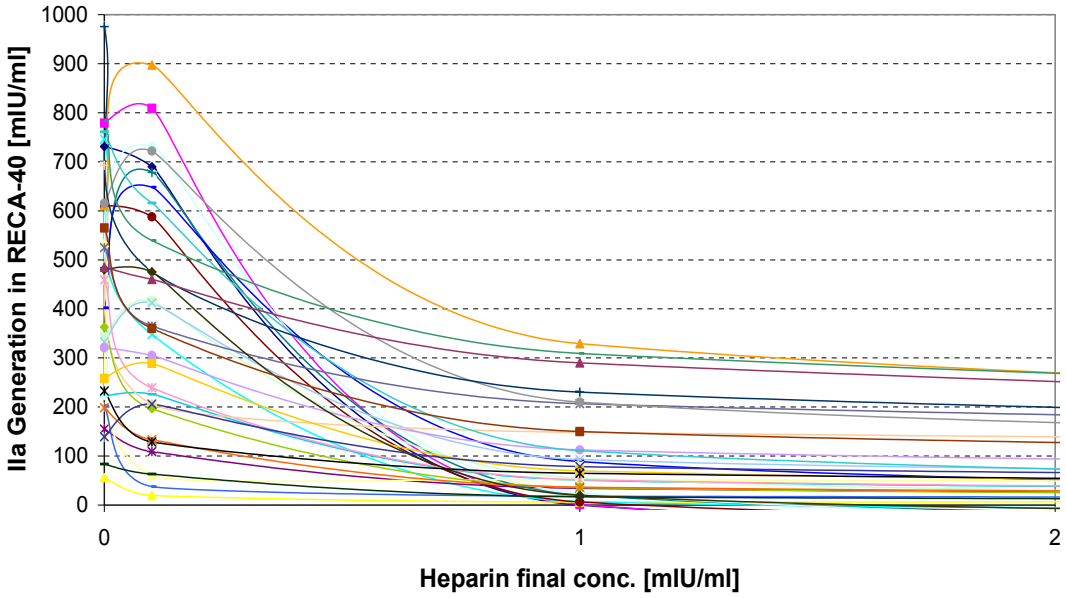

Figure 4.3.2a Inhibition of RECA-40 by increasing concentrations of unfractionated heparin (0-2 mIU/ml) in citrated patient plasmas (50 µl; n=32; each plasma has an individual symbol).

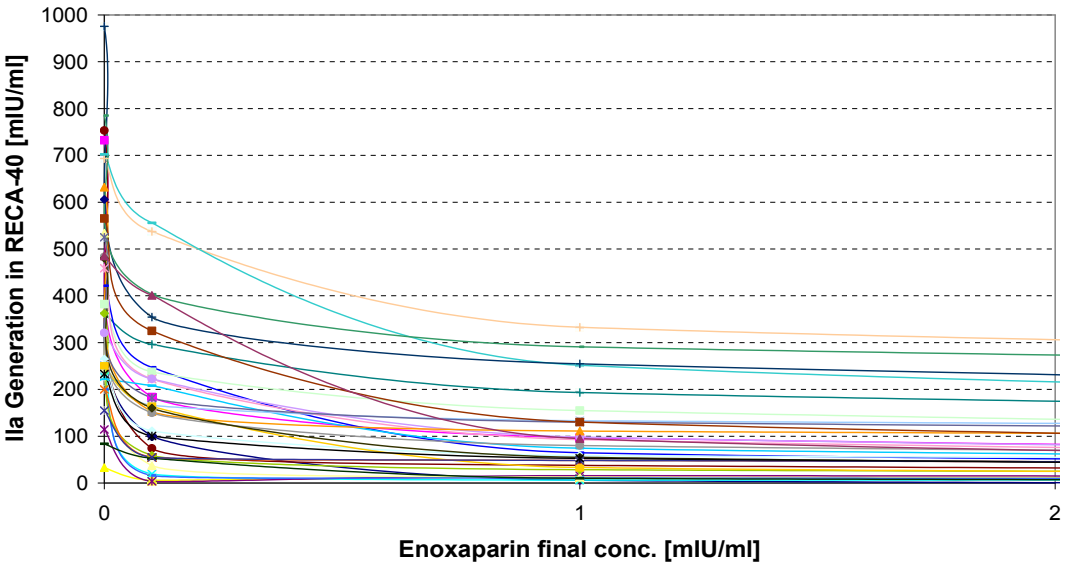

Figure 4.3.2b inhibition of RECA-40 by increasing concentrations of LMWH-enoxaparin (0-2 mIU/ml) in citrated patient plasmas (50 µl; n=32; each plasma has an individual symbol).

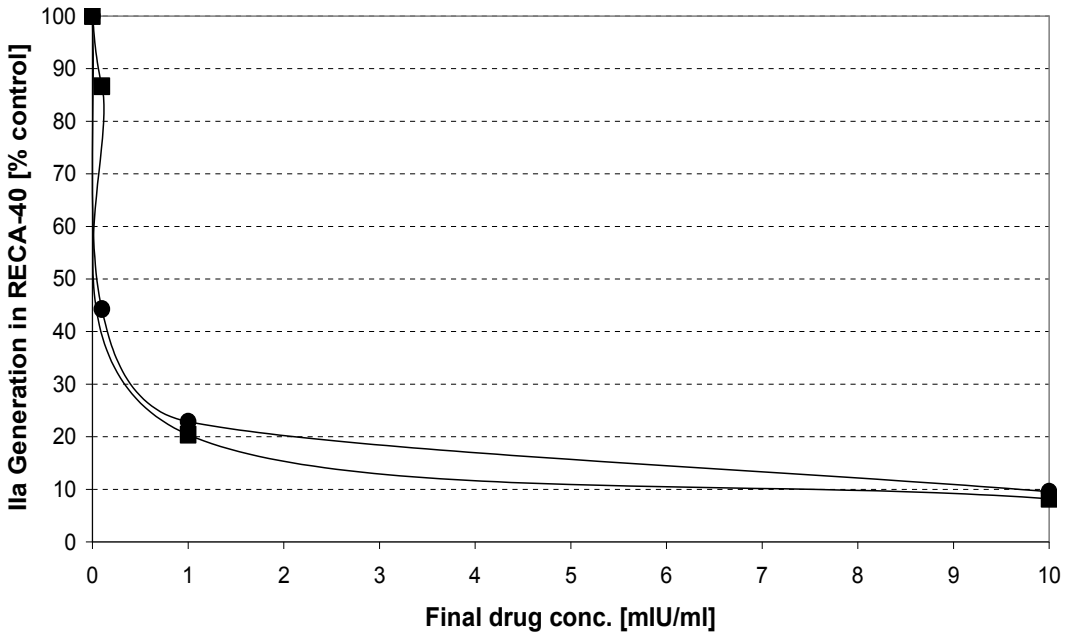

Figure 4.3.2c Inhibition of mean values in RECA-40 by increasing concentrations of heparin (■) (0-10 mIU/ml) or enoxaparin (●) (0-10 mIU/ml) in 50 µl citrated patient plasmas.

5. Discussion

The estimated total number of symptomatic VTE (Venous thromboembolism) events per annum within the six EU countries (France, Germany, Italy, Spain, Sweden, United Kingdom) was 466,000 cases of deep-vein thrombosis (DVT), 296,000 cases of pulmonary embolism (PE), and 370,000 VTE-related deaths. Of these deaths, an estimated 7% were diagnosed as being antemortem; 34% were sudden fatal PE, and 59% followed undiagnosed PE. (Cohen et al. 2007).

Acute coronary syndromes (ACSs) are traditionally divided into two separate categories based on the presenting electrocardiogram ECG: STMI (ST segment elevation >0.1 mV in all leads other than V2-V3; for leads V2-V3 ≥0.2 mV in men ≥40 years, ≥0.25 mV in men <40 years, or ≥0.15 mV in women) and NSTMI (ESC/ACCF/AHA/WHF committee). By patients with STMI a 1% absolute decrease in mortality was observed for each hour of decreasing time to treatment (Dalen JE, 1988). Ratio mortality in STMI-patients treated within 70 minutes from onset of symptoms when compared to that of those treated between 70 minutes and 3 hours was 1.2%, 8.7% respectively (Weaver; 1993). Most of patients with VTE or ACSs events initially have been either treated or prevented with the traditional anticoagulants subcutaneously or intravenously unfractionated heparin (UFH) or with low-molecular-weight-heparin (LMWHs) subcutaneously such as enoxaparin, a most common used anticoagulants for patients within hospitals.

The anticoagulant potency or activity of unfractionated heparin can be measured by the activated partial thromboplastin time (APTT), however the effects of LMWHs cannot be acceptably measured using the activated partial thromboplastin time (APTT). The aim of the present work was to measure of sensibility of individual plasma to unfractionated heparin (UFH) or low-molecular-weight-heparin (LMWH) enoxaparin using extrinsic coagulation activity assay (EXCA), a new chromogenic thrombin generation assaye for measurement of anticoagulation efficiency of UFH and LMWHs. Anticoagulants (UFH, LMWH) in this present research were added to citrated plasma directly out of a siliconized original vial to obtain the final plasma concentration without further plasma dilution steps, which imitates the continuous intravenously infusion or the subcutaneous administration of these anticoagulants.

The results of present study show that the slightest prophylactic anticoagulation, equivalent to about 40 % of normal EXCA, occurred at about 0.3 IU/ml (LMWH) enoxaparin concentration. The therapeutic anticoagulation, equivalent to about 10-20 % of normal EXCA, occurred at about 0.6 IU/ml - 1 IU/ml (LMWH) enoxaparin concentration, i.e. the dosing of LMWH must be individualized according to patient need (prophylactic or therapeutic) and anticoagulant response as reflected by the EXCA. Target EXCA for prophylactic intention is 20-40 % of normal thrombin generation whereas for therapeutic intention and prophylactic anticoagulation in high-risk patients (e.g. orthopaedic knee surgery) (Stief TW. 2007b) it is 10-20 % of normal thrombin generation.

The mean approximate 50 % inhibitory concentration (IC50) of unfractionated heparin or low-molecular-weight-heparin (LMWH) enoxaparin in citrated plasma samples of normal healthy donors was 0.08 IU/ml, 0.14 IU/ml in EXCA respectively.

0.01 IU/ml heparin or enoxaparin reduced normal thrombin generation in EXCA by about 10 % or 20 %, respectively. But 1 IU/ml (LMWH) enoxaparin concentration reduced the normal thrombin generation in EXCA to about 13 % of normal whereas at 1 IU/ml heparin the normal thrombin generation was completely inhibited.

This means that LMWH enoxaparin at 1 IU/ml concentration is a strongly therapeutic anticoagulant, unfractionated heparin at 0.01 IU/ml concentration is anticoagulantly too weak whereas at 1 IU/ml heparin there was almost no thrombin generation (toxic range).

At anticoagulant concentrations in the range about 10 mIU/ml enoxaparin is more effective than the potential contact activator heparin (Stief TW. 2007a).

Long-term anticoagulation with use nonphysiologic antiVit.K-anticoagulants (Phenprocoumon (Marcumar), Coumadin (Warfarin) and Acenocoumarol (Sintrom) derivatives) with target INR rang of 2-3 results in about 10 % of patients in severe bleeding or in thrombo-embolic complications (Olsson SB. 2005; Indredavik B. 2005; Cheung CM. 2005; Reynolds MW. 2004), i.e. an INR of 2-3 is in some patients too high and in others it is too low. This might be due to a lack of relationship between INR and the plasmatic serine proteases F2 and F10 (Sarode R. 2006). 25 % of patients treated with vitamin K epoxide reductase inhibitors develop intracranial hemorrhage by target INR rang less than 2.5 (Toyoda K. 2007; Rosand J. 2004; Lee SB. 2006). Therefore, it is suggested to replace vitamin k antagonists by LMWHs and to monitor

their efficiency via an ultra-specific thrombin generation assay, such as the EXCA (Stief TW. 2008c).

The present data of study support the concept that the LMWH dosage of each individual patient should be adjusted with an ultra-specific thrombin generation assay.

The present study demonstrates that unfractionated heparin at 0.1 mIU/ml concentration does not decrease thrombin generation in RECA-40; instead it even increased the thrombin generation in RECA-40 by about 20 % higher than that in unsupplemented plasma, whereas 0.1 mIU/ml enoxaparin significantly decreased thrombin generation in RECA-40 by about 30%- 60%.

Polynegative sulphated glycosaminoglycans (SGAG) sized 15000 to 18000 Daltons activate antithrombin-3 (AT-3) and prekallikrein when compared with low molecular weight heparin enoxaparin sized 4500 Daltons that only activate AT-3 without any activated influence on prekallikrein, a trigger intrinsic pathway of hemostasis (Stief TW. 2007a), i. e. heparin at a temperature higher than 23 °C and lower than 37°C has a dual function: anticoagulant (through activation AT-3) and procoagulant (through activation prekallikrein into kallikrein).

In ultra-low concentrations, the ability of UFH to activate prekallikrein is superior to its ability of AT-3 activation. Enoxaparin does not show this phenomenon, wherefore enoxaparin might be the clinically preferred drug to avoid these complications of heparin. It is suggested to monitor the correct dosage of enoxaparin for each individual patient (Fareed J. 1990; Dawes J. 1990) by innovative thrombin generation assays. In ultra-low concentrations, as e.g. occurring in the end of heparin infusion, heparin might dangerous especially for some susceptible patients. It is suggested to add enoxaparin to heparin at the end of heparin infusion with administration of enoxaparin, and to monitor the correct dosage of enoxaparin in each individual patient by specific thrombin generation assays.

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7.2. Verzeichnis der akademischen Lehrer

Meine akademischen Lehrer waren die Damen und Herren in Marburg:

Prof. Dr. med. Harald Renz

PD Dr. med. Thomas Stief

PD Dr. med. Nadia Al-Fakhri

Dr. med. Angelika Helwig-Rolig

Meine akademischen Lehrer waren die Damen und Herren in Syrien:

Prof. Dr. med. Mufid Jokhadar

Prof. Dr. med. Gasan Homos

Prof. Dr. med. Anas Sapah

Prof. Dr. med. Pahgat Tanus

Prof. Dr. med. Hiam Bshara

Prof. Dr. med. Murhaf Ajib

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7.4. Ehrenwörtliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel

„The Plasma Sensibility to Heparin and Enoxaparin“

im Institut für Laboratoriumsmedizin und Pathobiochemie und molekulare Diagnostik des Universitätsklinikums Marburg (Direktor: Prof. Dr. med. H. Renz) unter der Anleitung und Unterstützung von PD Dr. med. T. Stief ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation aufgeführten Hilfsmittel benutzt habe. Ich habe bisher an keinem in- oder ausländischem Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht, noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.

Teile der vorliegenden Arbeit wurden in folgenden Publikationen veröffentlicht:

-Thomas W. Stief, Salem Ajib and Harald Renz. **The sensibility of individual plasma to heparins.** ***Hemostasis Laboratory*** 2008; 1: 143-57.

-Thomas W. Stief, Salem Ajib and Harald Renz. **Loss of anticoagulant action by ultra-low-dose Heparin.** ***Hemostasis Laboratory*** 2008; 1: 135-42.

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