THROMBOPHILIA AND DIRECT THROMBIN INHIBITOR LEPIRUDIN - CLINICAL AND MONITORING ASPECTS

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ABBREVIATIONS

Anti-FIIa	anti-Factor IIa assay
APTT	activated partial thromboplastin time
BCS	Budd-Chiari syndrome
CHD	coronary heart disease
CI	confidence interval
CLI	critical limb ischemia
CPB	cardiopulmonary bypass
CRP	C-reactive protein
DM	diabetes mellitus
DTI	direct thrombin inhibitor
dTT	plasma diluted thrombin time
DVT	deep vein thrombosis
ECA	ecarin chromogenic assay
ESRD	end-stage renal disease
F	coagulation factor
HD	hemodialysis
HIT	heparin-induced thrombocytopenia
HR	hazard ratio
INR	international normalized ratio
IV	intravenous
LA	lupus anticoagulant
LMWH	low molecular-weight heparin
MI	myocardial infarction
PAD	peripheral arterial disease
PAR	protease-activated receptor
PCI	percutaneous coronary intervention
PiCT®	prothrombinase-induced clotting time
PTA	percutaneous transluminal angioplasty
SC	subcutaneous
TAFI	thrombin-activatable fibrinolysis inhibitor
TF	thrombophilia
TFPI	tissue factor pathway inhibitor
TT	thrombin time
UFH	unfractionated heparin
VKA	vitamin K antagonist

ABSTRACT

Background and objectives

Thrombophilia (TF) predisposes mainly to venous and to a lesser extent to arterial thrombosis at a young age. TF may be involved in severe thrombosis associated with antiphospholipid antibody syndrome, Budd-Chiari syndrome (BCS), or inoperable critical limb ischemia (CLI), and it may associate with inappropriate response to heparin treatment. Lepirudin, a potent direct thrombin inhibitor (DTI), is currently indicated for treatment of heparin-induced thrombocytopenia (HIT) and HIT-related thrombosis. Recombinant hirudins, like lepirudin, are at least as efficient as heparins, for instance in treatment of deep vein thrombosis (DVT) and acute coronary syndrome, but their wider use is limited by high costs and need for laboratory monitoring due to their narrow therapeutic range.

The first two studies of this thesis evaluated off-label use of lepirudin in severe thrombosis after traditional anticoagulation had failed and no other treatment options seemed feasible except for an invasive liver procedure or lower extremity amputation. Lepirudin-treated patients were relatively young and had a TF or thrombogenic background associated with heparin-resistant thrombosis either in hepatic veins (one patient having BCS) or in inoperable CLI (six patients). Furthermore, in the third study we assessed lepirudin dose responses by specific laboratory monitoring methods in repeated plasma samples obtained from additional five lepirudin-treated patients and in lepirudin-spiked plasma pools. Our aim was to overcome the limitations of activated partial thromboplastin time (APTT) and the clinically relevant confounding effects of warfarin and lupus anticoagulant (LA).

The impact of TF is not fully established in the thrombosis or stenosis of hemodialysis (HD) vascular access in patients with end-stage renal disease (ESRD). Thrombosis or stenosis of vascular access are common and expensive complications, and are associated with need for hospitalizations and with high morbidity among HD patients. The fourth study aimed to evaluate the prevalence of TF in patients with ESRD and its impact upon thrombosis- or stenosis-free survival of the vascular access.

Methods

Lepirudin treatments (n=12) were monitored clinically and with repeated APTT measurements, and daily hematocrit, platelet count, and prothrombin time (PT) assessments to ensure treatment safety. APTT was compared in vitro with prothrombinase-induced clotting time (PiCT®), ecarin chromogenic assay (ECA), anti-Factor IIa assay (Hirudin activity assay) (Anti-FIIa), and plasma diluted thrombin time (dTT) in lepirudin-spiked normal plasma, warfarin, and LA-containing plasma pools. Warfarin effects were assessed as an international normalized ratio (INR) of 1.5-3.9.

Altogether 237 ESRD patients were prospectively screened for TF and thrombogenic risk factors prior to HD access surgery in 2002-2004. Patient records were reviewed with a mean follow-up of 3.6 years (range 2.3-5.8) after access surgery to analyze the impact of TF and thrombogenic risk factors upon thrombosis- and stenosis-free access survival.

Results

Preliminary studies with lepirudin in thrombotic calamities appeared safe, and no bleeds occurred. Effective thrombin inhibition with lepirudin seemed to calm the thrombotic processes, and all patients gradually recovered. Further antithrombotic treatments after lepirudin were individually tailored during the follow-up (mean 4 years). Only one lower limb amputation was performed 3 years later.

The laboratory monitoring study revealed that assessment of lepirudin can be improved by use of chromogenic ECA or anti-FIIa assessments, as they correlated precisely (r=0.99) with each other and with spiked lepirudin in all plasma pools: normal, warfarin, and LA-containing plasma. Both PiCT® and APTT were limited by non-linear dose responses throughout the therapeutic range, showing the strong co-effect of warfarin, and impairment due to the presence of LA. In addition, dTT was disturbed neither by warfarin nor by LA, but dTT was less precise and more laborious than the chromogenic methods.

Of the 237 ESRD patients, TF was evident in 43 (18%), more often in males (23 vs. 9%, p=0.009). Known gene mutations of FV Leiden and FII G20210A occurred in 4%. Vascular access sufficiently matured in 226 (95%). The 1-year thrombosis- and stenosis-free access survival was 72%. Thrombosis or stenosis of vascular access occurred in 79 patients (35%) with matured access. These events occurred early, as 30% of them were encountered before initiation of HD. Female gender (hazards ratio, HR, 2.5; 95% confidence interval, CI, 1.6-3.9) and the presence of TF (HR 1.9, 95% CI 1.1-3.3) were independent risk factors in a Cox proportional hazards model for the shortened thrombosis- and stenosis-free survival. In a separate analysis of the 123 predialysis patients, independent risk factors were also female gender (HR 3.5, 95% CI 1.8-6.5) and presence of TF (HR 2.6, 95% CI 1.3-5.4), but in univariate analysis also elevated fibrinogen implied shorter thrombosis- and stenosis-free access survival than did normal fibrinogen (37 ± 3, CI 31-43 vs. 53 ± 5, CI 44-62 (months), p=0.04). Among 104 patients on regular preoperative dialysis, the only independent risk factor for the decreased event-free access survival was female gender (HR 2.0, 95% CI 1.0-3.8).

Conclusions

Lepirudin offers a potent and safe option for treatment of severe thrombosis, possibly also in indications other than HIT-related thrombosis. The chromogenic methods ECA and Anti-FIIa were the most precise methods for lepirudin monitoring. The global coagulation test APTT may be useful to perform in parallel in challenging clinical situations, as it remains a global routine test of the coagulation cascade. A multidisciplinary approach is required to ensure appropriate patient selection, interpretation of laboratory monitoring, and a balance between adequate treatment of thrombosis and avoidance of bleeding complications. TF seems to be a risk factor for thrombosis or stenosis of vascular access. Routine TF screening before access surgery is not warranted, but in association with repeated access complications or history of prior unprovoked thromboembolic events, it may aid in the choice of an individually enhanced antithrombotic and surveillance approach. In these studies, TF seemed to be associated with complicated thrombotic events, in venous (BCS), arterial (CLI), and vascular access systems. Multi-centered randomized trials are necessary to assess the possible role of DTIs in complicated thrombotic events as well as the optimal treatment for prevention of access complications.

LIST OF ORIGINAL PUBLICATIONS

I Salmela B, Nordin A, Vuoristo M, Mäkisalo H, Numminen K, Lassila R. Budd-Chiari syndrome in a young female with factor V Leiden mutation: Successful treatment with lepirudin, a direct thrombin inhibitor. Thromb Res 2008;121:769-772.

II Salmela B, Albäck A, Räike P, Lepäntalo M, Lassila R. A direct thrombin inhibitor, lepirudin, for thrombophilic patients with inoperable critical limb ischemia. Thromb Res 2009;123:719-723.

III Salmela B, Joutsi-Korhonen L, Saarela E, Lassila R. Comparison of monitoring methods for lepirudin: impact of warfarin and lupus anticoagulant. Thromb Res 2010; 125:538-44.

IV Salmela B, Hartman J, Peltonen S, Albäck A, Lassila R. Thrombophilia and Thrombogenic Risk Factors Impair Vascular Access Survival in End-Stage Renal Disease. Submitted.

INTRODUCTION

Most coagulation disorders are associated with thrombin generation. Moreover, despite their specific mechanisms, all anticoagulants, antiplatelet agents, and antithrombotics eventually lead to decreased generation of thrombin. Furthermore, the prevalence of TF may lead to increased thrombin generation and be associated in rare cases with the treatment resistance of thrombosis to conventional oral anticoagulants and heparins. Thrombosis occurring unprovoked, at an atypical location, at a young age, or with simultaneous arterial and venous thrombosis should raise the suspicion of underlying TF (Rosendaal 1999, de Moerloose et al. 2007, Lindhoff-Last et al. 2008). Furthermore, TF multiples the risk for thrombosis in females having a provoking factor for thrombosis, for instance use of oral contraceptives (Rosendaal 1999). Screening for TF under these circumstances, in both venous and arterial thrombosis and possibly also in vascular access thrombosis in HD patients, could help to estimate patients' risk for thrombosis and determine whether extended antithrombotic treatment is required.

A special unmet medical need exists among thrombophilic patients in whom traditional anticoagulation has failed and therapeutic surgical or radiological interventions are impossible. Successful experience in these rare, but disastrous cases can be gained only by preliminary reports, compatible with treatment of severe bleeding with recombinant activated coagulation factor VII (Hedner 2000). Thereafter, a multi-center prospective randomized trial can be designed, but still such trials may be impossible in certain rare, but life-threatening thrombotic events (Martinelli et al. 2008).

Direct inhibition of thrombin by lepirudin is an efficient and safe treatment in thromboprophylaxis and thrombosis, especially in patients having HIT-related thrombosis (Lubenow et al. 2005, Greinacher et al. 2008). Official indications for lepirudin are limited, and the treatment requires obligatory accurate laboratory monitoring to ensure the relatively narrow therapeutic ranges (Greinacher et al. 2008). The gold standard for monitoring lepirudin, activated partial thromboplastin time (APTT), has several limitations (Nowak 2001, Fenyvesi et al. 2002b, Gosselin et al. 2004a, Warkentin et al. 2008b). Establishment of another option for lepirudin monitoring has been needed to ensure appropriate lepirudin dosing and to overcome the important limitations of APTT, i.e. prothrombin deficiency or coexistence of lupus anticoagulant, which are encountered relatively often in patients having thrombosis.

Vascular access represents a life-line for patients on regular HD. Unfortunately, a failure of vascular access, mainly due to thrombosis or stenosis of the access, is common and is associated with high costs, need of hospitalizations, and mortality (Feldman et al. 1996). The impact of TF in access failure is not, however, established because many studies are relatively small; with no comprehensive TF screen included (Fodinger et al. 1996, LeSar et al. 1999, Manns et al. 1999, Valeri et al. 1999, Adler et al. 2001, Atac et al. 2002, Mallamaci et al. 2005). We aimed to further evaluate the potential importance of TF and of thrombogenic risk factors for vascular access survival, because, to our knowledge, only one representative study of 419 patients has been performed which included comprehensive screening for TF (Knoll et al. 2005).

REVIEW OF THE LITERATURE

Thrombin

Structure

Thrombin is the ultimate coagulation factor, as it is the final proteinase generated in the coagulation cascade. It is homologous to other serine proteinases like chymotrypsin, which have a serine residue in the active site cleft (Bode 2005); figure 1 demonstrates its structure. The active site is surrounded by hydrophobic 60-loop, hydrophilic γ–loop, and charged patches, i.e. exosites (Crawley et al. 2007). In addition, thrombin has a loop with a sodium ion-binding site; the binding of a sodium ion modulates allosterically the activity of thrombin (Page et al. 2005), the half-life of which, in plasma, is ca. 10-15 seconds, as it is inhibited with antithrombin and eventually cleared from the circulation (Crawley et al. 2007).

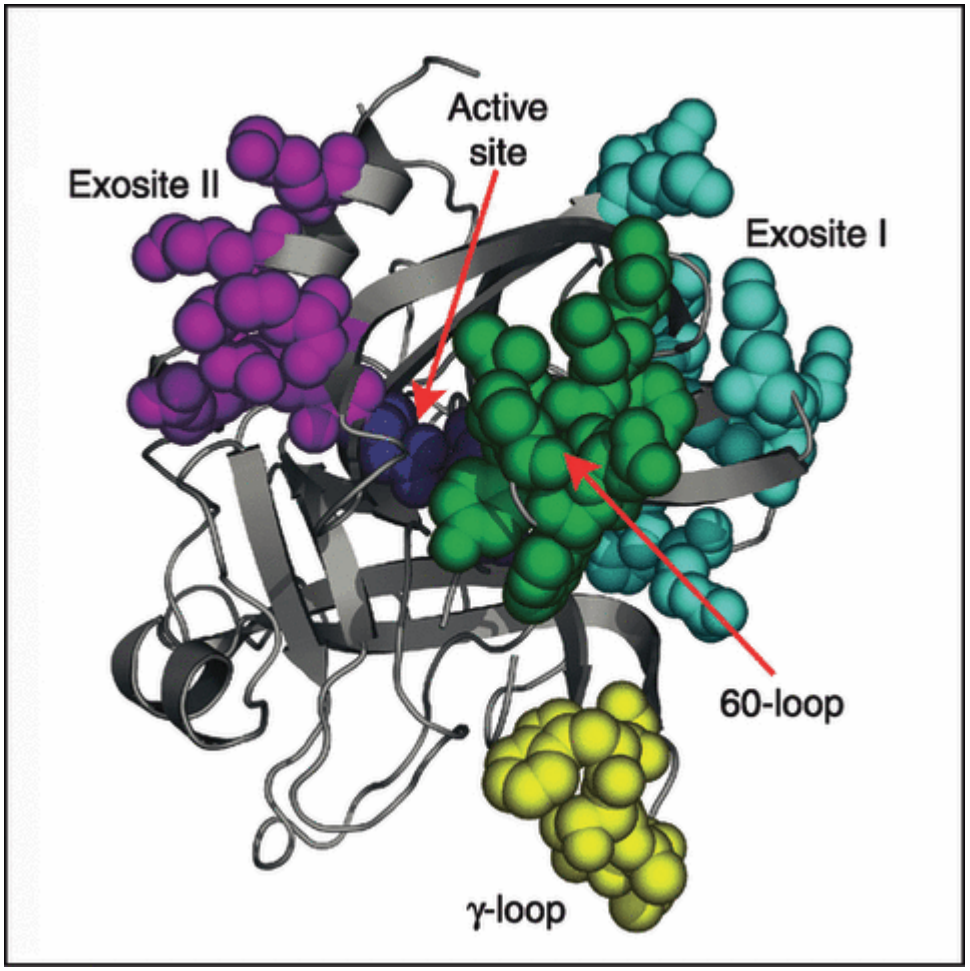

Figure 1. *Structure of thrombin: active site, exosites I and II, and γ- and 60-loops are shown. Crawley JT et al. J Thromb Haemost, 2007, reprinted with permission from the copyright holder.*

Exosites

Thrombin's two anion-binding exosites (I and II) interact with negatively charged regions on cofactors and substrates of thrombin (Crawley et al. 2007). The thrombin cofactors compete for their exosites, and their subsequent specific occupation determines the resulting coordinated reactions of thrombin (Huntington 2005, Lane et al. 2005). The substrate concentration and relative affinity of the exosite for the substrate determine the specificity and regulation of thrombin (Crawley et al. 2007). For example, thrombomodulin overcomes fibrin in binding to exosite I, and as a result thrombin is removed from the clot, because FXIII activation by fibrin ceases. This superiority of thrombomodulin assures that the clot formation ceases on the intact endothelium because thrombomodulin is present at a high concentration on the undamaged endothelium (Crawley et al. 2007). The thrombin-thrombomodulin complex activates the anticoagulant protein C pathway. In fact, the activation of protein C by the thrombin-thrombomodulin complex is greatly enhanced in comparison with what thrombin is capable of alone (Esmon 2006). Furthermore, exosite II hastens the reactions involving heparan sulfate on the endothelial cell surface and glycoprotein Ibα on the platelet surface, and the competition is solved based on the higher amount of cofactor available (Crawley et al. 2007). Similar to the competition for exosite I, the victory of heparan sulfate binding over glycoprotein Ibα to thrombin, is facilitated due to high amounts of heparan sulfate on the undamaged endothelial surface (Crawley et al. 2007). As a result, inhibition of thrombin by antithrombin is enhanced when it is bound to heparan sulfate.

Thrombin substrates

Thrombin has over 12 substrates and 5 cofactors (Crawley et al. 2007). Several hemostatic substrates of thrombin are summarized in Table 1. Efficient proteolysis occurs even without cofactors when thrombin cleaves fibrinogen, FV, and FVIII by multiple interaction sites (both exocites, sodium ion-binding loop, and the active site) (Crawley et al. 2007).

Table 1. *Various hemostatic substrates of thrombin (Crawley et al. 2007, Mann et al. 2003a, Bouma et al. 2004, Coughlin 2005, Mosesson 2005, Esmon 2006). Anticoagulant effects of thrombin in blue.*

Substrates of thrombin	Function of thrombin	Result
Fibrinogen	Catalysis of fibrinogen to fibrin	An extensive meshwork surrounds the aggregated platelets and seals the vascular injury
FV and VIII	Feedback activation of the factors	Function enhancement of FXa and IXa: generation of thrombin accelerates
FXIII	Proteolytic activation of XIII enhancement by fibrin	Deposited fibrin fibrils stabilize by catalyzing covalent cross-linking
Protease-activated receptors (PAR-1 & -4)	Binding and cleaving PAR molecules	Receptor-mediated (intracellular signaling) platelet activation occurs
Glycoprotein V	Proteolysis	This results in hyperresponsive platelets
Platelet bound FXI	Proteolytic activation	In the presence of activated platelets, accelerated cleavage of FXI
Protein C	Activation of thrombin-thrombomodulin complex	Down-regulates further generation of thrombin
TAFI	Activation of thrombin-thrombomodulin complex	Stabilizes fibrin clots
Antithrombin	Thrombin locks into an irreversible complex	Clears thrombin from circulation

F, coagulation factor; TAFI, thrombin-activatable fibrinolysis inhibitor

Regulation of thrombin

Thrombin plays a definitive role in all four stages of hemostasis: in initiation, amplification, propagation, and attenuation and also in fibrinolysis (Crawley et al. 2007). It is therefore understandable that excessive thrombin activity leads to thrombosis, whereas insufficient thrombin predisposes to bleeding. As a result, the coagulation system has to be tightly regulated by stoichiometric and dynamic inhibition systems (Mann et al. 2003b). Coagulation is regulated by the stoichiometric inhibitors, tissue factor pathway inhibitor (TFPI), and antithrombin, and dynamically by natural anticoagulants, the protein C and S system, (van't Veer et al. 1997a, van't Veer et al. 1997b, Esmon 2003, 2006). The TFPI mainly blocks the tissue factor-FVIIa-FXa product complex, which neutralizes the extrinsic FXase and eliminates catalyst generation of FXa and FIXa (Girard et al. 1989, Mann et al. 2003b).

When disruption of the endothelium exposes tissue factor to clotting factors in circulating blood, thrombin is generated mainly by the tissue factor pathway (Mann et al. 2003a). Thrombin generation occurs mainly on the activated platelet surface and the thrombin produced recruits platelets to the damaged surface and stabilizes the platelet thrombus. Thrombin, the most potent physiological platelet agonist, activates platelets by binding and cleaving protease-activated receptors (PARs) (Coughlin 2005). The activated platelets further accelerate thrombin generation by providing negatively charged phospholipids, i.e. on the procoagulant cell surface, to which coagulation complexes can attach (Monroe et al. 2002). In the initiation phase of coagulation, nanomolar amounts of thrombin are generated by FXa, whereas during the propagation phase, the most thrombin is formed by the prothrombinase complex (Mann et al. 2003b). This results in an insoluble fibrin clot by cleavage of soluble fibrinogen and an increase in its stability (Mann et al. 2003a). Thrombin amplifies its own generation by activating FVIII and FV (Mann et al. 2003a, Lane et al. 2005).

Normally, the intact endothelium inhibits thrombin formation through thrombomodulin as described above in the Exosites paragraph. As a result, protein C is activated by the thrombin-thrombomodulin complex, and the anticoagulant protein C downregulates thrombin generation by inactivating FVa and FVIIIa (Esmon 2003, 2006). Protein S serves as a cofactor for this reaction on membrane surfaces; this protein C anticoagulant pathway blocks the amplification of the coagulation system (Esmon 2006).

Multiple roles of thrombin in coagulation, inflammation, and cell proliferation

Thrombin can act both as a pro- and as an anticoagulant in hemostasis (Fig. 2). Procoagulant actions of thrombin include fibrin formation, platelet activation, and feedback activation for its generation (Weitz et al. 1990, Crawley et al. 2007). Fibrin-bound thrombin is protected from inactivation by fluid-phase inhibitors, and it retains local activity towards platelets, FV, and VIII, and converts fibrinogen to fibrin (Weitz et al. 1990, Kumar et al. 1994, 1995). Furthermore, anti-fibrinolytic properties of thrombin serve also to enhance coagulation because fibrin-bound thrombin activates Factor XIII, which crosslinks fibrin and α2-antiplasmin onto fibrin, thereby raising the resistance of the thrombus to fibrinolysis (Table 1) (Crawley et al. 2007). In addition, fibrin-bound thrombin activates procarboxypeptidase B, i.e. thrombin-activatable fibrinolysis inhibitor (TAFI); TAFI prevents binding of plasminogen and plasmin (Binette et al. 2007, Walker et al. 2007).

In addition to the coagulation pathway, thrombin plays a role in the inflammation pathway and activates PARs on monocytes, smooth muscle cells, and endothelial cells (Coughlin 2000). Both pro- and anti-inflammatory effects can also occur partly independently of the thrombin receptor, PAR-1 (Crawley et al. 2007) (Fig. 2). Additionally, protein C, activated by thrombin, has many anti-inflammatory activities linking coagulation and inflammation (Esmon 2006). Furthermore, inflammatory mediators modulate plasma coagulation and platelet reactivity, thus causing a potent prothrombotic state (Esmon 2003, 2006).

In addition to hemostasis and inflammatory mechanisms, thrombin can act as a potent mitogen, can stimulate leukocytes, endothelial cells, vascular smooth muscle cells, and fibroblasts (Mann 2003, Tsopanoglou et al. 2004, Snyder et al. 2008). Consequently, cell proliferation and release of cytokines, vasoactive agents, and growth factors occur (Esmon 2003, 2006, Crawley et al. 2007).

Given the central role of thrombin in coagulation and inflammation pathways, it provides an intriguing target for antithrombotic treatment in several diseases in which thrombin generation is essentially involved in the disease pathogenesis.

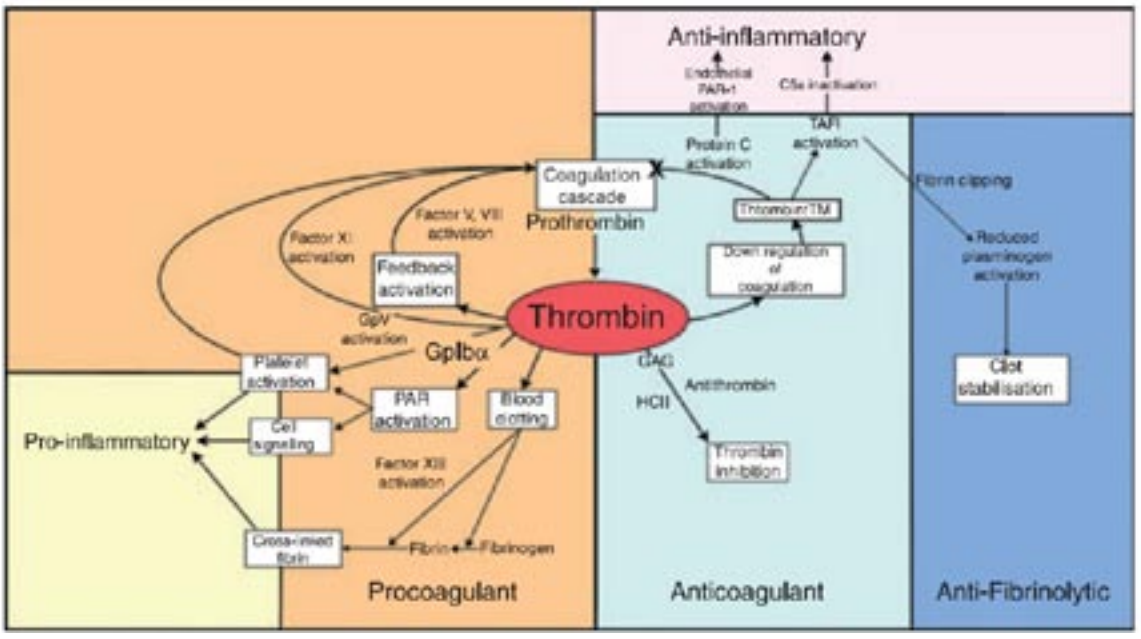

Figure 2. *Multiple roles of thrombin in proacoagulant, anticoagulant, antifibrinolytic, and pro- and anti-inflammatory pathways. Reprinted with permission from the copyright holder, Crawley JT et al. J Thromb Haemost, 2007.*

Direct thrombin inhibitors (DTIs)

Direct inhibitors of FIIa (thrombin) and FXa exert inhibition towards individual serine proteases. These direct inhibitors offer an advanced method for prophylaxis and treatment of thrombosis, as they target the end products of the coagulation cascade (Ansell 2007, Weitz 2007). In comparison, the effect of conventional warfarin, vitamin K antagonist (VKA), is mediated through decreased K vitamin- dependent synthesis of coagulation factors (FII, VII, IX, and X) (Ansell et al. 2004). Figure 3 demonstrates the main targets of VKA, direct inhibitors of thrombin and FXa, and heparins in the coagulation cascade. Direct thrombin inhibition seems an efficient and delicate option to treat thrombosis considering the central role of thrombin in hemostasis (Crawley et al. 2007).

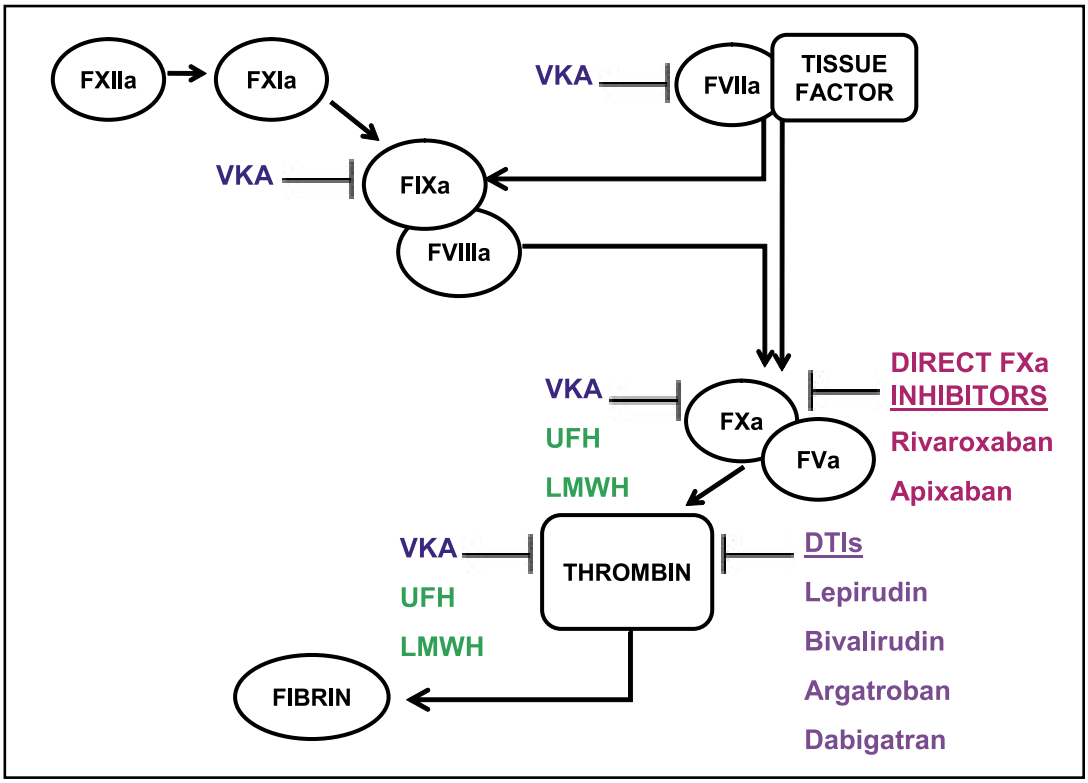

Figure 3. *Coagulation targets of direct thrombin inhibitors, heparins, inhibitors of coagulation factor Xa, and vitamin K antagonist (VKA). F, coagulation factor; DTIs, direct thrombin inhibitors; UHF, unfractionated heparin; LMWH, low molecular-weight heparin. Modified after Maegdefessel et al. Vascular health and Risk Management, 2010.*

Direct thrombin inhibitors (DTIs) are either univalent, interacting with the active (catalytic) site of thrombin, or bivalent, capable of recognizing also the fibrinogen-binding site of thrombin (Fig. 1) (Table 2). Figure 4 demonstrates different binding mechanisms of various DTIs to thrombin.

The recombinant hirudins lepirudin and desirudin were studied extensively in the 1990s, and bivalirudin, argatroban, and dabigatran are synthetic drugs, which seem to overcome some drawbacks of hirudins such as highly kidney-dependent metabolism and narrow therapeutic range (Di Nisio et al. 2005, Gajra et al. 2008, Greinacher et al. 2008, Liesenfield et al. 2006, Sanford et al. 2008, Warkentin et al. 2008a, van Ryn et al. 2010). Table 2 demonstrates the distinct properties of various DTIs. Affinity and specificity for thrombin is higher among bivalent than univalent DTIs (Table 2) (Gajra et al. 2008). The therapeutic window of thrombin inhibition is wider with the use of reversible rather than irreversible DTIs.

Great expectations have arisen as to whether old warfarin can be replaced with a new agent having less complicated administration and interactions than does warfarin (Ansell et al. 2004). Experiences gathered with recombinant hirudins and other previously available DTIs are of immense value for the development of new DTIs, for instance dabigatran, regarding both clinical use and monitoring aspects.

Table 2. *Comparative data on direct thrombin inhibitors (Di Nisio et al. 2005, Gajra et al. 2008, Greinacher 2008, Warkentin et al. 2008, Sanford 2008).*

Properties	Lepirudin (Refludan®) (Desirudin, Revasc®)	Bivalirudin (Angiox®)	Argatroban (Novastan®)	Ximelagatran WITHDRAWN	Dabigatran etexilate (Pradaxa®)
Origin	Recombinant hirudin	Synthetic oligopeptide	Synthetic, competetive DTI	Double prodrug	Double prodrug
Molecular mass (Da)	698 (696)	218	527	430	628
Bivalent binding	Yes	Yes	No	No	No
Affinity for human thrombin (Ki, nM)	0.0002	2.3	39 (5-39)	2	4.5
Binding	Irreversible (slowly reversible)	Transient	Reversible	Reversible	Reversible
Metabolism	Renal (90%)	Non-organ mechanism (proteolysis) & renal (20%)	Hepatobiliary (CYP3A4/ 5)	Renal (80%)	Renal (80%) & bile
Dosing	IV (SC)	IV	IV	Peroral	Peroral
Bioavailability	85-100% (SC)			20%	~ 6 %
T ½	60-80 min (IV) 120 min (SC)	25 min	~ 45 min	2-3 h	12-17 h
Peak level	1.3-2.5 h		1-3 h	2 h	2-4 h
Distribution volume (l/kg)	0.2-0.3	0-24	0.4± 0.15		60-70 l
Main indication	HIT: prophylaxis& treatment	PCI, CPB (also in HIT patients)	HIT prophylaxis and treatment	Withdrawn due to liver-related side-effects	Prophylaxis of DVT and of stroke in AF

IV, intravenous; SC, subcutaneous; PCI, percutaneous coronary intervention; CPB, cardiopulmonary bypass; DVT, deep vein thrombosis; AF, atrial fibrillation

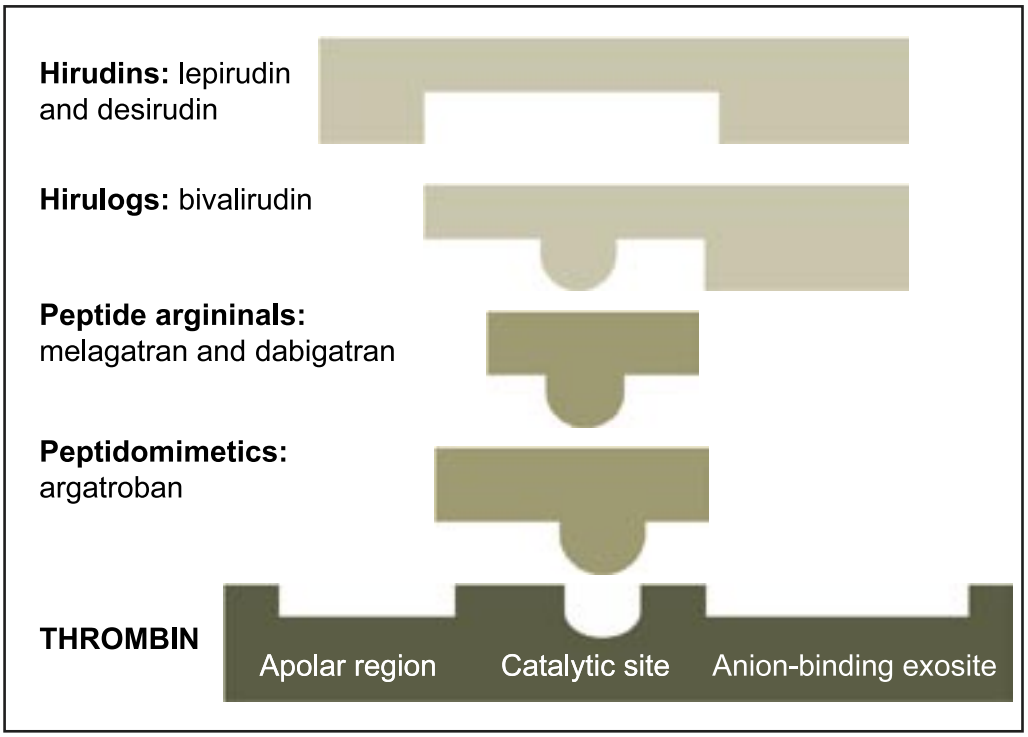

Figure 4. *Schematic view of thrombin reactions with different direct thrombin inhibitors. Modified after Markwardt F. Semin Thromb Haem, 2001.*

Lepirudin, a direct thrombin inhibitor

Structure and mechanism of action

The recombinant hirudin lepirudin (Refludan®, Pharmion, Cambridge, UK) is produced in yeast cells (Saccharomyces cerevisiae) by recombinant technology (Harvey et al. 1986). Hirudin was the first anticoagulant used in humans and was derived from leech (Hirudo medicinales) saliva in 1905 (Markwardt 2002). In comparison with natural hirudin, lepirudin has a leucine instead of isoleucine as the first N-terminal amino acid residue ([Leu1, Thr2]-63-desulfatohirudin). Lepirudin lacks sulfation of the tyrosine residue at position 63, resulting in an approximately 10-fold increase in the Ki value (Markwardt 2002) (Table 2). The other recombinant hirudin, desirudin, has a similar molecular weight, but has different N-termini: a-valine-valine instead of a leucine-threonine structure (Markwardt 2001, 2002).

Lepirudin forms an equimolar 1:1 noncovalent complex with thrombin; it inhibits thrombin activity by bivalent binding with high affinity (Table 2) to the active (catalytic) site as well as to the fibrinogen (anion-binding exocite) site of thrombin (Fig. 1) (Markwardt 2001, 2002). As a result, it inhibits clot-bound thrombin in addition to free thrombin independently of antithrombin in comparison with heparins (Weitz et al. 1990, 1998). In addition, inhibition of platelet activation occurs, but it demands higher concentrations than obtained during anticoagulation (Glusa et al. 1990, Harenberg et al. 2002).

Indications

The official indications for lepirudin are heparin-induced thrombocyopenia (HIT) and associated thromboembolic disease, and heparin allergy (Greinacher et al. 2008). The other recombinant hirudin, desirudin, is used only subcutaneously (SC) for thromboprophylaxis following joint replacement surgery (Greinacher et al. 2008). The usability of these recombinant hirudins is limited by their greater costs in comparison with heparin's.

HIT may occur between days 5 and 14 during and after unfractionated heparin (UHF) (Warkentin et al. 1995, 2008b, Fischer 2004). It occurs in 1 to 3% of the patients when UHF is used over 5 days, and it occurs more infrequently (1:10) when low molecular-weight heparin (LMWH) is used (Warkentin et al. 1995, 2008b, Fischer 2004). It is an autoimmune life-threatening disorder in which an anticoagulant switches to a procoagulant. In HIT, anti-platelet factor 4-heparin antibodies bind to specific receptors (FcγIIa) on platelets (Kelton et al. 1988). This leads to platelet activation and clumping, which causes thrombocytopenia, profound thrombin generation, and also venous or arterial thrombosis or both (Kelton et al. 1988, Greinacher 2009). Cessation of heparin is mandatory, but unfortunately thrombin generation continues, and the procoagulant state persists (Warkentin et al. 1995). Another nonheparin anticoagulant like danaparoid, lepirudin, argatroban, fondaparinux, or bivalirudin, but not warfarin, is required to prevent and treat further thromboembolic events (Warkentin et al. 2008b). Several studies have demonstrated the efficacy of lepirudin in HIT in terms of platelet recovery and effective anticoagulation, when it prevented limb amputations, new thromboembolic events, and mortality (Warkentin et al. 2004, 2008b, Lubenow et al. 2005).

The efficiency of recombinant hirudins is observable also in association with acute coronary syndrome, cardiac surgery, and deep vein thrombosis (Table 3) (Antman 1994, Eriksson et al. 1997, Potzsch et al. 1997, Schiele et al. 1997, Organization to assess strategies for ischemic syndromes (OASIS) investigators 1997, OASIS-2 investigators 1999, Koster et al. 2000, Direct Thrombin Inhibitor Trialists' Collaborative Group 2002, Di Nisio et al. 2005, Gajra et al. 2008, Greinacher et al. 2008). Overall, lepirudin was equal to or more efficient than was heparin (Table 3), but bleeding complications were more frequent than in controls in association with thrombolysis and also in HIT studies (Lubenow et al. 2005). The underlying reason for lepirudin-associated bleeding complications in HIT studies was that the lepirudin dosing was higher than recommended later (Warkentin et al. 2004, 2008b) and furthermore, the historical controls had received no antithrombotics (Lubenow et al. 2005). Successful off-label use of lepirudin has been reported in thrombotic calamities in mesenterial thrombosis (Puurunen et al. 2006) and in a pediatric patient (Sturm et al. 2007). Bivalirudin overcomes the drawbacks of lepirudin in cardiovascular procedures by having a shorter half-life and only transient thrombin inhibition in comparison with lepirudin's (Table 2), and as a result it can be used at higher and potentially more effective doses (Bates et al. 1998, Warkentin et al. 2008a).

Table 3. *Studies assessing efficacy of recombinant hirudins for treatment of HIT and related thrombosis, DVT, and acute coronary syndrome studies (including of acute transmural myocardial infarct, of non-Q wave myocardial infarct, thrombolysis, and of percutaneous coronary interventions).*

Study	Indication	Patients in r-hirudin group	Comparison	Efficacy	Bleeding risk	Comment
HAT-1, HAT-2, HAT-3 studies	HIT	399	Historical control groups	New thrombosis reduced (12 vs. 32%, p = 0.0008)	Increased, RR 2.31 (95% CI 0.94-5.71)	Placebo control group considered unethical, ACCP recommended lower dosing 2008
Lubenow 2002, post-marketing study	HIT	496	-	New thrombosis 5.2%	Major bleeds 5.4%	Considered effective and safe
Eriksson BI et al. 1996-1997(desirudin)	Prophylaxis of DVT in hip surgery	792* (2 studies)/ 802	UFH (2 studies)/ enoxaparin	More effective	Equal	Cost-effectiveness not studied
Schiele F et al. 1997	Treatment of DVT	117 (3 doses)	UFH	Equally effective	Equal, more bruising in lepirudin groups	Short duration (5 days)
Direct Thrombin Inhibitor Trialists' Collaborative Group (meta-analysis)	Acute coronary syndrome	14 563	UFH	Death and MI risk reduced OR 0.81 (95% CI 0.73-0.91)	More major bleeds, OR 1.28 (95% CI 1.06-1.55)	Studies combining thrombolysis partly explain the high bleeding risk

* Three doses in the first study 10, 15, and 20 mg, whereas in the two other studies the dose was 15 mg twice daily. HIT, heparin-induced thrombocytopenia; DVT, deep venous thrombosis; RR, risk ratio; UFH, unfractionated heparin; MI, myocardial infarct

Pharmacokinetics and pharmacodynamics

Lepirudin is administered parenterally, mainly intravenously. It is rapidly distributed extracellularly, as only 20% is found in the plasma, and after an intravenous (IV) bolus plasma half-life is only 8 to 12 minutes (Glusa et al. 1990, Fischer 2004). Normally, r-hirudin follows a 2-compartment model in plasma pharmacokinetics, and after an IV bolus of 0.01 to 0.5 mg/kg, terminal plasma elimination half-life is 0.8 to 1.7 hours, and a continuous infusion for 6 hours leads to a 1.1 to 2.0 hour half-life (Markwardt et al. 1988). It takes about 30 to 60 minutes of IV infusion to reach therapeutic levels. SC lepirudin is mainly used twice daily, and its peak concentration is reached within 2 to 3 hours (Schiele et al. 1997).

Renal clearance and degradation account for 90% of the systemic clearance of lepirudin, and impaired renal function may prolong its half-life even 50-fold (up to 150 hours) (Nowak et al. 1992, Vanholder et al. 1997, Fischer 2002). A very low dose (0.005-0.01 mg/kg/h) is obligatory in renal insufficiency where accumulation occurs, and the consequent risk for bleeding complications is high (Greinacher et al. 2008). In females, the renal clearance of lepirudin is 25% lower than in males, and the clearance is also 20% lower in those aged over 65 (Gajra et al. 2008).

Lepirudin in hemodialysis

Natural hirudin was the first anticoagulant used for hemodialysis (HD) in 1924 (Greinacher et al. 2008). Patients on HD may also develop HIT, when heparin is used in association with HD. Lepirudin has been used safely and effectively for HD, but the appropriate dosing requires meticulous monitoring, preferably bedside (Vanholder et al. 1994, 1997, Nowak et al. 1997). Lepirudin concentrations in plasma depend on HD clearance and the type of the hemodialyzer membrane, and also on residual kidney function, (Vanholder et al. 1994, 1997, Bucha et al. 1999, Fischer 2002, Benz et al. 2007).

Safety and tolerability

Tolerability of adequately dosed lepirudin is excellent, based on trials in thousands of patients (Table 3), but its long-term tolerability is not as well studied, because most treatment protocols were only short term (Fischer 2004). Major drawbacks of lepirudin are its narrow therapeutic window, i.e. balancing efficient thrombosis treatment and avoidance of bleeding complications and a highly kidney-dependent metabolism. Consequently, it is difficult to estimate the proper dosing regimen beforehand, especially in the elderly, in patients with other comorbidities, or in those critically ill. In these patients, creatinine may be initially normal, but transient renal impairment often occurs (Greinacher et al. 2008). As a result, bleeding risk factors for lepirudin use include impaired renal function, long duration of the treatment, underlying diseases, and co-medications, especially thrombolytics (Antman 1994, Lubenow et al. 2005).

Recently, a lower than earlier dosing schedule was recommended (avoidance of bolus of 0.2-0.4 mg/kg in most circumstances, and an infusion rate of 0.05-0.1 mg/kg/h vs. bolus of 0.4 mg/kg and an infusion rate of 0.15 mg/kg/h) (Warkentin et al. 2004, 2008b). The international guidelines were changed, because bleeding complications and a need for reducing infusion rates occurred at the doses used earlier (Tardy et al. 2006, Tschudi et al. 2009). Furthermore, a mean dose over 0.07 mg/kg/h was shown to be an independent risk factor for bleeding complications (Tardy et al. 2006). Avoidance of bolus dosage is recommended also to reduce risk for anaphylaxis and especially in renal impairment to minimize bleeding risk (Greinacher et al. 2008, Warkentin et al. 2008b). Moreover, in intensive care patients, lepirudin dosing is recommended to be reduced to 0.05 mg/kg/h, because risk for renal impairment and bleeding events is high (Gajra et al. 2008). In obese patients, dosing is also problematic and should not be increased beyond the dose calculated for 110 kg according to the manufacturer.

No antidote is commercially available, and in severe overdoses, only hemofiltration or hemodialysis with high flux membranes clears hirudin (Bucha et al. 1990). Allergic and anaphylactic events are infrequent with lepirudin, but they more likely occur under re-exposure based mainly on case reports (Greinacher et al. 2008).

Induction of antibodies

Approximately 40 to 70% of lepirudin-treated patients develop antilepirudin antibodies, which cross-react 100% with desirudin due their similar structure, and 40% cross-react with bivalirudin (Eichler et al. 2000b, 2004, Greinacher et al. 2008). Patients re-exposed to lepirudin seem to develop antibodies in up to 70% of the cases (Lubenow et al. 2005). Antibodies are

usually noted on day 4, and the maximum level occurs on days 8 to 9 (Eichler et al. 2000b). These antibodies may enhance the hirudin effect (activated partial thromboplastin time, APTT, prolongs) and necessitate dose reductions, because the lepirudin-immunoglobin complexes reduce renal clearance of lepirudin (Eichler et al. 2000a, Fischer et al. 2003). An inhibitory hirudin-neutralizing effect may occur in only 2 to 3% of patients (Eichler et al. 2000a, Fischer et al. 2003). The main clinical impact of the antibody formation is that it emphasizes the need for regular laboratory monitoring of lepirudin treatment, even after the steady state has been achieved around day 5 (Greinacher et al. 2008).

Differences between lepirudin and heparins

DTIs are not limited by the same pharmacokinetic or biophysical effects as is heparin, as they are not bound to plasma proteins in a similar manner (Glusa et al. 1990, Bates et al. 2000, Weitz et al. 2010). Lepirudin inhibits thrombin independently of any cofactor, whereas heparins need antithrombin as a cofactor (Weitz et al. 2010). In addition, DTIs are able to access and inactivate both free soluble thrombin and also fibrin-bound thrombin (Weitz et al. 1990, 1998). Inability of heparin to inactivate thrombin bound to fibrin is its major limitation, especially in association with dissolution of arterial thrombosis, or during the procoagulant state after thrombolysis (Owen et al. 1988, Meyer et al. 1998, Weitz et al. 1998). Lepirudin shows more sustained antithrombotic activity on the clot surface after its plasma clearance than does heparin, which explains why lepirudin is effective even when used for only a short time (Agnelli et al. 1992, Biemond et al. 1996). Furthermore, careful monitoring of UFH is required to establish an adequate anticoagulant effect, because heparin is neutralized by platelet factor 4, and additionally the anticoagulant response of heparin between patients varies (Bates et al. 2000).

Lepirudin is a potent antithrombotic which has certain advantages in comparison to heparin. Use of lepirudin as an antithrombotic agent does not promote any life-threatening procoagulant state as use of heparins do in the form of HIT-related thromboses. Furthermore, in contrast to heparins, lepirudin's capacity also to inhibit clot- and fibrin-bound thrombin results in downregulation of thrombin generation. The drawbacks of lepirudin, highly kidney-dependent metabolism and narrow therapeutic range, make precise laboratory monitoring of lepirudin elementary to ensure treatment safety and efficacy.

Laboratory monitoring of direct thrombin inhibitors – lepirudin in focus

Antithrombotic and anticoagulant therapies always imply a potential bleeding risk. Bleeding complications may result from too-high dosing, emergency need for surgery or other interventions, or a patient-related bleeding tendency in addition to thrombosis. It is mandatory to weigh the expected benefit of thromboprophylaxis or treatment over the estimated bleeding risk of the individual patient. Lepirudin may be a better DTI option than argatroban for instance in liver insufficiency, and vice versa in kidney insufficiency, based on their metabolism (Table 2). However, caution is still necessary when using lepirudin in association with liver failure, as coagulation factors produced by the liver may also be decreased and predispose to bleeding tendency.

The therapeutic range of lepirudin is narrow (ca. 0.6-1.0 µg/ml), as thrombin inhibition needs to be controlled at the platelet surface; complete inhibition would induce bleeding complications (Nowak 2001, Greinacher et al. 2008). Inhibition of thrombin, however, must be strong enough to allow the DTIs to compete with the high-affinity thrombin substrates in the circulation. Adequate lepirudin dosing depends on clinical situation, inflammation, thrombohemorrhagic balance, platelet counts, thrombin generation, renal clearance, co-medication (antiplatelet agents, bridging with anticoagulants), age, and the amount of thrombin generated (Fischer 2004).

Because DTIs disturb, even at low doses, thrombin-dependent coagulation assays, e.g. prothrombin time (PT) and thrombin time (TT), tests that are more specific are required. It is noteworthy that DTIs also disturb thrombin-dependent determinations of various coagulation factors, for instance fibrinogen.

Coagulation time-based assays

Prothrombin time (PT)

Blood clotting occurs in PT after addition of an extrinsic tissue factor source such as thromboplastin (Mann et al. 2003b). PT is a complex test which activates the coagulation cascade at a high stage (FVII, extrinsic system). PT shows linearity with DTI doses in an insufficient range and is therefore inappropriate for monitoring DTIs. Lepirudin raises international normalized ratio (INR) values from 1.1 to 1.5 at a concentration of 1.2 µg/ml (near the upper therapeutic limit), which underestimates the lepirudin effect. The DTI effect on INRs is dependent upon reagent sensitivity. The effects of bivalirudin and argatroban on PT are more pronounced than is lepirudin's (Gosselin et al. 2004a), and in fact argatroban produces nearly linear PT dose responses in its therapeutic range (Fenyvesi et al. 2002b).

Thrombin time (TT) and plasma diluted thrombin time (dTT)

TT, a highly sensitive method, directly assesses the activity of thrombin present in a plasma sample, and even low concentrations of DTIs prolong it to above its measurement range (140 s). TT as such is not suitable for DTI monitoring due to its poor linearity over a very small range (Love et al. 2007). For measurements in this small range, plasma needs to be diluted in several steps, and this preanalytical prerequisite may make measurements inaccurate (Nowak 2001). However, one study reported the suitability of plasma diluted thrombin time (dTT) measurements for assessing lepirudin effects (Love et al. 2007).

PT and TT are unsuitable for DTI monitoring as such, but they may function as indicators that the patient is on a DTI, because a strong effect occurs even though dose response estimations are precluded.

Activated partial thromboplastin time (APTT)

APTT is based on the presentation of a foreign surface to plasma (Mann et al. 2003b). This initiates coagulation and results in clotting, i.e. fibrin formation. Several coagulation factors XII, VIII, V, and II influence APTT (Fenyvesi et al. 2002b). In other words, the coagulation cascade

is activated at a high stage, and several confounding factors may affect drug monitoring, for instance, deficiencies in certain coagulation factors. Table 4 summarizes various limitations of APTT (Nowak 2001, Fenyvesi et al. 2002b, 2004, Gosselin et al. 2004a, Warkentin et al. 2008b).

Table 4. *Limitations of activated partial thromboplastin time (APTT) for monitoring of lepirudin therapy (Nowak 2001, Fenyvesi et al. 2002, Gosselin et al. 2004b, Mismetti et al. 2010).*

Limitations of APTT
Affected by many pre-analytic and analytic factors
Prolongs, for instance, in association with hepatic dysfunction, warfarin ad ministration, disseminated intravascular coagulation, antiphospholipid antibodies, hemodialysis
Reproducibility is limited
Dependence on test procedure and reagents (nearly 300 different ones exist)
Distinct sensitivity of the reagent towards the anticoagulant in use
Inter- and intra-individual variability
Non-linear dose response
Dose response curve flattens, starting from about concentration 0.5 µg/ml, and a plateau effect occurs
Underestimation of high doses and even overdoses

Intravenously administered lepirudin causes a maximum APTT response at 10 min after a bolus, and at 3 to 6 hours after continuous infusion for 6 hours. APTT should be measured every 4 hours until the steady state is reached, and 4 hours after every dose change, and at least once a day in the steady state, keeping in mind the possibility of antilepirudin antibodies and the possible need for dose reduction (Greinacher et al. 2008). It is important to note that in patients with impaired kidney function, APTT at 4 hours will be inaccurate, as it takes longer to achieve the steady state in these patients, and APTT responses are nonlinear (Gajra et al. 2008). Prolongation of APTT over 2.0-fold is not recommended in general, as beyond that point, the dose responses are nonlinear, so overdoses can be missed, and bleeding risks increase (Nowak 2001, Warkentin et al. 2008b).

Prothrombinase-induced clotting time (PiCT®)

PiCT® is a clotting assay which measures both activated FXa and FIIa (Schoni 2005, Calatzis et al. 2008). As PiCT® involves a low-stage activation of the coagulation cascade, the sensitivity of this method is better than with APTT, because variation in plasma factor levels and other disturbing variables is reduced. PiCT® is based on adding to platelet-poor plasma a reagent (1:1) that contains factor Xa, Russell viper venom factor V activator, and phospholipids. During incubation, the Russell factor V activator activates FV to FVa, and then the plasma is recalcified with CaCl2. Measured clotting time is proportional to the amount of thrombin or FXa inhibitor present in the sample. The result is given as clotting time, or it is also possible to provide the results in anti-FXa or anti-FIIa units, after calibration against the WHO standards (Schoni 2005, Calatzis et al. 2008).

PiCT® may overestimate lepirudin concentrations in coexistence with either recently administered UFH or warfarin (Fenyvesi et al. 2002a), although at a lower concentrations

warfarin has little effect (Guy et al. 2008) (Table 5). PiCT® responds non-homogeneously to different DTIs (lepirudin, argatroban, and melagatran), but a common feature was a steep initial section of each dose-response curve, whereafter a flat linear section appeared in each (Fenyvesi et al. 2002b).

Table 5. *Comparison of monitoring options for recombinant hirudin, lepirudin, and advantages and disadvanteges of the methods (Hafner et al. 1995, Nowak et al. 1997, Novak et al. 2001, Fenyvesi et al. 2002, Nowak 2003, Fenyvesi et al. 2003, Lange et al. 2005, Gray et al. 2005, Love et al. 2007, Calatzis et al. 2008, Guy et al. 2008).*

Method	Advantage	Disadvantages
APTT	Widely available, global coagulation test, "the gold standard"	Several (see Table 4)
PiCT®	Suitable for both FIIa and FXa inhibitors Independent of thrombin-mediated feedback reactions for FV activation Linearity is modestly improved compared with APTT, but inferior to ECT and ECA	Limited by the prevalence of LA, heparin, or high warfarin content High deficiency of FV, prothrombin or fibrinogen (< 25% of normal) produces prolonged clotting times
dTT	Availability	Needs several dilutions
ECT	Not effected by fibrinogen deficiency Available for blood or plasma testing, Insensitive to heparin and warfarin, Independent of calcium and phospholipids Available for bedside monitoring	Limited by prothrombin deficiency
ECA	Not effected by fibrinogen or prothrombin deficiency Insensitive to plasma heparin and warfarin Independent of calcium and phospholipids	Not widely available
Anti-FIIa	Presumably like ECA above	Interlaboratory variation, only for scientific use

APTT, activated partial thromboplastin time; PiCT®, prothrombinase-induced clotting time; F, coagulation factor; LA, lupus anticoagulant; dTT, plasma diluted thrombin time; ECT, ecarin clotting time; ECA, ecarin chromogenic assay; Anti-FIIa, Anti-Factor IIa assay (Hirudin activity assay)

Methods using chromogenic substrates

Determination of DTI quantities in plasma is possible by use of chromogenic substrate-based methods, like ecarin clotting time (ECT) and ecarin chromogenic assay (ECA), or anti-FIIa assay (Hirudin activity assay, Anti-FIIa).

Ecarin clotting time (ECT)

Ecarin, snake venom (Echis carinatus) metalloprotease, degrades prothrombin and activation products mainly to meizothrombin, and ECT can thus be seen as a "meizothrombin generation test" (Nowak 2001, 2003). A defined quantity of ecarin is added to hirudin containing citrated whole blood or plasma. Ecarin generates meizothrombin (a prothrombin-thrombin-like intermediate), which has proteolytic activity similar to that of thrombin (Nowak 2001, 2003). ECT and also ECA methods are based on specific activation of prothrombin to meizothrombin

(Fig. 5), which reduces interference with other coagulation factors in comparison with APTT and PT (Fenyvesi et al. 2002b, 2003, Gosselin et al. 2004b). The limitation of ECT is that a low prothrombin concentration indicates falsely high DTI levels in the sample (Nowak 2003). This limitation should disappear when the sample is diluted 1:1 with normal human plasma, as in dTT (Nowak 2003). Table 5 shows the advantages of ECT which make it suitable for monitoring of lepirudin, and possibly other DTIs, also during a changeover from other anticoagulants (Nowak 2001).

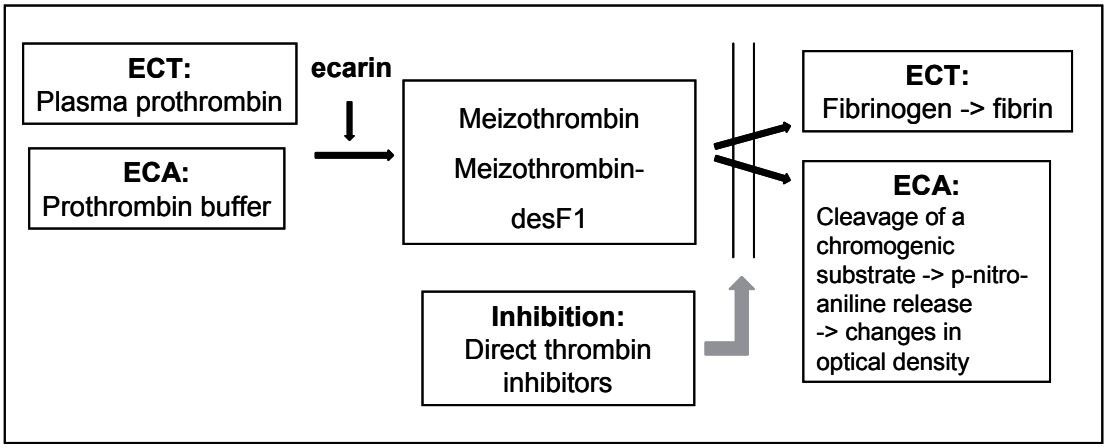

Figure 5. *Principles of ecarin clotting time (ECT) and ecarin chromogenic assay (ECA). Ecarin serves as the activator of prothrombin in these reactions. Meizothrombins are inhibited concentration- dependently by direct thrombin inhibitors like hirudins. Modified after Lange U et al. Pathophysiol Haemost Thromb, 2003.*

Ecarin chromogenic assay (ECA)

Deficiency of prothrombin is relatively common in association with warfarin use, in critically ill patients, and for instance in disseminated intravascular coagulation (DIC) (Greinacher et al. 2008). These circumstances indicate falsely high levels of lepirudin if assessed with APTT or ECT, if the samples are not adequately diluted for ECT measurements (Nowak 2003, Greinacher et al. 2008). ECA overcomes the limitation of ECT, as it contains an excess of prothrombin; as a result, ECA is independent of both prothrombin and fibrinogen levels in the sample (Lange et al. 2005). Figure 5 demonstrates the principle of ECA for DTI quantifications, and Table 5 summarizes the advantages of ECA in comparison with other monitoring methods.

Anti-FIIa assay

An anti-FIIa assay (Hirudin activity assay, Anti-FIIa) can also serve to quantify lepirudin activity in plasma: a method independent of antithrombin and fibrinogen levels (Hafner et al. 1995). Plasma sample and substrate are mixed, and the reaction is started with a thrombin reagent, which forms a 1:1 complex with lepirudin. Residual thrombin activity is determined by the increase in absorbance by kinetic testing (Hafner et al. 1995). Anti-FII has been compared with other methods in only one study, and it seemed to have more interlaboratory variation than for instance ECT (Gray et al. 2005), thus each laboratory needs to generate its own standard dose-response curve (Table 5).

Coagulation-based methods, APTT and PiCT®, have several limitations in laboratory monitoring of DTIs (Table 5), but on the other hand, these global methods may reflect coagulation defects and help to assess dose adjustments, whereas more precise and reproducible chromogenic methods register only DTI concentrations. Comparative studies of these methods are limited; no studies exist which compare all ECA, Anti-FIIa, PiCT®, and dTT with APTT, the gold standard for laboratory monitoring of lepirudin (Gray et al. 2005, Guy et al. 2008).

Thrombophilia and thrombogenic risk factors

Hereditary and acquired

Thrombosis is usually influenced by three main factors postulated first by Virchow: stasis of the blood, abnormalities in the vessel wall, and blood composition (Virchow R, Cellular Pathology. London, Churchill, 1860). Table 6 summarizes the hereditary and acquired hemostatic alterations which may cause abnormalities in the blood constituents (Poort et al. 1996, Martinelli et al. 1998, Dahm et al. 2003, Weitz et al. 2004). These abnormalities associate commonly with venous thromboembolism, as it is more often related to disturbances in plasma coagulation than is arterial thrombosis. In addition to abnormalities in the blood constituents, several important clinical factors predispose to thrombosis risk: smoking, obesity, aging, hypertension, use of oral contraceptives, hormonal replacement therapy, pregnancy, surgery, trauma, immobilization, acute diseases, infection and inflammation, cancer and its treatment, nephrotic syndrome, and immunological and complement activation (Rosendaal 1999, de Moerloose et al. 2001, 2007, Goldhaber 2010). All of these factors play an established role in venous thrombosis, whereas their role in arterial thrombosis is less evident except for smoking, hypertension, obesity related to metabolic syndrome, hormonal therapies, and aging (Holst et al. 2010). The prothrombotic state, determined by the activation markers of coagulation and fibrinolysis, seems to be influenced by both genetic and environmental factors (Ariens et al. 2002). The established thrombotic risks within a patient can be transient or continuous, and risks change during a lifetime in association with pregnancy, surgery, obesity, bleeding complications, and advanced age; and the net risk needs to be re-evaluated accordingly.

The pathogenesis of arterial disease (coronary artery disease, ischemic stroke, and peripheral arterial disease, PAD) is complex, involving inflammatory changes in the atherosclerotic vessel wall, various genetic and environmental factors, thrombogenesis, and regulation of vascular tone (Robbie 2001, Libby 2008, 2009, Rocha 2009). Arteriosclerosis is clinically associated with hypertension, diabetes, dyslipidemia, genetic factors, obesity, and smoking. Platelet interaction with the damaged vessel wall under high shear conditions plays the main role in arterial thrombosis in contrast to low shear conditions prevailing in association with venous thrombosis. However, also arterial thrombosis seems to be more common in patients with than without thrombophilia (TF), especially when thrombotic complications occur at a young age (Badimon et al. 1994, de Moerloose et al. 2007). The role of TF in arterial thrombosis is less prominent than in association with venous thrombosis, but at least antiphospholipid antibodies predispose to both entities: to arterial events including stroke, myocardial infarction (MI), and

Table 6. *Hereditary and acquired thrombophilia and thrombogenic risk factors and a simplified classification of their potential effect on risk for first venous thromboembolism (VTE) in comparison to individuals without a thrombophilic defect (Weitz et al. 2004, Poort et al. 1996, Martinelli et al. 1998, Rosendaal 1999, Dahm et al. 2003).*

Thrombophilia or thrombogenic factors	Estimated risk for a first VTE (Relative risks)
Hereditary	
Antithrombin deficiency	Strong (8-10)
Protein C deficiency	Strong (7-10)
Protein S deficiency	Strong (8-10)
Homozygous FV Leiden or Prothrombin mutation	Strong (> 30)
Double heterozygous FV Leiden and prothrombin G20210A mutation	Strong
FV Leiden mutation (heterozygous)	Moderate (3-7)
Prothrombin G20210A mutation (heterozygous)	Moderate (3)
Non-O ABO blood type	Moderate
Fibrinogen 10034T	Moderate
FXIII variant	Weak
XI variant	Weak (2)
Acquired (mainly)	
Lupus anticoagulant	Strong (11)
Anticardiolipin antibodies	Weak (all 1.6, only high titres 3.2)
Elevated FVIII (dose-dependent)	Moderate (2-11)
Elevated FIX	Weak (2-3)
Homocysteine	Weak (2.5)
Low tissue factor pathway inhibitor	Weak

F, coagulation factor

peripheral arterial disease (PAD) (Linnemann et al. 2008, Neville et al. 2009). Additionally, antiphospholipid antibody syndrome can be found in association with recurrent spontaneous abortions and intra-uterine fetal death (Empson et al. 2005). Elevated FVIII:c levels also show an association with MI and recurrent arterial events (Linnemann et al. 2008). The presence of FV Leiden and prothrombin G20210A mutations seems to play a role in young patients who have an idiopathic MI or stroke with only minor vascular lesions or in patients needing repeated revascularizations. In general, population studies in adults have shown only weak associations or results contradictory to these gene defects in arterial thrombosis (de Moerloose et al. 2007, Foy et al. 2009).

PAD severity correlates with fibrinogen and D-dimer levels (Lassila et al. 1993, Makin et al. 2002), and in young patients, hypercoagulability associates with PAD and risk for recurrent thrombosis after revascularization (Eldrup-Jorgensen et al. 1989). PAD often progresses to critical limb ischemia (CLI), which associates with high mortality and risk for lower extremity amputation (Lepäntalo et al. 1996, Dormandy et al. 1999a, 1999b, Aulivola et al. 2004). If

revascularization is impossible, other treatment options are of only limited benefit, although DTIs have led to promising results in treatment of CLI such as acute coronary syndrome (Allie et al. 2005, Shammas 2005).

Thrombosis represents a multicausal disease resulting from dynamic interaction of both genetic and acquired risk factors (Rosendaal 1999). It is often associated with accumulation of risk factors, which in turn shifts the regulated hemostatic mechanisms towards unregulated thrombosis. This phenomenon is often encountered by clinicians supporting the "two-hit theory." In fact, although in children thrombosis is rare, it is often encountered along with three to four risk factors (Rosendaal 1999, Rask et al. 2010). Furthermore, several risk factors potentiate one another. As an example, females with Factor V Leiden mutation who use oral contraceptives are estimated to be at much higher risk for thrombosis than do females having this mutation, but taking no oral contraceptives (risk ratio 36.9 vs. 5.7) (Rosendaal 1999).

Thrombophilia and vascular access for hemodialysis

Vascular access represents a lifeline in end-stage renal disease (ESRD) patients on HD. The maintenance of HD access is a major challenge in ESRD patients, as thrombosis and stenosis often result in access failure, which causes hospitalizations, high expanses, morbidity and mortality (Feldman et al. 1996). Furthermore, as high as 72% of the patients on HD for 2 years have been hospitalized due to access complications (Feldman et al. 1993). What is especially challenging is to balance the elevated risks of bleeding and of thrombotic complications among ESRD pattients with ongoing HD. No optimal therapy to prevent access thrombosis or stenosis has been established, despite extensive efforts using clopidogrel or dipyridamol plus aspirin or heparins (Kaufman 2000, 2003, Dember et al. 2008, Dixon et al. 2009), and recurrent thrombosis of vascular access is especially common in some patients, who often seem to have hypercoagulable states (LeSar et al. 1999, Mitsiev et al. 1999, O'Shea et al. 2003).

Three types of vascular access exist: a forearm fistula (Brescia-Cimino) connecting the cephalic vein with the radial artery, an expanded polytetrafluoroethylene bridge graft also connecting a vein and artery in the arm, and a central venous catheter (either temporary or permanent, i.e. tunnelled). The thrombosis rate of the arteriovenous fistula is about 10 to 35% annually and 30 to 65% of the polytetrafluoroethylene graft, however, the rate of thrombosis of the access is the highest in the central venous catheters, and as a result, their life span is reduced to 1 year (Suhocki et al. 1996). In Finland, according to the Finnish National Registry for Kidney Diseases (Finnish Registry for Kidney Diseases. Report 2008. Helsinki, Finland, 2009; available at: http://www.musili.fi/smtr/english), arteriovenous fistula (AVF) is the most commonly used access type in Finland (79%), in comparison with grafts (3%) and permanent (tunneled) catheters (16%). This high share for AVF in Finland coincides with the "Fistula First" campaign in the USA, as fistulas require fewer interventions, have fewer infections, lower costs, and longer patency than do grafts or catheters (Vascular Access Work Group 2006, Allon 2007). Unfortunately, inadequate maturation (Dixon 2006) and early thrombosis and stenosis limit fistula use for HD.

HD vascular access unites venous and arterial systems in unique high-shear vascular

circumstances, in which both venous and arterial risk factors may predispose to access thrombosis and stenosis. The impact of TF on access thrombosis and stenosis is unclear, as most of the studies have been small, including no prospective comprehensive TF screening (Fodinger et al. 1996, LeSar et al. 1999, Manns et al. 1999, Valeri et al. 1999, Atac et al. 2002, Mallamaci et al. 2005). One study of 419 patients found TF (FV Leiden or prothrombin mutations, FXIII genotype, methylenetetrahydrofolate, LA, anticardiolipin antibody, homocysteine, and lipoprotein(a) concentrations) in 43% of ESRD patients on regular HD and the presence of TF elevated risk for access thrombosis (odds ratio 1.91; 95% CI 1.2-3.0) (Knoll et al. 2005).

Relevance of thrombophilia screening

One key objective in screening for TF is to detect strong risk factors like antithrombin deficiency, especially in association with unprovoked thrombosis (Foy et al. 2009). However, routine screening of patients with provoked venous thrombosis or arterial thrombosis with established arteriosclerotic risk factors is not recommended (de Moerloose et al. 2007, Lindhoff-Last et al. 2008, Foy et al. 2009). Indeed, a consensus prevails that selective screening based on personal or family history of thromboembolism is cost-effective in contrast to unselective screening (de Moerloose et al. 2001, Wu et al. 2005, 2006).

The role of TF has been questioned in many clinical entities, and we are likely still far from its establishment. Additionally, in certain rare thromboses, adequately powered randomized controlled trials seem impossible, as patients are distributed widely between single centers (Martinelli et al. 2008). Budd-Chiari syndrome, for instance, is a relatively rare disorder with hepatic venous obstruction, mainly caused by thrombotic etiologies (Janssen et al. 2000, Valla 2002, Plessier et al. 2006). It is important to note that the risk for thrombosis caused by TF varies, some defects having stronger impact than others (Table 5). For instance, estimated relative risk for first venous thromboembolism is 8 to 10 in association with antithrombin deficiency (considered a strong risk factor) whereas it is 3 in association with heterozygous prothrombin 20210A mutation (a moderate risk factor) (Poort et al. 1996, Martinelli et al. 1998, Weitz et al. 2004). TF penetrance and risk for thrombosis varies among families (Rosendaal 1999). The sum effect of TF also depends on other acquired or inherited thrombotic factors simultaneously present (Rosendaal 1999). Moreover, risk for recurrence in venous thromboembolism differs in association with various forms of TF, ranging from 1.4 to 11, with elevated FVIII:C and antiphospholipid antibodies representing the strongest risk factors for recurrence (Weitz et al. 2004).

An additive effect of TF on risk for thrombosis is evident, the relative risk ratio for venous thromboembolism ranging from 2 to 11 depending on the severity of the TF defect; this means that TF should always be noted in association with other risk factors like pregnancy, surgery, advancing age, and cancer (Weitz et al. 2004). Such patients are definitely more liable to experience a thrombotic event than are patients without TF, and therefore require tailored and extended thromboprophylaxis. TF screening results should influence aspects of antithrombotic treatment such as prophylaxis, its duration, risk of recurrence, medical counseling, and patient education (i.e. prevention or hormone replacement therapy options for affected females) (Wu et al. 2005, 2006).

AIMS OF THE STUDY

Severe inherited or acquired TF and thrombogenic risk factors potentiate the risk for venous and arterial thrombosis at a young age. The impact of TF in inoperable CLI and in HD vascular access failure is not established. Hypothetically, the presence of TF may cause uncontrolled thrombin generation and lead to an impaired response to traditional anticoagulation. This study aimed at describing the clinically complicated young patients who have TF and severe thrombosis. Their thromboses did not respond to heparin and were treated with a DTI, lepirudin, which inhibits both clot- and fibrin-bound thrombin. Various laboratory monitoring methods were compared for lepirudin treatment. Additionally, TF and thrombogenic risk factors were evaluated among ESRD patients in relation to thrombosis or stenosis of vascular access.

The specific aims of the studies were:

1) to describe the efficacy and safety of lepirudin in severe thrombosis, i.e. in hepatic venous thrombosis (Budd-Chiari syndrome, BCS) and in inoperable CLI in patients with TF or thrombogenic risk factors.

2) to compare five different specific laboratory monitoring methods for lepirudin and to analyze the impact of common confounding factors, warfarin or lupus anticoagulant, upon these methods. This aim is compatible with improving the safety and efficacy of lepirudin administration in clinical practice.

3) to assess the prevalence of and impact of TF and coagulation disorders on thrombosis- and stenosis-free access survival in ESRD patients. Both arterial and venous risk factors may uniquely contribute to vascular access occlusion.

MATERIALS AND METHODS

Subjects

Patients with TF and heparin-resistant thrombosis were treated with lepirudin, one for hepatic venous thrombosis (BCS) (I), and six for arterial thrombosis with CLI (II). For evaluation and assessment of the lepirudin treatment results in the CLI patients, we analyzed the data of patients aged ≤55 (21 patients) among all 426 patients who underwent a major lower extremity amputation during the same follow-up period (II). In the third study, repeated blood samples (34) were collected from an additional five consecutive patients on lepirudin therapy and later reanalyzed to assess the optimal laboratory monitoring method for lepirudin (III). TF and thrombogenic risk factors were routinely and prospectively screened during 2002-2004 before HD access surgery. A total of 237 consecutive ESRD patients were included in our study that assessed the impact of TF on thrombosis- and stenosis-free access survival. (IV). In addition, we measured the dynamic markers of coagulation in 10 consecutive patients before and after HD (unpublished data).

The preliminary studies (I, II) followed the Institutional Guidelines of Good Clinical Practice. A gastroenterologist (I), liver transplantation (I) or vascular surgeons (II), a radiologist, and consultants in coagulation disorders jointly selected the patients for lepirudin treatment, when other treatment options (surgery, radiological interventions or antithrombotic therapy) were unsuitable or had failed. The patients were informed of treatment-related bleeding risks and their monitoring, and possible clinical benefits of the off-label lepirudin use. The other studies (III, IV) were approved by the hospital's Institutional Review Board and Ethics Committee.

Data collection

Data on clinical characteristics and endpoints came from medical records. The patients were followed on average for 4 years (I), 4.5 years (II), and 3.6 years (IV).

Blood samples: collection and processing

Blood samples were collected by antecubital venipuncture into vacuum tubes containing 3.2% sodium citrate (109 mM). Thereafter, plasma was prepared by centrifugation (2,000 × g, 10 min), and only the upper or middle third of the plasma was collected. After routine laboratory measurements, the remaining plasma was stored in aliquots at -70°C (III).

Thrombophilia screening (I-IV)

Both inherited and acquired disorders were included in the routine assessments of TF: resistance to activated protein C (APC) and associated factor V (R506Q) Leiden mutation, prothrombin G20210A mutation, activity of protein C, antithrombin III activity, free antigen of protein S, lupus anticoagulant with cardiolipin IgG and β2-glycoprotein I IgG antibodies, and homocysteine. Prothrombin time (PT), thrombin time (TT), coagulation activity of factors VIII

and V, fibrinogen, and D-dimer were also included in the analysis as dynamic measurements of coagulation (I, II, IV).

Automated coagulation analyses were performed with the Behring Coagulation System® -XP analyzer (Siemens) and the measurement of cardiolipin and Beta-2-glycoprotein I IgG antibodies with the Evolis- analyzer (ELISA microplate system, Bio-Rad). D-dimer and homocysteine were analyzed with Modular Analyzer (Roche Diagnostics).

Definition of thrombophilia and thrombogenic risk factors (I-IV)

Thrombophilia was defined as the presence of either homo- or heterozygous factor V Leiden or prothrombin mutation and positive antiphospholipid antibodies (aPL) (lupus anticoagulant, cardiolipin or β2-glycoprotein IgG antibodies). Additionally, low antithrombin activity (under 60%), or decreased activity of natural anticoagulants in patients without warfarin were noted in the lowest 5th percentile (i.e. protein C below 74% and protein S antigen below 60% in males). The cut-off values for other thrombogenic risk factors were: the 75th quartiles (clearly above the reference ranges) in FVIII:C (>206%), fibrinogen (>5.9 g/l), D-dimer (≥2.0 mg/l), and homocysteine (in ESRD patients >35 μmol/l), and the lowest quartile of thrombin time (≤16 s). The lowest 10th percentile of antithrombin (<77%) was registered as a thrombogenic risk factor.

Plasma spiking with lepirudin (III)

Three types of pooled plasma were spiked to achieve a wide concentration range of lepirudin (0–4.0 μg/ml):

1) Normal control plasma (Standard Human Plasma, Siemens Healthcare Diagnostics, Marburg, Germany) containing coagulation factors in the standard range.

2) Warfarin plasmas (Coumadin® plasma, CliniSys Associates Ltd, Atlanta, GA, USA) at three INR levels: 1.5, 2.5, and 3.9.

3) Three LA-positive plasma pools were combined from 8 to 21 patient samples screening positive or strongly positive in two LA tests. In the three LA-positive plasma pools (LA1–LA3), levels of cardiolipin and β2-glycoprotein I antibodies (normal for both <15 U/ml) were, respectively 70 and > 100 U/ml (LA1), 40 and 30 U/ml (LA2), and 30 and 50 U/ml (LA3).

Lepirudin therapy and laboratory monitoring (I-III)

APTT served for laboratory monitoring of lepirudin treatments in all 12 patients (I-III) as recommended by the manufacturer and from clinical guidelines (Warkentin et al. 2004). Furthermore, other hemostatic markers were followed to minimize bleeding risks. The objectives were to maintain the following criteria: hematocrit above 0.3 (normal 0.35-0.46 for females, 0.39-0.5 for males), prothrombin time above 70% (normal 70-130%), and platelet

count above 100x109/l (normal 150-360 x109/l). Blood products and vitamin K (to ensure sufficient synthesis of vitamin K-dependent coagulation factors) were to be administered, if needed to maintain these values.
Additionally, we developed our own diluted plasma thrombin time (dTT) method, in which the plasma samples were diluted with normal plasma at 1:8 and 1:16, so that TT became measurable (i.e. <140 s) (III). When assessing the optimal monitoring methods for lepirudin (III), we tested specific direct prothrombin activation methods of the ecarin chromogenic Assay (ECA -H, HaemoSys®, JenAffin GmbH, Jena, Germany) and prothrombinase-induced clotting time (PiCT®, Pefakit®, Pentapharm, Basel, Switzerland), and another quantitative method in addition to ECA: the chromogenic anti-FIIa Assay (Anti-FIIa, Siemens). All analyses were performed with a BCS® XP coagulation analyzer (Siemens).

Definition of vascular access thrombosis- and stenosis-free survival (IV)

The assessment of thrombosis- and stenosis-free survival of vascular access was defined from the creation of an arteriovenous fistula or graft. Access angiography was performed for any suspicion of malfunction or when duplex scanning revealed inaccurate volume flow or focal increase in peak flow velocity, even without clinical signs of insufficient dialysis. During HD, access was followed clinically and by measurements of venous pressure, blood flow, and recirculation. Eleven patients whose access failed to mature within 8 weeks were excluded from further analysis, if the reason for insufficient maturation was unknown or was other than thrombosis or stenosis of vascular access. Functional (unassisted) patency of the vascular access was determined from the initiation of the first successful HD, i.e. no interventions were needed for maintaining the access patency.

To assess any possible effects of HD on the coagulation markers, the blood samples of 10 consecutive patients were analyzed before and after HD. Both antithrombin and FVIII levels were relatively unchanged, whereas variability was evident in both fibrinogen and D-dimer levels (Fig. 6) (unpublished data).

Statistical analyses

Statistical analyses were performed with PASW Statistics 18 software (SPSS Inc., Chicago, IL, USA). The non-parametric Wilcoxon signed-rank test served for repeated measurements of ankle-brachial pressure index and toe blood pressure (TBP) in patients having inoperable CLI (II) and in analysis of coagulation markers before and after HD (IV, unpublished data).
In laboratory monitoring of lepirudin (III), either intraclass correlation (ICC; One-Way Random model, with 95% confidence intervals) or Spearman's correlation coefficients (r, 2-tailed significance) were determined for each method and the respective lepirudin concentration.
Factors having p-values of ≤0.2 established in correlation analysis by Chi square testing were included in univariate Kaplan Meier and multivariate Cox's regression analysis of thrombosis- and stenosis-free access survival and functional patency (IV). Breslow's method (thrombosis- and stenosis-free survival) and Log rank testing (functional patency) served for significance analysis (IV). Significance was set at $p < 0.05$ in all studies.

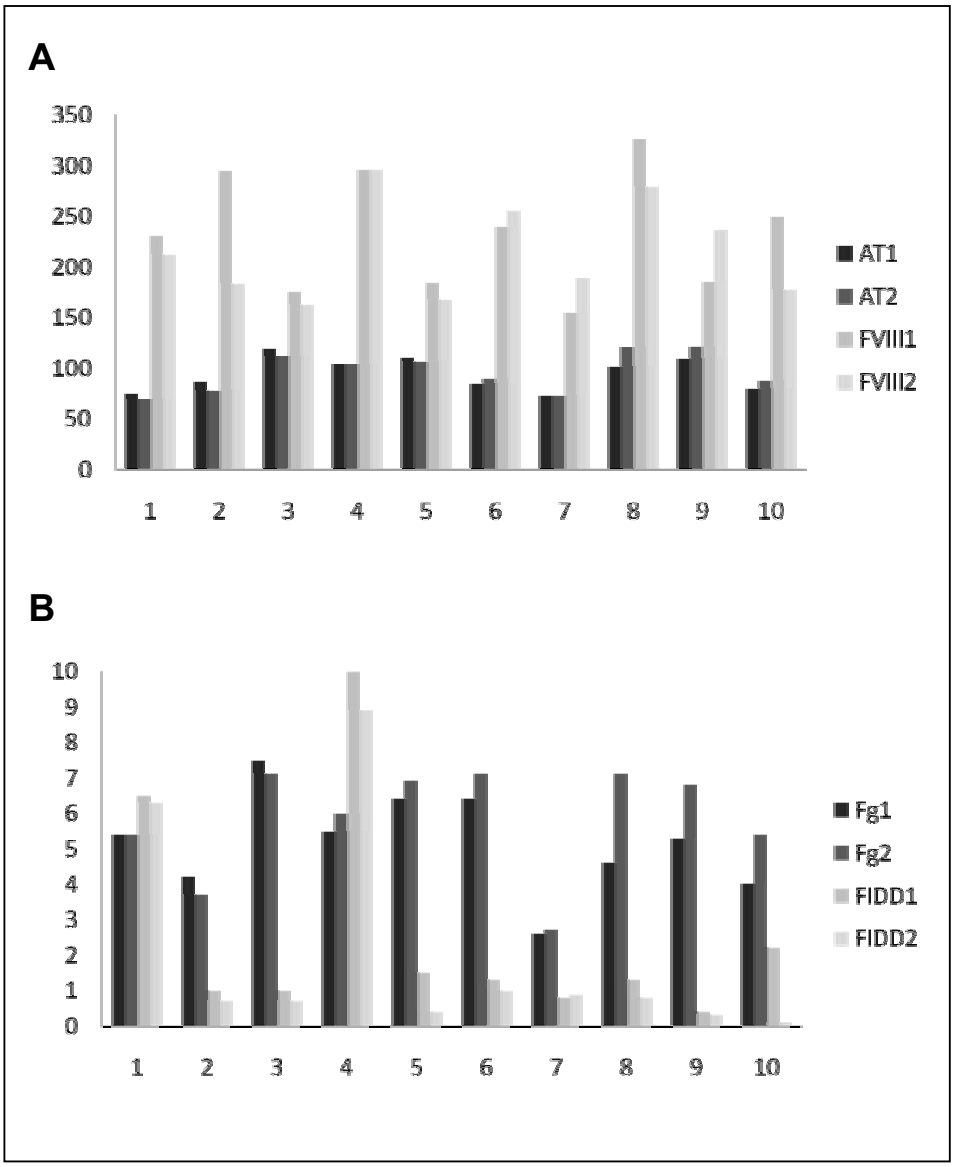

Figure 6. *Measurements of antithrombin, coagulation factor VIII, fibrinogen, and D-dimer levels (mean ± SD) before (1) and after (2) hemodialysis in 10 consecutive patients. The X axis indicates individual patients numbered from 1 to 10.*

A) Antithrombin (AT) (94 ± 16 vs. 96 ± 19 (%), p=ns) and coagulation factor VIII (FVIII) (234 ± 59 vs. 216 ± 48 (%), p=ns) levels were stable.

B) Fibrinogen (Fg) levels mainly increased (5.2 ± 1.4 vs. 5.8 ± 1.6 (g/l), p=0.05) and D-dimer (FIDD) levels decreased after hemodialysis (p=0.008).

RESULTS

Lepirudin therapy in severe progressive thrombosis in thrombophilic patients (I-III)

Budd-Chiari syndrome (I)

Lepirudin was successfully used in a young patient having factor V Leiden mutation with BCS, i.e. hepatic venous outflow obstruction. The thrombosis had eventually occluded median and left hepatic veins of the liver (Fig. 7) and led to intractable ascites and renal insufficiency. Initial treatment had been inadequate, because warfarin therapy was not accompanied by LMWH. Thereafter, the anticoagulant therapy was switched to LMWH monotherapy, but the patient's condition did not improve. After careful consideration with a gastroenterologist, transplantation surgeons, and a consultant in coagulation disorders, lepirudin was initiated at 2.5 months after the initial hospitalization. Dosage of lepirudin was markedly reduced from the normal regimen due to kidney insufficiency (creatinine clearance was 0.22 ml/ min/ 1.73 m2) (Table 7). Lepirudin therapy was followed meticulously with repeated APTT measurements. After 10 days of IV treatment, lepirudin was continued SC for 16 months. The patient recovered, as thrombosis did not progress, the right hepatic vein remained patent, and remnant liver tissue and kidney function had time to recover. Invasive procedures could also be avoided. No bleeds occurred. During the further 4-year follow-up, liver enzymes, kidney function, and D-dimer normalized and remained normal (I, Table 1).

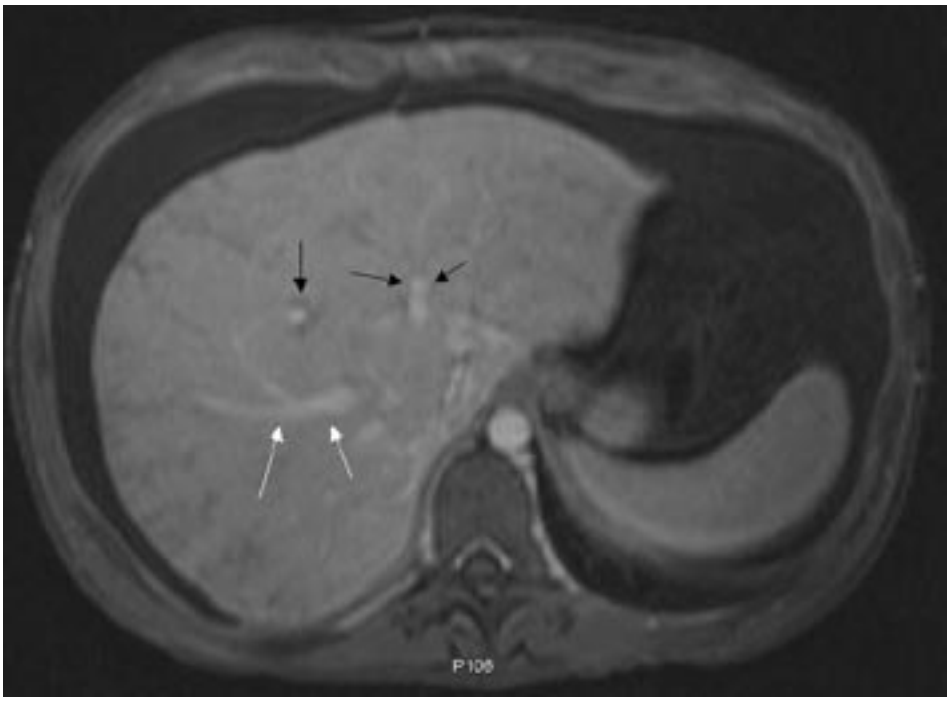

Figure 7. *Contrast-enhanced T1-weighted magnetic resonance image showing open right hepatic veins (white arrows); middle and left hepatic veins were occluded, but a collateral network (black arrows) was visible. Figure provided by radiologist Kirsti Numminen (Helsinki University Central Hospital) and printed with her permission.*

Table 7. *Clinical demographics of 12 patients treated with off-label lepirudin in Studies I-III.*

Age	Indication (Study I-III)	Comorbidities and risk factors	Thrombophilia or thrombogenic risk factors	Median dosage* (mg/kg/h) (range)
24	BCS (**I**)	-	FV Leiden (Het), low FV	0.04 (0.02-0.05)
38	CLI (**II**)	Popliteal artery aneurysm	FV Leiden (Het)	0.06 (0.04-0.15)
36	CLI (**II**)	Recurrent arterial thrombosis	FII G20210A mutation (Het)	0.16 (0.1-0.2)
54	CLI (**II**)	DVT x 2, smoker	FVIII:c† Short TT (15 s)‡	0.13 (0.07-0.18)
42	CLI (**II**)	Thrombangitis obliterans (Buerger's disase)	Hemoconcentration (plasmapheresis)	0.12 (0.08-0.14)
43	CLI (**II**)	Thrombotic microangiopathia, Crohn's disease	Essential thrombosis	0.06 (0.05-0.08)
50	CLI (**II**)	Severe PAD, hypertension	Prior HIT, smoker Short TT (16 s)‡	0.15 (0.12-0.17)
24	Bilateral PE (**III**)	Dilative cardiomyopathy	Not found	0.2 (0.13-0.23)
28	BCS (**III**)	-	Paroxysmal nocturnal hemoglobinuria	0.07 (0.05-0.11)
79	CLI (**III**)	DM	Antiphospholipid antibodies	0.06 (0.06-0.09)
51	CLI (**III**)	DM, CHD, atrial fibrillation, renal transplant	FVIII:c†	0.04 (0.01-0.12)
38	CLI with aortic thrombosis (**III**)	Horton's neuralgia	FVIII:c†	0.11 (0.1-0.15)

* Lepirudin was started at 0.1–0.15 mg/kg/h without a bolus dose owing to prior anticoagulation therapy.

† FVIII:c, factor VIII coagulant activity was high > 200% (normal range 52–148%).

‡ TT, thrombin time (normal range 17-25s)

BCS, Budd-Chiari syndrome; CLI, (inoperable) critical leg ischemia; PAD, peripheral arterial disease; PE, pulmonary embolism; DM, diabetes mellitus; CHD, coronary heart disease

Peripheral arterial disease (II)

Inoperable CLI (Fontaine stage III-IV) occurred in six relatively young (mean age 43) patients without typical atherosclerotic risk factor profiles, except for patient #6. These patients were at high risk for lower extremity amputation, because no revascularizations were possible based on the angiography and duplex examinations. TF or thrombogenic risk factors (II, Table 1) were found in addition to a history of prior vascular revascularizations. The patients were treated with a combination of antithrombotic therapies, antiplatelet agents, and anticoagulation (LMWH or warfarin in one patient) (II, Table 1). Lepirudin was initiated (IV) based on careful multidisciplinary clinical judgment, when the conventional double or triple antithrombotic therapy was deemed insufficient, and no vascular interventions could be offered. Platelet inhibitors were re-administered only after lepirudin was ensured to have reached the steady state without hemostatic compromises. Lepirudin infusions were occasionally withheld for 2

hours if the APTT ratio exceeded 2.5 (average per patient, 2 times, range 0-6). No bleeding or thromboembolic complications occurred. Patients' subjective and objective symptoms (pain, discoloration) were resolved, and TBP improved (II, Fig. 1) compatible with the distal involvement of the thrombosis. Both TBP and ankle-brachial pressure index measurements remained relatively unchanged during the first year after lepirudin treatments. Further antithrombotic therapy was individually tailored after lepirudin combining LMWH and antiplatelet agents, and patients were regularly followed in our tertiary unit. Only one below-knee amputation was performed 58 months later for patient #6 (II, Table 3), who also was noncompliant towards antithrombotic therapy. Amputation-free survival continued among other patients by the end of 4 years of follow-up.

During 2001 to 2004, major lower limb amputations were performed in our tertiary unit, due to unreconstructable distal limb ischemia or otherwise unsalvageable lesions (i.e. osteitis), on 426 patients. Only the 21 patients aged ≤55 (5% of all 426 patients), like the six lepirudin-treated patients above, were included in the further analysis (Table 8). We assessed the similarities between these patient groups. DM was common, but non-diabetic angiopathy, as well, had led to lower extremity amputation in 10 patients, who in age and in being non-diabetic were similar to our lepirudin-treated patients. Three of the patients without DM had suffered from severe thrombogenic conditions: HIT, disseminated intravascular coagulation, and cancer exacerbating bilateral DVT, in addition to arterial thrombosis without obvious PAD. Among these 21 patients, occasional TF and thrombogenic risk factor screening had been performed in 8 (38%), of which only one screen was normal. Table 8 shows these patients' primary diagnoses and their thrombogenic risk factors. After limb amputation, eight patients with DM and three without died. Mean survival was shorter in patients without than with DM (24 vs. 4 months). Those patients having a lower extremity amputation at a relatively young age, as in our treatment group, seemed highly thrombogenic based on either thrombogenic etiology of their leg ischemia (3), on positive TF screening (7 of 8), or on a common history of either stroke or myocardial infarction at a young age (8) (Table 8).

Clinical and laboratory monitoring of lepirudin (in I and in II, and in III, patient data)

Laboratory monitoring of lepirudin was performed by use of APTT. Under meticulous monitoring and repeated reassessments, lepirudin treatments appeared to be safe and efficient, but based on APTT results, the initial dosing of lepirudin was markedly reduced (III, Table 1). On some occasions, APTT was limited by certain factors, for instance, when bridging lepirudin with warfarin, or in a patient with LA whose APTT became nonmeasurable even at low doses of lepirudin. Additionally, APTT failed to respond to high doses of lepirudin in one patient, and as a result the dose was further increased, nearly exceeding the recommended maximum dosage (Table 7, patient with bilateral pulmonary embolism). Plasma dTT was developed because of a need for another laboratory monitoring method for lepirudin. It was used along with APTT to ascertain that the dosing of lepirudin remained in the therapeutic range before more specific methods could be tested. Based on experience from our lepirudin administration in clinical practice, it appeared that use of lepirudin was occasionally suboptimal, especially due to the limitations of APTT.

Table 8. *Characteristics of those 21 patients aged ≤ 55 years and underwent lower extrem-ity amputation during 2001-2004.*

Age	Primary diagnosis	Secondary diagnosis	Died	Follow-up (months)	Thrombophilia
46*	Bilateral DVT	Gastric cancer	x	4	-
55	Sepsis, DIC	Reiter's disease, HTA		67	-
53	Sepsis, DIC, MOF	Otherwise healthy	x	0.5	-
51	PAD	CHD, HTA, CABG, MI	x	6	-
53	PAD	CHD, ESRD, stroke (2)		64	FVIII↑
52	PAD	Prosthetic aortic valve		79	Normal
32	Aortic dissection	HTA		66	-
38	Burger's disease	-		30	FVIII ↑
53*	PAD	Alcohol abuse, CHD		55	-
54*	Osteitis	Alcohol abuse		43	-
39	DM, PAD	CHD, hemodialysis	x	6	FVIII ↑
42	DM, PAD	CHD, stroke	x	14	FVIII ↑
44	DM, PAD	ESRD	x	6	FVIII, low PC and AT
46	DM, PAD	HTA, stroke	x	78	Short TT
48	DM	CHD, uremia, stroke	x	18	-
49*	DM, HIT	Stroke	x	1	-
54	DM, PAD	-		62	-
55	DM, PAD	CHD, cardiomyopathy, HTA		50	-
55	DM, PAD	Stroke		58	FVIII ↑, short TT, low PS
55*	DM	Stroke, AMI	x	0.5	-
55	DM, PAD	CHD, heart failure, HTA	x	68	-

* Above-knee amputation, - not examined

DVT, deep vein thrombosis; DIC, disseminated intravascular coagulation; HTA, arterial hypertension; MOF, multi organ failure; PAD, peripheral arterial disease; CHD, coronary heart disease; CABG, coronary artery bypass surgery; MI, myocardial infarct; ESRD, end-stage renal disease; F, coagulation factor; DM, diabetes mellitus; AT, antithrombin; TT, thrombin time; PC, protein C; PS, protein S

Comparison of laboratory monitoring methods for lepirudin (III)

Patients

Ex vivo samples were obtained from five patients treated with lepirudin for heparin-resistant thromboses (III, Table 1). These samples were reanalyzed with the more specific comparative methods (Fig. 8). This analysis demonstrated the limitations of APTT and PiCT® in comparison with the chromogenic methods ECA and Anti-FIIa (III, Fig. 1). Both coagulation-based methods indicated falsely high lepirudin doses in patients with paroxysmal nocturnal hemoglobinuria (Fig. 8, patient #2) or LA (Fig. 8, patient #3). In addition, ECA and Anti-FIIa results indicated that both over- and underdosing of lepirudin (Fig. 8, patients #1 and #4) had occurred while the clinical treatment had been based on APTT and dTT at dilution 1:8 (III, Fig. 1). Lepirudin was successfully maintained in its therapeutic range based on all four methods in patient #5 (Fig. 8). Additionally, at 1:16 dilution the dTT was capable of capturing all lepirudin concentrations (Fig. 8, Patient #5).

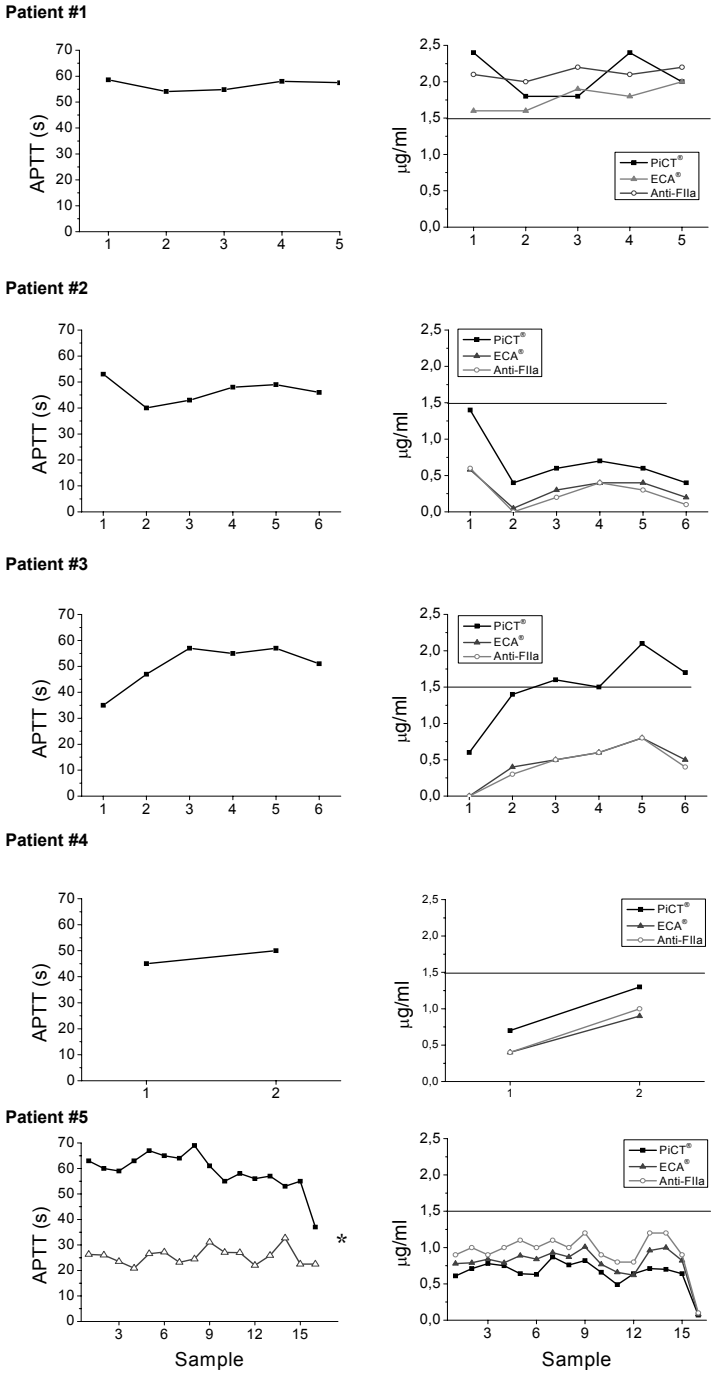

Figure 8. *Monitoring of lepirudin treatment in five patients by means of APTT, ECA, PiCT® and Anti-FIIa methods. Repetitive plasma samples (the x-axis refers to sample numbers) were obtained during i.v. lepirudin therapy for 3 to 8 days. Left-hand panels show APTT (s) and right-hand panels respectively ECA®, PiCT® and Anti-FIIa (µg/ml) results in individual patients. The therapeutic range for lepirudin concentration is marked by horizontal lines. Patient sample analysis demonstrates examples of overdosing (patient #1), underdosing (patient #2), interference of lupus anticoagulant (patient #3), insensitivity of APTT to dose escalation (patient #4), and successful laboratory monitoring (patient #5). * Triangles (patient #5) indicate dTT (1:16 dilution) in the therapeutic range. Notably, the last sample was taken after lepirudin infusion was stopped, and TT was measurable without additional dilution.*
APTT, activated partial thromboplastin time; ECA, ecarin chromogenic assay, PiCT®, Prothrombinase-induced Clotting Time; Anti-FIIa, anti-Factor IIa assay; dTT, plasma diluted Thrombin Time.

Comparison of monitoring methods in normal plasma

ECA and Anti-FIIa were the most precise methods for normal plasma. In contrast, a plateau effect was apparent even in the therapeutic range of lepirudin when assessed with APTT or PiCT® (III, Figs. 3-5, 7). Data analysis revealed that dTT needs to be diluted to 1:16 for to cover the therapeutic range of lepirudin, and the upper therapeutic limit at 1:16 dilution was about 45 to 50s (III, Fig. 6).

Effects of warfarin

The presence of warfarin additionally prolonged both APTT and PiCT® responses to lepirudin concentration. PiCT® was prolonged at INR 1.5 from the point at which lepirudin concentration exceeded 0.5 µg/ml (the lower limit of the therapeutic range), whereas ECA, Anti-FIIa, and dTT remained unaffected (III, Fig. 3-6). Prolongation of both coagulation-based methods, APTT and PiCT®, was more pronounced at higher INR levels. Administration of lepirudin in the therapeutic range to the warfarin-containing plasma pool (at INR 1.5), a situation mimicking overlapping anticoagulation, caused no notable changes in INR.

Effects of lupus anticoagulant

The presence of LA prolonged both coagulation-based assays, APTT and PiCT®, impairing the value of these methods for assessment of lepirudin dosage in the presence of LA. These methods became unsuitable for lepirudin monitoring when the baseline measurement was not within the reference ranges. The coexistence of LA did not disturb ECA, Anti-FIIa, or dTT, and they produced results similar to those obtained in the normal plasma pool. The interclass correlation efficient for both ECA and Anti-FIIa was 0.99 ($p<0.001$).

In summary, the chromogenic methods ECA and Anti-FIIa seemed the most precise and reliable methods for assessing lepirudin effects, as they were not influenced by the coexistence of either warfarin or LA. In addition, these two methods were capable of measuring even the supratherapeutic levels of lepirudin used in association with CPB surgery. Table 9 highlights the advantages and disadvantages of the specific monitoring methods for lepirudin, based on our comparison study.

Table 9. *Comparison data on monitoring methods for lepirudin in normal plasma, lupus anticoagulant, and warfarin-containing plasma.*

Method	Normal plasma pool linear response: upper limit	Lupus plasma pools	Warfarin plasma pools
APTT	ca. 0.4-0.5 µg/ml	Non-applicable	Impaired usability
PiCT®	ca. 0.7-1.0 µg/ml	Non-applicable	Enhanced coeffect
ECA	ca. 3.8 µg/ml	Unaffected	Unaffected
Anti-FIIa	ca. 4 µg/ml	Unaffected	Unaffected
dTT (1:16)	ca 1.0 µg/ml	Unaffected	Unaffected

Thrombophilia and vascular access survival in 237 end-stage renal disease patients (IV)

Results of screening for thrombophilia and thrombogenic risk factors

Of the 237 patients, TF was found preoperatively in 43 (18%) in association with HD access surgery (IV, Table 3). In females, TF appeared in 9%, in males in 23% (p=0.009). Gene mutations of FV Leiden and FII G20210A were encountered in nine patients (4%). Levels of the natural anticoagulants protein C activity and protein S antigen were low in 3% of the patients, the latter was found to be low only for males. In three patients, both the natural anticoagulants protein C and S were decreased. Low antithrombin levels (≤ 60%) were encountered only in association with another TF in four patients. The thrombogenic risk factors, i.e. fibrinogen, D-dimer, FVIII: C, and homocysteine, were clearly above their reference ranges in the vast majority of the patients (IV,Table 3). In addition, thrombin time was shorter than the reference in half the patients.

Thrombosis or stenosis of vascular access

Sufficient maturation occurred in 226 (95%) of all 237 vascular accesses. Access thrombosis or stenosis occurred during the follow-up (mean 3.6, range 2.3-5.8 years) in 79 (35%) of the 226 patients (IV, Fig. 1). Thrombosis or stenosis of vascular access tended to be more common among patients with than without TF (43 vs. 33%, p=ns). Thrombosis or stenosis of vascular access occurred early, as 30% of them were encountered before the initiation of HD, and up to 80% (63 events) ensued during the first year. The thrombosis- and stenosis-free access survival at 1 year was 72% (IV, Fig. 1). Table 10 shows access maturation rates, thrombosis- and stenosis-free survivals, and functional patency data separately for different vascular access types.

Table 10. *Access maturation, thrombosis- and stenosis-free survival, usability for hemodialysis, and functional (unassisted patency) separately for different types of vascular access.*

Vascular access type n = 237	Maturation (%)	Thrombosis- and stenosis-free survival (months) (mean ± SD)	Used in HD (%)	Functional patency (months) (mean ± SD)
Lower fistula (radial) n= 199	97	20.2 ± 17.6	82	21.1 ± 17.0
Upper fistula n= 20	80	23.5 ± 18.9	80	16.9 ± 17.8
Graft n= 18	94	9.1 ± 8.1	83	7.4 ± 8.9

HD, hemodialysis

Impact of thrombophilia on vascular access outcome

Female gender (HR 2.5, 95% CI 1.6-3.9, p<0.001) and presence of TF (HR 1.9, 95% CI 1.1-3.3, p=0.02) were independent risk factors in multivariate analysis for shortened thrombosis- and stenosis-free access survival (IV, Fig. 2). In females, the thrombosis- and stenosis-free survival of vascular access was significantly shorter than in males (29 ± 4, CI 22-36 vs. 45 ± 2, CI 41-50 (months), p=0.002). TF was not a risk factor for the functional patency of vascular access, but

in 23% of the patients having TF the access could not be used for HD, and these patients were excluded from this analysis. Both elevated D-dimer (p=0.03) and elevated CRP (p=0.02) were associated with functional patency failure.

Patients with and without dialysis at the time of vascular access surgery

Risk factors for thrombosis or stenosis of vascular access differed between patients with or without preoperative HD. A separate analysis of the 123 predialysis patients indicated that the higher the creatinine quartile, the better the thrombosis- and stenosis-free access survival (p=0.008) (Fig. 9), whereas the second urea quartile predicted the longest access survival, and the first urea quartile was associated with the poorest outcome (p=0.03). Among the predialysis patients, female gender (p<0.001) and presence of TF (p=0.009) were also independent risk factors for access thrombosis- and stenosis. In univariate analysis, elevated fibrinogen, but not D-dimer, implied shorter access survival than did normal fibrinogen (p=0.04) (IV, Fig. 3).

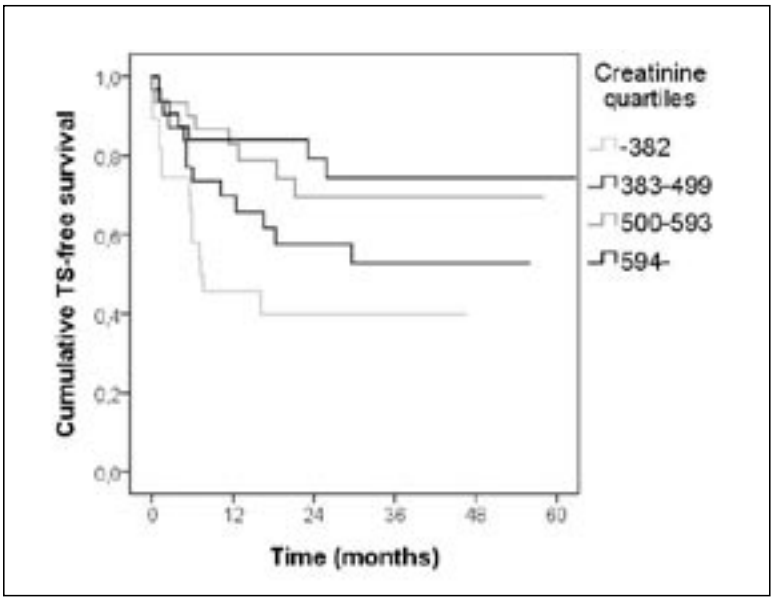

Figure 9. *Thrombosis- and stenosis-free survival of vascular access in predialytic (n=123) patients according to creatinine quartiles (µmol/l). The higher creatinine levels seemed to be associated systematically with better vascular access survival.*

Among the 103 dialysis patients (at the time of access surgery) an independent risk factor for shortened thrombosis- and stenosis-free access survival was similarly female gender (p=0.047). In addition, the shorter thrombosis- and stenosis-free access survival in univariate analysis was associated with elevated CRP (75th quartile, p=0.02), low homocysteine (under 50th percentile, p=0.01), and absence of medication for blood pressure (p=0.003).

Patients on LMWH therapy

At the time of the study, regular low-molecular-weight heparin (LMWH) therapy was administered to 30 of the ESRD patients with known TF or a history of thromboembolic complications. The etiology of ESRD in patients on LMWH was inflammatory in 37% and was either diabetes or renovascular in 23%. In comparison with patients without LMWH, these patients more often had TF (43 vs. 15%, $p<0.001$), coronary artery disease ($p=0.04$), a history of prior access operation ($p=0.001$), or elevated preoperative CRP ($p=0.006$), and more often administration of B-vitamin or folic acid ($p=0.04$). In addition, their FVIII:C levels coincided in the highest quartile (i.e. >206%, $p=0.05$), and they tended to have a common history of venous embolism (13 vs. 5%). Those 28 patients on LMWH whose access matured had shorter thrombosis- and stenosis-free survival than did those without LMWH medication ($p=0.011$).

DISCUSSION

Direct thrombin inhibitors like lepirudin provide a tempting and targeted management option for severe thrombosis, because thrombin and its regulation play a central role in the disease processes. This thesis aimed to describe the clinically complicated young patients who have TF or thrombogenic risk factors and severe thrombosis despite conventional anticoagulation. These patients were treated with a DTI, lepirudin, which inhibits both clot- and fibrin-bound thrombin. Various laboratory monitoring methods for lepirudin treatment were compared to overcome the limitations of APTT. Additionally, TF and thrombogenic risk factors were assessed among ESRD patients in relation to thrombosis or stenosis of vascular access. Figure 10 summarizes the thesis projects.

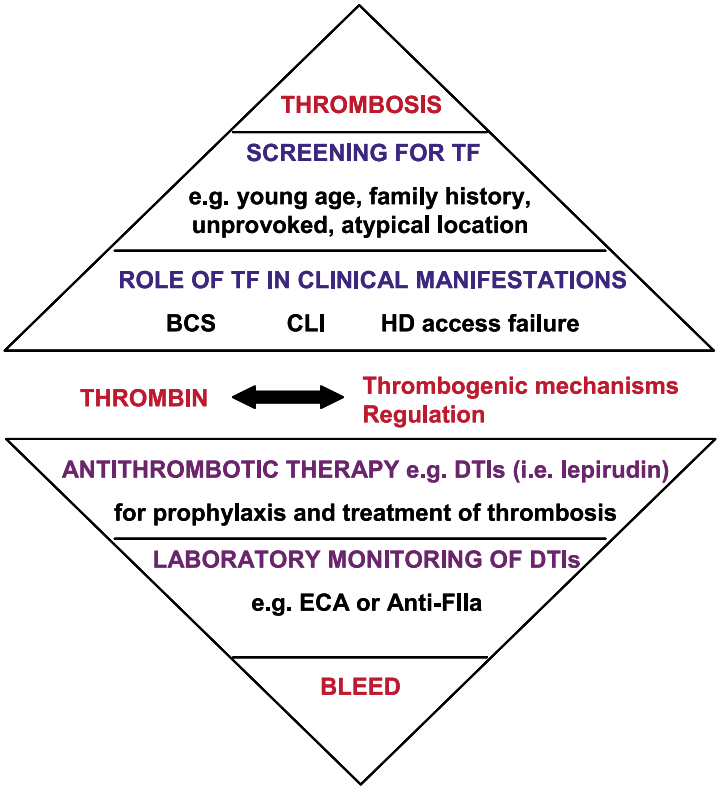

Figure 10. *Summary of thesis projects. Thrombosis with thrombophilia (TF) or thrombogenic risk factors occurred in Budd-Chiari syndrome (BCS), inoperable critical leg ischemia (CLI), or in end-stage renal disease (ESRD) patients with vascular access for hemodialysis (HD). Direct thrombin inhibitors (DTIs) like lepirudin provide a tempting and targeted management option, because thrombin and its regulation play a central role in these disease processes. Laboratory monitoring with either ecarin chromogenic (ECA) or anti-Factor IIa (Anti-FIIa) assays in addition to global coagulation test (e.g. activated partial thromboplastin time, APTT) is, however, essential in balancing efficient thrombosis management and avoidance bleeding complications.*

The novelty of our findings is that off-label lepirudin treatment was successful and safe for the thrombotic calamities occurring after more conventional antithrombotic therapy failed (I, II). The patients were relatively young, and therapeutic intervention possibilities were limited to liver transplantation or limb amputation. Patients with inoperable CLI have high unmet medical needs. Success with lepirudin, a potent DTI, in these preliminary studies without a control group is naturally anecdotal, but if the results can be confirmed, lepirudin possibly offers a great improvement over an otherwise poor outcome. In fact, the situation seems comparable with earlier reported compassionate use of recombinant activated coagulation FVII (Novoseven®) in severely bleeding patients (Hedner 2000). Preliminary studies are needed before randomized controlled trials can even be designed. On the other hand, in certain rare but life-threatening cases of venous thrombosis, randomized trials may be impossible (Martinelli et al. 2008).

Critical leg ischemia and end-stage renal disease

Approximately 3% of the patients having lower extremity amputation at a relatively young age had no diabetes and resembled our lepirudin-treated patients in having inoperable CLI without a typical atherosclerotic risk profile (II). In patients with chronic kidney disease, risk for limb amputation is extremely high (Eggers et al. 1999). Such a risk is even 10-fold higher in DM patients with than without chronic kidney disease. Alarmingly, over 60% of chronic kidney disease patients have, in one study, died within two years after amputation (Eggers et al. 1999). Similar high mortality has occurred after lower limb amputation in PAD patients (Aulivola et al. 2004). In fact, both PAD and ESRD significantly raise cardiovascular mortality (Shlipak et al. 2005, Golomb et al. 2006). Consequences of lower limb amputation for quality of life are also major. DM is an acknowledged risk factor for PAD and is associated with inflammation, impaired fibrinolysis, and endothelial damage (Knobl et al. 1993, Juhan-Vague et al. 1996, Thor et al. 2002). Enhanced antithrombotic therapy can be beneficial in combination with antiplatelet agents in patients with DM and CLI. In fact, LMWH has improved chronic diabetic neuroischemic ulcers in PAD patients (Kalani et al. 2003, 2007).

Anticoagulation in PAD has been widely studied (Clagett et al. 2004, Hackam et al. 2007), but still its role may be underestimated. Although it is possible that a DTI may provide specific beneficial effects in CLI patients (Allie et al. 2005, Shammas 2005), what cannot be excluded is that some of the effect seen with lepirudin was driven by raising the anticoagulation level as such. On the other hand, enhanced thrombin generation occurs in PAD, and disease severity associates with fibrinogen and D-dimer levels (Lassila et al. 1993, Herren et al. 1994). Additionally, marked thrombin generation occurs even without platelets or microparticles on vascular tissue on the surface of smooth muscle cells (Pathak et al. 2006). This capability of vascular tissue to generate thrombin could in part explain the efficacy of lepirudin in CLI, where co-administration of heparin and antiplatelet agents was insufficient to calm the thrombotic process. Both free soluble and clot-bound thrombin can be inhibited with lepirudin in comparison with the effect of heparins (Weitz et al. 1990, 1998). Furthermore, lepirudin or some other DTI might be effective also in patients with DM and CLI, or maybe even with ESRD, when determined with the aid of precise laboratory monitoring with ECA or Anti-FIIa assays.

Thrombophilia and risk for thrombosis

Thrombosis is a multicausal disease associated with the complicated dynamic interaction of several risk factors. TF is more commonly found in association with a family history of venous thrombosis, recurrent or idiopathic venous thrombosis, occurrence at a young age or at an unusual site, unusual pregnancy complications, and in combination with both arterial and venous thrombosis than it is in patients without such a history (Rosendaal 1999, de Moerloose et al. 2007, Lindhoff-Last et al. 2008). For instance, even as high as 50% of the patients have a thrombophilic disorder in association with idiopathic venous thromboembolism (Weitz et al. 2004). The additive effect of TF on thrombosis varies between different TF entities and also depends on other risk factors present (Rosendaal 1999). Additionally, the net thrombotic risk is not stable over time, as patients grow older, use contraceptives, become pregnant, or undergo surgery, which results in a need for repeated individual risk assessments.

TF alone is rarely the only causative factor, but it may associate with more severe manifestations of thrombosis in comparison with the situation in patients without TF but with otherwise similar risk factors. Antithrombotic treatment may sometimes be delayed and the thrombosis progress be due to difficulties in clinical diagnosis, as in our patient with BCS. Furthermore, the results of a TF screen take some time, but in general the initial treatment of a TF patient is similar to that for a patient without TF (Gallus 2005). Patients having thrombosis and some resistance to conventional antithrombotic treatment comprise a minority of patients with thrombosis, but the consequences may be severe. Underlying TF may call for extended anticoagulation in association with for instance antithrombin deficiency or antiphospholipid antibodies (Lindhoff-Last et al. 2008, Foy et al. 2009). TF screening at present is incomplete, as some factors are still likely to be uncovered. Moreover, it is important to note that a patient even without TF who is experiencing severe unprovoked thromboembolism may also need prolonged or indefinite anticoagulation.

Vascular access thrombosis and stenosis

TF and female gender were important independent risk factors for decreased thrombosis- and stenosis-free access survival in ESRD patients (IV). Normal CRP, fibrinogen, and D-dimer seemed to associate with the better event-free access survival. Among ESRD patients, one particular subgroup was at high risk for access thrombosis or stenosis, despite LMWH treatment. These 30 patients more often had TF, had an inflammatory etiology of ESRD (IV, Table 1), a history of earlier access failure, or a preoperatively elevated CRP than did patients without LMWH. Inflammatory mechanisms are known to up-regulate procoagulants, suppress natural anticoagulants, and impair fibrinolytic activity (Esmon 2003, 2006), of which all could predispose to access thrombosis. Despite the administration of LMWH, this small subgroup seemed to have shorter thrombosis- and stenosis-free survival than did others. Dosing with LMWH was not uniform, and our study design allows no further conclusions.

Routine preoperative risk assessment is used in orthopedic surgery to estimate a patient's individual risk for thrombosis and appropriate thromboprophylaxis. Development of a risk

index could also benefit patients having HD access surgery. Risk factors for access occlusions could include at least the following factors: TF, female gender, prior access complication, ongoing inflammatory progress, a possible prior unprovoked thromboembolic complication, especially at a young age, or other thrombogenic comorbities, for instance cancer (Miller PE et al. 1999, Gibson et al. 2001, Miller CD et al. 2003, Yevzlin et al. 2006), but further studies are needed for establishment of risk factors. However, evaluation of the net benefit-risk balance among ESRD patients is mandatory, because risk for bleeding complications is also high. Unfortunately, the optimal antithrombotic strategy for prevention of access occlusions is not yet established despite extensive efforts (Kaufman 2000, Kaufman et al. 2003, Dember et al. 2008, Dixon et al. 2009).

Laboratory monitoring of antithrombotic drug effects

Global coagulation based tests like PT and TT are unsuitable for DTI monitoring, because they are too sensitive, but they may serve as indicators that the patient is on a DTI. The specific coagulation-based monitoring tests APTT and PICT® were unable to assess lepirudin in normal plasma precisely at the upper therapeutic range, and they both became invalid at the supratherapeutic lepirudin concentration used during CPB surgery. Furthermore, in the presence of LA, any assessment of lepirudin with APTT or PiCT® was impossible, as was earlier reported regarding APTT (Perry et al. 2003). A more sensitive APTT reagent for drug monitoring may be less sensitive for screening of LA, which underlines the important fact that the limitations of the chosen APTT reagent should be known. However, it is more important to note a bleeding risk with an anticoagulant-sensitive APTT reagent, as the diagnosis of LA is based on other more specific tests than APTT. When interpreting APTT results in drug monitoring it is vital to be aware of the several limitations of APTT to avoid false conclusions and potentially harmful dose adjustments (Table 4) (Nowak 2001, Fenyvesi et al. 2002b, 2004, Gosselin et al. 2004a, Warkentin et al. 2008b). A multidisciplinary approach will help to guarantee safe and optimal therapy with potent DTIs like lepirudin. Cooperation between clinicians, clinical chemists, and a hemostasis laboratory is necessary for interpreting monitoring results in complicated situations. An optimal method for DTIs would also be suitable for bedside use, allowing fast dose-assessments in acute situations and during CPB surgery. ECA and Anti-FIIa methods using chromogenic substrates seemed suitable for clinical use, also in automated systems. Even if PiCT® and APTT are less specific than amidolytic methods for anti-Xa and anti-IIa assessments, they may provide clinically relevant information on the sum effects of anticoagulants as such.

Monitoring of DTIs is essential during overlapping anticoagulation with warfarin along with DTIs. For instance, in HIT-related thrombosis, overlapping is recommended for 5 days after recovery of the platelet count (Warkentin et al. 2008b). In these rare but vulnerable patients, chromogenic assays together with INR would possibly provide a better assessment of both regimens, to ensure a safer combination therapy than one based on APTT or INR or both. Furthermore, during CPB surgery, ECA or Anti-FIIa could provide suitable monitoring for DTIs, because they are unaffected by any often-occurring prothrombin or fibrinogen deficiency that is due to high consumption of coagulation factors during the surgery.

Furthermore, adequate monitoring of other DTIs like bivalirudin, argatroban, or dabigatran may be required in certain challenging situations: These include bleeding complications (to distinguish whether the underlying reason is overdose, accumulation or spontaneous bleeding due for instance to some tissue defect and to determine optimal management), kidney or liver insufficiency, and during any changeover from other anticoagulants to DTIs (or vice versa) for estimating residual anticoagulant activity.

Personalized antithrombotic treatment

DTIs, like lepirudin, bivalirudin, and dabigatran, are potent and efficient antithrombotic drugs. Lepirudin's potential may be currently underused for fear of bleeding complications, higher costs than for heparins, and a lack of specific and reliable monitoring methods. Bleeding complications with lepirudin were noted when it was used at high doses combined with thrombolytics (Antman 1994, Fischer 2004), which was an extremely potent combination, given the essential role of thrombin in hemostasis and fibrinolysis, and given the nearly irreversible inhibition of thrombin by lepirudin. In HIT trials, the lepirudin dose was higher than later recommended, which probably meant exposure to a higher risk for bleeding in comparison to their historical control groups, who in fact did not receive any antithrombotics (Warkentin et al. 2004, 2008b, Lubenow et al. 2005). In addition to HIT and heparin allergy, which are the official indications for lepirudin, some intriguing occasions seem to occur, where individually chosen potent thrombin inhibition with lepirudin or another DTI could be of benefit. Individually tailored anticoagulation seems reasonable, especially in treatment of rare and severe thrombosis, as well as in prevention of access occlusions in HD patients. Furthermore, thrombosis and bleeding risks must be individually evaluated.

New oral anticoagulants

The first oral anticoagulants, VKAs, have been used for decades, and their efficacy in preventing venous thrombosis, pulmonary embolism, and stroke has been shown in several clinical studies (A review: Ansell et al. 2004). A major drawback of VKA is the need to repeatedly adjust the optimal dosing by regular laboratory monitoring. Appropriate VKA doses vary significantly even in the same patient due to multiple food and drug interactions (Ansell et al. 2004). Unfortunately, even in highly controlled and selected populations the therapeutic range may be reached only 60% of the time (Connolly et al. 2009). In other words, new potent and safe, but less demanding drugs in terms of dosing and laboratory monitoring certainly will be highly valued.

The usability of lepirudin is limited by several factors, i.e. no possibility for oral administration, need for laboratory monitoring to assess the optimal dosing, and its strong dependence on kidney function (Greinacher et al. 2008). An ideal anticoagulant would require no monitoring of its effect nor would it have either inter- or intra-individual variability (Bounameaux et al. 2010, Mismetti et al. 2010). New anticoagulants seem to overcome some of the drawbacks of lepirudin. DTIs and direct FX inhibitors have a shorter half-life than VKAs, and they directly target an individual but essential coagulation factor in the complex and multifactorial coagulation cascade. As a result, they may be easier to use, because their pharmacokinetics

and pharmacodynamics are less variable (Mueck et al. 2008, Stangier, 2008). Currently the direct inhibitors dabigatran and rivaroxaban are marketed for selected thromboprophylaxis with the advantage of not needing routine monitoring of their effects (Mueck et al. 2008, Stangier 2008, Bounameaux et al. 2010).

Measuring the antithrombotic responses and adjusting the individual dosage of the new anticoagulants, i.e. dabigatran or rivaroxaban, may be warranted in certain clinical situations. These circumstances may include bleeding complications, overdosage, emergency surgery, extreme obesity or malnutrition, renal insufficiency, pregnancy, and use for children (Bounameaux et al. 2010). Experience in and assessment of monitoring options for lepirudin are of value in clinical practice when unselected patients are treated with these new anticoagulants for instance with dabigatran, and such situations occur. It is important to note that in clinical trials, many of the typical patients on warfarin therapy in emergency care are excluded, i.e. the elderly, patients with moderate to severe kidney insufficiency, and patients with heart insufficiency (Eriksson et al. 2008, Lassen et al. 2008, Levi et al. 2008, Connolly et al. 2009). Experience of treating these patients with new anticoagulants is gathered gradually and requires specific alertness when administering new anticoagulants to these vulnerable patients. It would, however, be a magnificent advantage if the new options for anticoagulation turn out be safe also in unselected patients. As a result, INR monitoring and counseling could become more infrequent, and vast amounts of clinical resources could be redirected (Bounameaux et al. 2010, Mismetti et al. 2010).

Inhibition of thrombin-induced mechanisms other than hemostasis

Clinical studies have shown that with cautious clinical and laboratory monitoring, short-term thrombin inhibition is safe and effective. DTIs may also inhibit the protein C anticoagulant pathway, which regulates thrombin generation and links thrombosis with inflammation (Esmon 2003, 2006, Crawley et al. 2007). This inhibition may be acceptable, with thrombin's procoagulant activities becoming inhibited simultaneously by DTIs. Thrombin also promotes cell proliferation, and inhibition of thrombin presumably affects these processes as well (Mann 2003). Inhibition of thrombin effects and cell proliferation, some speculate, is associated with anti-cancer effects of anticoagulants (Tsopanoglou et al. 2004, Piccioli et al. 2006, Snyder et al. 2008), but the implications of these effects are not yet established in clinical practice. Additionally, dabigatran has antifibrotic effects on lung fibroblasts (Bogatkevich et al. 2009).

Strength and limitations of the study

The strength of our study comparing monitoring options for assessing lepirudin therapy is that we included four specific methods for comparison with APTT, the gold standard (III). A limitation of the preliminary lepirudin studies (I, II) is that no markers of thrombin generation, ones like thrombin-antithrombin complexes or prothrombin fragment 1+2, were clinically available to assess the effect of lepirudin on thrombin generation per se under thrombotic circumstances. Indirectly, however, D-dimer, a marker of ongoing fibrinolysis, reflects thrombin generation, because thrombin cleavage of fibrinogen and thrombin-induced FXIII activation

are necessary before plasmin can generate D-dimer. Repeated D-dimer measurements were included in the studies assessing lepirudin efficacy (I, II). No control groups were included in the preliminary lepirudin studies (I, II), and multicenter international studies will be required to ensure sufficient study power to confirm our findings, because BCS and inoperable CLI occur relatively rarely.

HD seemed to be associated with changes in fibrinogen and D-dimer levels. Fibrinogen (340 kDa) and D-dimer (200 kDa) are molecules too large to be dialyzed, but some contact activation probably occurred on the filters or in the vascular accesses (IV, unpublished data). As a result, timing of these measurements in patients on HD should have been standardized, i.e. tests run for instance immediately before the next dialysis. However, although normal levels of fibrinogen and D-dimer seemed to be associated with functional access patency, the importance of the elevated levels needs reassessment. An additional limitation of Study IV was that positive antiphospholipid antibodies were not controlled for in the nephrology unit 12 weeks after the first sample, as recommended by the present guidelines (Miyakis et al. 2006). Sample size should have been further increased to analyze the impact of all individual TF on vascular access outcome.

Future considerations

Lepirudin therapy with ECA or Anti-FIIa assay monitoring options in addition to APTT seems worth studying in patients having CLI without revascularization options, and also in patients with DM, in prospective randomized trials. Several participating centers would be needed to guarantee sufficient study power, because inoperable CLI occurs relatively rarely. The lack of an oral administration option for lepirudin limits its usability; a tempting idea would therefore be to study whether dabigatran or some other oral DTIs, would aid in the treatment of inoperable CLI after the initial lepirudin infusion, and would help to avoid amputation and improve patient survival in the long term.

The suitability of ECA and Anti-FIIa assays for lepirudin monitoring has encouraged us to assess the possibility of using these chromogenic methods for bivalirudin monitoring. As a result, we have analyzed plasma samples from 10 consecutive patients during elective PCI performed with bivalirudin. In routine PCI, such monitoring is unnecessary, but it may become clinically important in association with overdosing, CPB surgery, combined use of antithrombotics, or with bleeding complications. The results could facilitate the possibility to use bivalirudin instead of UFH in certain clinical situations, if its dose responses could be precisely assessed.

Usability of UFH is severely limited by its adverse effects like HIT and by its unpredictable dosing requirements in patients. In contrast, bivalirudin, a transient DTI, could provide a tempting option for short-term thrombosis treatment, as it is less immunogenic and its dose response is more predictable (Warkentin et al. 2008a). Another advantage of bivalirudin is that after binding to thrombin, it is also cleaved by thrombin, and the active-site functions of thrombin are restored (Parry et al. 1994). This is important because what is crucial is that ample

free thrombin is made available for normal hemostasis and for other non-hemostatic-related functions of thrombin. In comparison with lepirudin, the therapeutic range of bivalirudin is wider: It inhibits thrombin only transiently, and its metabolism is less dependent on kidney function (Greinacher et al. 2008, Warkentin et al. 2008a).

Presumably, thrombogenic risk factors in the majority of the ESRD patients are associated with the high cardiovascular morbidity in such patients. Consequently, we are assessing the impact of TF and thrombogenic risk factors on the mortality of these 260 ESRD patients screened for our Study IV and have also gathered data on causes of death from the official register (Statistics Finland).

TF is strongly associated with vascular access thrombosis or stenosis, which suggests that it may have some influence in other vascular circumstances. Similarly, we have screened TF and thrombogenic risk factors in patients with severe chronic heart failure who are being evaluated for heart transplantation. We are aiming to assess in these patients the impact of TF and thrombogenic risk factors on postoperative complications, i.e. thromboembolic complications, kidney insufficiency, heart rejection, and patient survival.

CONCLUSIONS

Our comparison study of various monitoring methods for lepirudin (III) revealed that clinical management of these severe thrombotic disorders (I, II) was possibly occasionally even too cautious, when involving the monitoring on APTT alone. In some patients, even modestly higher doses of lepirudin could have been administered safely, but on the other hand, use of APTT led, in one patient, almost to an overdose of lepirudin (III). Consequently, either ECA or Anti-FIIa, the most precise lepirudin monitoring options, seems best to ensure treatment safety and to further improve the efficacy of lepirudin therapies. A multidisciplinary approach is required to guarantee safe and optimal therapy with potent DTIs like lepirudin.

TF and female gender were the most important risk factors for shortened thrombosis- and stenosis-free HD access survival (IV). TF screening is not a routine requirement before access surgery, but certain risk factors like previous access failure, personal or family history of unprovoked venous thromboembolism, or arterial thrombosis, especially at a young age, should raise the suspicion of TF, and lead to laboratory testing. Preoperatively normal fibrinogen indicated reduced risk for vascular access thrombosis or stenosis in predialysis patients. In addition, normal preoperative CRP or D-dimer indicated lower risk for functional patency failure of the access. Establishment of individualized risk estimations and effective prophylactic antithrombotic treatment to prevent access complications among ESRD patients is still an unmet medical need.

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