PHARMACOLOGICAL CHARACTERIZATION OF UNCONVENTIONAL HEPARINS

Salvador Rico Amaro

Universitat Autònoma de Barcelona

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Pharmacological characterization of unconventional heparins
(Caracterización farmacológica de heparinas no convencionales)
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CARACTERIZACIÓN FARMACOLÓGICA DE HEPARINAS NO CONVENCIONALES

Salvador Rico Amaro

Trabajo realizado para optar al grado de **Doctor en Farmacología** bajo la dirección del **Dr. Ignasi Gich Saladich** y del **Dr. Jordi Fontcuberta Boj**, en el Centre d'Investigació del Medicament, Institut d'Investigació Biomèdica, Hospital de la Santa Creu i Sant Pau

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Salvador Rico Amaro recibió apoyo del Consejo Nacional de la Ciencia y Tecnología (CONACYT), México, para realizar sus estudios de Doctorado en Farmacología.

“Si j'avais un conseil à donner à un être jeune et dont je respectais l'intelligence, l'ardeur ou le courage, je lui dirais: «Ne t'attache pas. Ne t'attache jamais. Tu ne rencontreras dans ta vie que trop de servitudes pour t'en forger librement, et au hasard, et sans savoir où te mèneras l'engagement pris. Pour le bien d'autrui comme pour le tien, ne t'attache pas. Le malheur est qu'il faut avoir été souvent et beaucoup attaché pour savoir le prix de ne pas l'être.»

L'attache extérieure n'est sentie, dans tous le cas, que lorsque le lien intérieur s'est usé ou brisé.

Mais d'autre part qui ne s'attache pas ne connaît jamais que le plus superficiel des êtres.”

Marguerite Yourcenar, Sources II

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ABBREVIATIONS

95% CI	95% Confidence interval
ACS	Acute coronary syndrome
ACCP	American College of Chest Physicians
ACT	Activated clotting time
ADP	Adenosine diphosphate
A_{max}	Peak activity; maximal plasma anti-activated Factor Xa or Factor IIa
Anti-FIIa	Activated anti-FIIa activity
Anti-FXa	Activated anti-FXa activity
AP-1	Activator protein 1
aPL	Antiphospholipid antibody
APS	Antiphospholipid syndrome
aPTT	Activated plasma thromboplastin time
ARD	Absolute risk difference
AT	Antithrombin
AUC	Area under the curve
BMPs	Bone morphogenetic proteins
BMPR	Bone morphogenetic protein receptor
BID	"*Bis in die*", twice a day
Cl	Clearance
CKD	Chronic kidney disease
Cl_{Cr}	Creatinine clearance
COPD	Chronic obstructive pulmonary disease
CV	Coefficient of variation
Cyr61	Cysteine-rich angiogenic inducer 61
Da	Daltons
DILI	Drug-induced liver injury
DTH	Delayed-type hypersensitivity
DTI	Direct thrombin inhibitor
DVT	Deep vein thrombosis
ECG	Electrocardiogram
ECGF	Endothelial cell growth factor
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
E_{max}	Maximum effect
EP	European Pharmacopoeia
ESCs	Endometrial stromal cells
EVTC	Extravillous trophoblast
FDA	US Food and Drug Administration
FGF	Fibroblast growth factor
FIIa	Factor IIa
FOBT	Fecal occult blood test

FTIH	First-Time-in-Human
FXa	Activated factor X
GAG	Glycosaminoglycan
GFR	Glomerular filtration rate or estimated glomerular filtration rate
GlcA	β-D-glucuronic acid
GlcA(2S)	2-O-sulfo-β-D-glucuronic acid
GlcNAc	α-D-N-acetylglucosamine or 2-acetamido-2-deoxy-α-D-glucose
GlcNAc(6S)	α-D-N-acetylglucosamine-6-O-sulfate
GlcNS	α-D-N-sulfoglucosamine
GlcNS(6S)	α-D-N-sulfoglucosamine-6-O-sulfate or 2-deoxy-2-sulfamido-α-D-glucose 6-sulfate
GPI	Glycosyl phosphatidyl inositol
h	Hour
HA	High affinity
HB-EGF	Heparin-binding epidermal growth factor-like growth factor
HCII	Heparin cofactor II
HDL	High density lipoproteins
HIT	Heparin-induced thrombocytopenia
HL	Hepatic lipase
HPSE1	Endo-β-glucuronidase heparanase
HR	Hazard ratio
HRG	Histidine-rich glycoprotein
HS	Heparan sulphate
HSPG	Heparan sulphate proteoglycans
HUVEC	Human umbilical vein endothelial cells
IDL	Intermediate density lipoproteins
IdoA	α-L-iduronic acid
IdoA(2S)	α-L-iduronic acid 2-sulfate or 2-O-sulfo-α-L-iduronic acid
IFN-γ	Interferon gamma
IGF-1	Insulin-like growth factor 1
IGFBP-1	Insulin-like growth factor-binding protein
IU	International units
IV	Intravenous
ka	Absorption rate constant
LMWH	Low molecular weight heparins
LPL	Lipoprotein lipase
MAPK	Mitogen-activated protein kinase
MMP-2	Matrix metalloprotease-2
MRT	Mean retention time
MW	Molecular weight
NCAM	Neuronal cell adhesion molecule
NF-κβ	Nuclear factor kappa-light chain-enhancer of activated B cells
NIBSC	National Institute for Biological Standards & Control
NK	Natural killer cells

NMR	Nuclear magnetic resonance
NNT	Number needed to treat
NNH	Number needed to harm
NOAC	New oral anticoagulants
NS	Not significant
OR	Odds ratio
OSCS	Oversulfated chondroitin sulphate
PAI	Plasminogen activation inhibitor
PD	Pharmacodynamic
PE	Pulmonary embolism
PECAM-1	Platelet endothelial cell adhesion molecule-1
PF4	Platelet factor 4
PK	Pharmacokinetic
pNA	Para-Nitroaniline
PO	"*Per os*", oral administration
PRL	Prolactin
PT	Prothrombin time
QD	"*Quaque die*", every day
RCL	Reactive center loop
RCT	Randomized clinical trial
RI	Renal insufficiency
RIETE	Registro Informatizado de la Enfermedad Tromboembólica
RR	Relative risk
SC	Subcutaneous or subcutaneously
SD	Standard deviation
SEM	Standard error of the mean
t ½	Elimination half-life
TAFI	Thrombin activatable fibrinolytic inhibitor
TCT	Thrombin clotting time
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
THR	Total hip replacement
TKR	Total knee replacement
TG's	Triglycerides
TGF-β	Transforming growth factor-beta
THA	Total hip arthroplasty
TID	"*Ter in die*", three times a day
T_{max}	Time to reach maximum concentration or biological activity
TNF-α	Tumor necrosis factor alpha
t-PA	Tissue plasminogen activator
Tp-TmT	Thromboplastin-thrombomodulin mediated time
TT	Thrombin time
UDP	Uridine diphosphate
UFH	Unfractionated heparin

ULMWH	Ultra-low molecular weight heparins
ULN	Upper limit of normal
USP	US Pharmacopoeia
Vd	Volume of distribution
VEGF	Vascular endothelial growth factor
VKA	Vitamin K antagonist
VLDL	Very low density lipoprotein
VTE	Venous thromboembolism
vWF	von Willebrand factor
WHO	World Health Organization

Chapter 1

Introduction

CHAPTER 1 – GENERAL OUTLINE & INTRODUCTION

A. General outline

Discovered by James McLean in 1916 (McLean, 1916), heparin (also referred to as unfractioned heparin, UFH) came into clinical use in the 1930s (Wardrop and Keeling, 2008). It is still a life-saving compound, crucial for conditions such as treatment of venous thromboembolism (VTE); comprising deep vein thrombosis (DVT) and pulmonary embolism (PE); and acute coronary syndrome (ACS), as well as for anticoagulation in surgery, interventional cardiology, and hemodialysis (Marder et al., 2012). Low molecular weight heparins (LMWHs) are depolymerized heparin derivatives developed in the 1980s, with pharmacologic profiles distinct from heparin (Hirsh and Raschke, 2004). LMWHs lack the non-specific binding affinities of heparin and, as a result, have greater bioavailability, produce a more predictable anticoagulant response than heparin, have a longer elimination half-life, do not generally require monitoring, and possess a better safety profile (i.e. less incidence of heparin induced thrombocytopenia and osteoporosis) (Garcia et al., 2012). Because of these advantages, over the past 20 years LMWHs have replaced heparin for most clinical indications (Hirsh and Raschke, 2004).

Despite the more recent development of other therapeutic options, such as the synthetic pentasaccharide fondaparinux, direct thrombin inhibitors (DTIs), or direct factor Xa (FXa) inhibitors, heparin and LMWHs continue to play a major role in the management of thrombotic and cardiovascular disorders (Fareed et al., 2008). Thanks to their polypharmacology, the use of LMWHs is expanding to other indications, such as cancer-associated thrombosis (Khorana, 2012), and prophylaxis of adverse pregnancy outcomes in thrombophilic women with previous recurrent pregnancy loss, preeclampsia, intrauterine growth restriction, and sudden fetal death (Tersigni et al., 2012). Moreover, the marginal superiority of the newer compounds over heparins, and the higher cost associated with their use, will also contribute to the continued utilization of LMWHs in the near future (Fareed et al., 2008, Prescrire-International, 2009, Guyatt et al., 2012).

With their development, LMWHs overcame several of the disadvantages of UFH. LMWHs have superior pharmacodynamic (PD) properties, and a more favorable benefit-to-risk ratio than heparin when used to treat VTE. Nevertheless, in spite of being available for several years, their pharmacology has not been fully characterized. Firstly, there is a dearth of head to head comparisons of their PD and efficacy pro-

files that allows for the right selection of LMWH. Secondly, no evidence has been generated to support accurate dosing in special populations, such as the elderly or in patients with chronic kidney disease (CKD). Since LMWHs are predominantly cleared by the kidneys, inaccurate dosing could lead either to bioaccumulation and an increased risk of bleeding, or administration of subtherapeutic doses and an increased risk of VTE. Another area that deserves research attention is the impact of a lower molecular weight (MW) distribution (i.e. ultra-low MWHs (ULMWHs)) in the PD of these compounds and their potentially improved safety and efficacy profile.

The aim of this work is to present the results of clinical trials in healthy volunteers aimed at addressing these 3 areas. Three clinical trials were conducted: 1) a randomized, single-blind, cross-over study to compare the PD time-course, safety, and tolerability of bemiparin (a second generation LMWH) and enoxaparin at high prophylactic doses (Antonijoan et al., 2009), 2) an open-label, randomized, First-Time-in-Human (FTIH) ascending dose study with an alternating cross-over design to evaluate the safety and PD profile of RO-14, a novel ULMWH (Rico et al., 2011), and 3) a multi-center, open-label, 2-period, parallel study to evaluate the PD of prophylactic and therapeutic doses of bemiparin in healthy young and elderly volunteers, and in patients with varying degrees of renal impairment, as well as the evaluation of the potential need for dose adjustment in these patient populations (Rico et al., 2014).

B. Introduction: Heparins, low molecular weight heparins, and related compounds

1. The structure and biosynthesis of heparin

Heparin (from Ancient Greek ηπαρ (hepar), liver) is a family of heterogenous polydispersed mixture of sulfated glycosaminoglycans (GAGs) (Hirsh and Levine, 1992). Like all mammalian GAGs, it is absolutely linear in sequence, with no branches and a rod-like conformation (Mulloy et al., 1993). In spite of its apparent simplicity, as a member of the heparan sulphate (HS) family of GAGs, its structure is extremely complex through heterogeneity in both sequence and size (Mulloy, 2012).

The biosynthesis of heparin has been well described. Heparin is synthesized from uridine diphosphate-sugar precursors as a polymer of alternating disaccharides of uronic acid (in this case, β-D-glucuronic acid, GlcA) and hexosamine (in this case, α-D-N-acetylglucosamine residues, GlcNAc) (Rang and Dale, 2012, Goodman et al., 2011, Mulloy, 2012) as a heparin proteoglycan (750,000-1,000,000 Da). The disaccharide in heparin is mostly converted to a trisulphated form in which the GlcA has been epimerized to α-L-iduronic acid (IdoA). Multiple heparin chains of the proteoglycan, each containing 100 to 150 disaccharide units, are covalently linked to a core protein called serglycin. Once generated, the heparin proteoglycan is degraded by three different types of lysosomal enzymes: (a) proteases that cleave the polypeptide chain, (b) endoglycosidases that cut the heparin chain between glucuronic acid and glucosamine residues, and (c) exoglycosidases that remove monosaccharide units from the non-reducing ends of heparin oligosaccharides. The degraded products are then stored in the secretory granules of mast cells, which are widely distributed in a variety of organs, including the liver, heart, lungs, kidneys, and intestine (Tollefsen and Zhang, 2012).

The sugar residues may be O-sulfated at the C-6 and C-3 positions of the glucosamine and the C-2 position of the uronic acid. The main sugars occurring in heparin are: (a) α-L-iduronic acid 2-sulfate (IdoA(2S)), (b) 2-deoxy-2-sulfamido-α-D-glucose 6-sulfate (GlcNS(6S)), (c) β-D-glucuronic acid (GlcA), (d) 2-acetamido-2-deoxy-α-D-glucose (GlcNAc), and (e) α-L-iduronic acid (IdoA). These saccharides are present in decreasing amounts, usually in the order (b) > (a) > (d) > (c) > (e), and are joined by 1,4-glycosidic linkages forming polymers of varying sizes (Figure 1) (Mousa et al., 2007, Page, 2013). The most common disaccharide unit is composed of IdoA(2S)-GlcNS(6S). In total, there are at least 32 potential unique disaccharide units that together make this class of compounds one of the most information dense in biology (Nugent, 2000). Under physiological conditions, the ester and amide sulfate groups are deproto-

nated and attract positively charged counterions to form a heparin salt. It is in this form that heparin is usually administered as an anticoagulant.

Besides the uniform, highly sulphated domains which make up the larger part of heparin, there exist short unsulphated domains and more complex sequences, the most significant of which is the sequence with high affinity for the plasma serpin antithrombin (AT) (Mulloy, 2012) The structures of the repeating disaccharide motifs in heparin, and the pentasaccharide with high affinity for AT, are shown in Figure 2 (Gray et al., 2008).

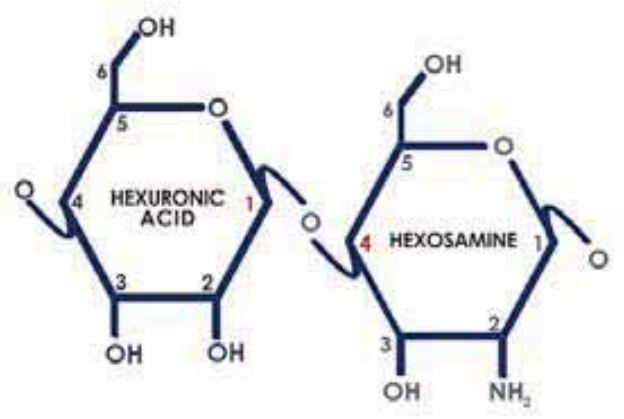

Figure 1. Generic disaccharide backbone structure of glycosaminoglycans with 1-4 linkage, common to heparin and heparan sulphate.
Numbers on the rings relate to the carbon position, hydroxyl, and amine groups can be sulphated and alternatively orientated. *Modified from Page et al. (2013) with permission.*

a) Heparin structure and its relationship to function

The anticoagulant activity of UFH *in vitro* is overwhelmingly dependent on the presence of the sequence with high affinity (HA) for AT. This sequence, characterized by its central, essential pentasaccharide motif containing the unusual 3,6 D-O-sulfated, 2-N-sulfated glucosamine residue, does not occur in every heparin molecule. In order to potentiate the inhibition of thrombin, a heparin molecule must contain the HA sequence and also have sufficient chain length to bind to both AT and thrombin. The combination of HA sequence with this extra chain length has been termed the "C-region", the "C" standing for Choay (Gray et al., 2008, Al Dieri et al., 2003, Hemker et al., 2003).

Full linear sequences for heparin (or heparan sulfate (HS)) molecules are not available – scarcely two molecules are alike – but an overall picture of the proportions of different residue and sequence types may be determined by several methods: degradative, such as enzymatic depolymerization and oligosac-

charide profiling (Sasisekharan et al., 2006); and non-destructive, such as monodimensional proton and carbon nuclear magnetic resonance (^{1}H or ^{13}C NMR) (Guerrini et al., 2001). Either type of assay can give an estimate for HA sequence content, but not of C-region. It is also possible to estimate the HA sequence content of a heparin sample by titration of the native fluorescence of purified AT, which is enhanced on binding to heparin (Gray et al., 2008, Al Dieri et al., 2003, Lin et al., 2001).

b) Three-dimensional structure and dependence on sequence

Like heparan sulphate (HS), heparin has two main domain types; those in which glucosamine residues are N-sulphated, alternating with IdoA (the "S" domains), and those in which glucosamine residues are N-acetylated and alternated with GlcA, having been unaltered by enzymes of post-polymerization transformation. In heparin, especially in commercial heparins, the S-domains overwhelmingly predominate, and are highly substituted with sulphate at C2 of iduronate and C6 of glucosamine. Unlike most polysaccharides, heparin behaves in solution as a rod-like molecule (Mulloy and Forster, 2000). A recent study using ultracentrifugation and X-ray scattering showed that relatively small fragments of heparin are almost rigid, and longer lengths a little more flexible (Khan et al., 2010). These results were in agreement with conformational studies of heparin by nuclear magnetic resonance (NMR), where data on distances between hydrogen atoms in the structure could not be interpreted on the basis of a globular or random-coil structure, but fitted very well for a linear, rod-like shape (Mulloy et al., 1993). That is not to say, however, that heparin is a static molecule. The iduronate residue is not, as are many hexopyranose sugars, stable in a chair form of its six-membered ring, but exists in a dynamic equilibrium between a chair form and a twisted skew-boat form, which may itself represent the average of a rapidly fluctuating ensemble of related structures. While variations in sulphate substitution were found to affect the overall conformation of heparin only moderately, they have a considerable effect on the conformational equilibrium of the iduronate residue (Mulloy et al., 1994, Mulloy, 2012, Ferro et al., 1990).

The solution structure of a heparin dodecasaccharide composed solely of six IdoA(2S)-GlcNS(6S) repeat units has been determined using a combination of NMR spectroscopy and molecular modeling techniques (Mulloy et al., 1993). Two models were constructed, one in which all IdoA(2S) were in the $^{2}S_{0}$ conformation (Figure 3 – A and B), and one in which they are in the $^{1}C_{4}$ conformation (Figure 3 – C and D). However, there is no evidence to suggest that changes between these conformations occur in a concerted fashion.

c) The size of heparin molecules

All heparin preparations are polydisperse linear polymers, so that their MWs cannot be described by a single number. A convenient way to express the MW profile of a polymer such as a heparin sample is to take the number average MW M_n and the weight average MW M_w. The ratio M_w/M_n (known as the polydispersity) expresses the spread of MWs in the sample. M_n for porcine mucosal UFH is about 12,000 to 16,000 Da and M_w about 17,000 to 20,000 Da, giving a polydispersity (M_w / M_n) of about 1.3–1.4. The polydisperse nature of heparin can be an important issue whenever a property of heparin depending on molar concentration is measured, including all measurements of binding and kinetic constants (Gray et al., 2008).

The number average MW, $\bar{M}_n$,is defined:

$$\bar{M}_n = \frac{\sum_i N_i M_i}{\sum_i N_i}$$

where N_i is the number of molecules at MW M_i.

The weight average MW, $\bar{M}_w$, is defined:

$$\bar{M}_w = \frac{\sum_i g_i M_i}{\sum_i g_i}$$

where g_i, is the weight of the sample at MW M_i.

The mean MW of heparin is around 12,000 to 15,000 Da, with individual polysaccharide heparin chains varying in MW from 3,000 to 30,000 Da with a mean of 15,000 Da, which corresponds to approximately 45 saccharide units (Hirsh and Levine, 1992, Andersson et al., 1976, Harenberg et al., 1989, Johnson et al., 1976).

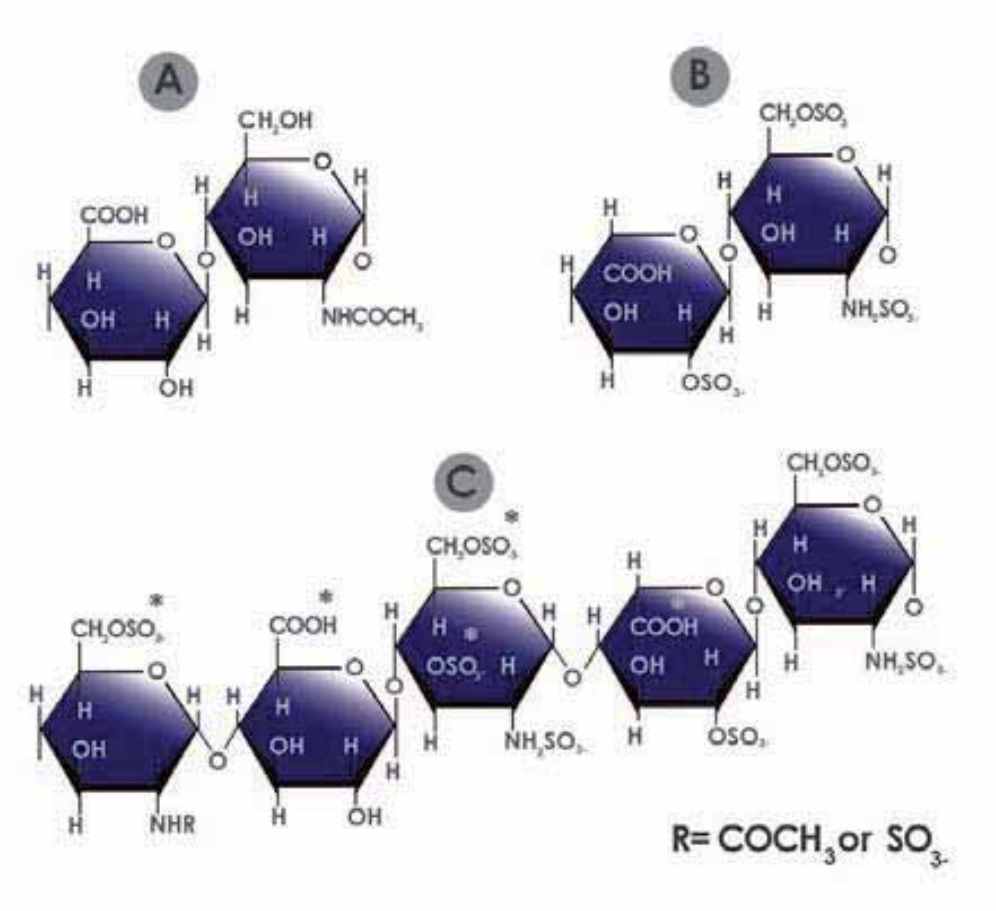

Figure 2. Structures of the repeating disaccharide motifs in heparin (and heparan sulphate), and the pentasaccharide with high affinity for AT.
A) The main repeating unit of heparan sulphate, –4)-β-D-GlcA-(1→4)-α-D-GlcNAc-(1–. This structure forms the precursor polysaccharide which is transformed by a series of enzymes into B) the main repeating unit of heparin -4)-α-L-IdoA($2SO_3^-$)-(1→4)-α-D-GlcNSO_3^-($6SO_3^-$)-(1-. The structural distinction between heparin and heparan sulphate is subtle; broadly, heparin contains 70% or more of structure B, heparan sulphate much less, with a higher proportion of the many intermediate structures arising from incomplete action of the postpolymerization enzymes. C) The pentasaccharide in heparin which is the minimal structure with high affinity for AT: - 4)-α-D-GlcNAc($6SO_3^-$)-(1→4)-α-D-GlcA-(1→4)-α-D-GlcNSO_3^- (3,6diSO_3^-)-(1→4)-α-L-IdoA($2SO_3^-$)-(1→4)-α-D-GlcNSO_3^- ($6SO_3^-$)-(1-. Substituents essential for this affinity are marked with an asterisk. *Modified with permission. Gray E, Mulloy B, Barrowcliffe TW. Heparin and low-molecular-weight heparin. Thromb Haemost (2008); 99: 807-18.*

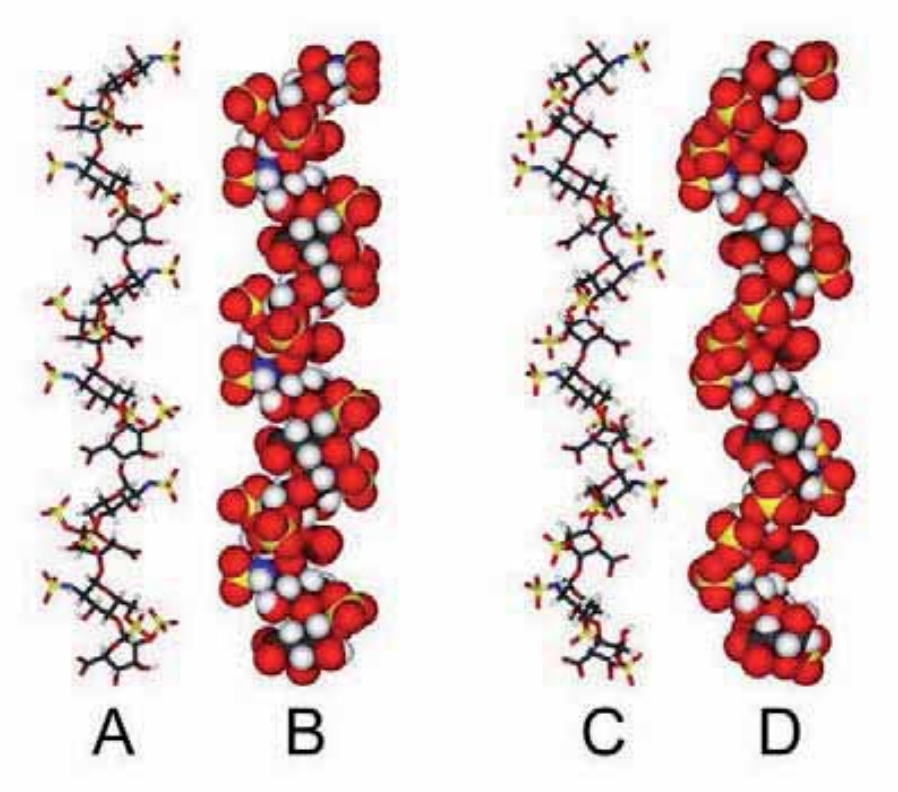

Figure 3. Models of three-dimensional structure of heparin.
A = 1HPN (all IdoA(2S) residues in $^{2}S_{0}$ skew-boat conformation), B = van der Waals radius space filling model of A, C = 1HPN (all IdoA(2S) residues in $^{1}C_{4}$ chair conformation), D = van der Waals radius space filling model of C (Jmol, 2011, Wikipedia-contributors, 2014).

2. Production and chemical processing of heparin

Commercial preparations of UFH are extracted from beef lung or porcine intestinal mucosa, which is rich in mast cells. The methods for the commercial preparation of heparin involve five basic steps: (1) preparation of the tissue at the slaughterhouse and its preparation for processing, (2) extraction of heparin from tissue at elevated temperatures and pressures using processes involving hydrolysis at alkaline pH aided by proteolytic enzymes, (3) recovery of raw heparin using anion exchange resin to enable the heparin-like GAGs to selectively adsorb onto the resin according to the charge density of the different GAGs, (4) purification of heparin through dissolution, filtration, oxidation and often by cation exchange chromatography, and (5) recovery of purified heparin after undergoing precipitation and vacuum-drying or re-dissolution in purified water, and filtration or freeze-drying. The yield of porcine intestinal heparin is typically 10-25 mg/g wet tissue corresponding to 30,000 to 50,000 IU/animal (Linhardt and Gunay, 1999).

Because preparations differ in potency, they are assayed biologically against an agreed international standard; doses are specified in units of anti-factor Xa (anti-FXa) activity rather than of mass (Rang and Dale, 2012). The anti-FXa assay involves monitoring the activity of FXa added to human citrated plasma with a synthetic FXa-directed substrate that changes color when cleaved by the enzyme (i.e. chromogenic). The higher the heparin concentration in the sample, the less the residual FXa activity detected. To determine heparin potency, residual FXa activity in the sample is compared with that detected in controls containing known concentrations of an international heparin standard and is expressed in international units per mg (IU/mg) (Goodman et al., 2011, Control, 2008).

3. Low molecular weight heparins and ultra-low molecular weight heparins

LMWHs are defined as a heterogeneous mix of polysaccharide chains of different lengths and weights derived from UFH by chemical or enzymatic depolymerization (Figure 4). The LMWHs are prepared from porcine mucosa-derived heparin and have approximately one-third the MW of the parent material (MW 3,000 to 10,000 Da) with a mean MW around 5,000 Da (Table 1). The different depolymerization processes also cause partial desulfation, reduction in charge density, and other changes in the heparin saccharide chains (Samama et al., 2012). Because they are prepared using different methods of depolymerization, the various LMWHs differ, at least to some extent, in their pharmacokinetic properties and anticoagulant profiles and in their recommended dosing regimens. Therefore, these drugs are not interchangeable on a unit-for-unit basis (Garcia et al., 2012).

Depolymerization of heparin yields low MW fragments that exhibit reduced binding to proteins and cells (Table 2). The reduced affinity for proteins and cells explains the anticoagulant, pharmacokinetic, and other biologic differences between UFH and LMWHs. Thus, compared with heparin, LMWHs have reduced ability to inactivate thrombin because the smaller fragments cannot bind simultaneously to AT and thrombin. Reduced binding to plasma proteins other than AT is responsible for the more predictable dose-response relationship of LMWHs (Anderson et al., 1993). Decreased binding to macrophages and endothelial cells explains the longer plasma half-life of LMWH relative to UFH, whereas reduced binding to platelets and platelet factor 4 (PF4) explains the lower incidence of heparin-induced thrombocytopenia (HIT) (Kelton et al., 2013). Finally, the decreased binding of LMWH to osteoblasts results in less activation of osteoclasts and less bone loss (Bhandari et al., 1998, Shaughnessy et al., 1995).

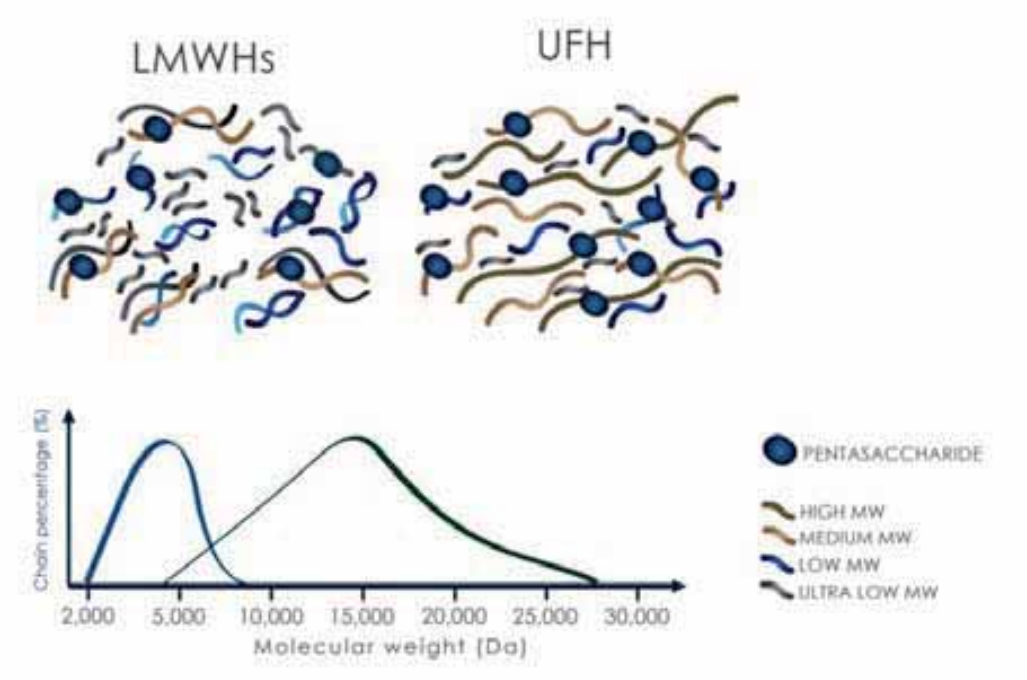

Figure 4. Varying molecular weight chain distribution of UFH and LMWHs.

Table 1. Biochemical and pharmacological characteristics of heparin and LMWH.

	Heparin	LMWHs
Mean MW (Da)	12,000 -15,000	4,000 -6,500
Saccharide units (mean)	40:50	13:22
Anti-FXa/anti-FIIa ratio	1:1	2:1 to 160:1
Bioavailability (IV)	30%	90-100%
Half-life (t ½)	1 h	3-4 h
Clearance mechanism	Hepatic clearance (first order kinetics)	Renal clearance
Dose-dependent clearance	Yes	No
Binding to endothelium	Yes	Low
Protein binding	Histidine-rich glycoprotein, fibronectin, vitronectin, platelet factor 4, von Willebrand Factor	Vitronectin
Inhibitable by Platelet Factor 4	Yes	No
Neutralization by protamine	Yes	Partial
Heparin Induced Thrombocytopenia	Rare	Very rare
Osteoporosis	Rare	Very rare

Table 2. Biologic consequences of reduced binding of LMWHs to plasma proteins and cells (Garcia et al., 2012).

Binding target	Biologic Effects	Clinical Consequence
Thrombin	Reduced anti-FIIa activity relative to anti-FXa activity	Unknown
Proteins	More predictable anticoagulant response	Coagulation monitoring unnecessary
Macrophages	Cleared through renal mechanism	Longer plasma half-life permits once-daily administration
Platelets and PF4	Reduced formation of HIT antibodies	Reduced incidence of HIT
Osteoblasts	Reduced activation of osteoclasts	Lower risk of osteopenia

a) Methods for preparing LMWHs

Many years of experience in the manufacture of pharmaceutical grade heparin have shown that it exhibits a surprisingly high level of physical and chemical stability, with a shelf life approaching a decade. Numerous processes have been used to prepare pharmaceutical grade heparins, involving the use of harsh conditions, including elevated temperature, pressure, shear, high ionic strength, acid, base, and organic solvents. Manufacturers, however, observed oxidative instability and microbial degradation of heparin, suggesting potential approaches to depolymerize it.

Heparin can be oxidatively broken down using a variety of oxygen containing reagents, like hydrogen peroxide, or by ionizing γ-irradiation. Each of these methods rely on the generation of oxygen radicals that are believed to act by oxidizing sensitive saccharide residues within the heparin polymer. Of the different oxidative methods, only hydrogen peroxide has been utilized to commercially prepare LMWHs (ardeparin sodium and parnaparin sodium) for clinical use (Linhardt and Gunay, 1999).

In addition to oxygen radical processes, it is possible to oxidatively depolymerize heparin through deamination. In these reactions, heparin is N-nitrosated, using either nitrous acid or another nitrosating reagent such as isoamyl nitrite, at the amino group of its N-sulfoglucosamine residues. Controlled deaminative cleavage is possible by controlling the process conditions (temperature, pH, time) or by limiting the amount of nitrosation reagent. The LMWH product formed using these controlled conditions is obtained in high yield, and has the appropriate chemical and biological properties. Several LMWHs prepared through deaminative cleavage are currently used clinically (Linhardt and Gunay, 1999).

Bacterial enzymes, heparin lyases I, II, and III (heparinases), are known to act on heparin. These enzymes have distinct substrate specificities and act in a random endolytic fashion through a β-eliminative cleav-

age mechanism. This enzymatic reaction can be mimicked chemically by esterifying the carboxyl group of the uronic acid residue and treating the resulting heparin ester with base, offering another possible method for heparin depolymerization. Two β-eliminative methods, one enzymatic and the other chemical, are used to commercially prepare LMWHs. In the enzymatic method, heparinase is used to depolymerize heparin. The depolymerization is stopped by removing or inactivating the enzyme. After recovery of the GAG from the enzyme and very low MW byproducts (i.e. disaccharides and tetrasaccharides), a LMWH is obtained that has the desired MW and activity properties. This method is used to prepare tinzaparin sodium. Chemical β-elimination can involve the direct treatment of heparin or its quaternary ammonium salt with base. Alternatively, the benzyl ester can be prepared by treatment of the benzethonium salt of heparin with benzyl chloride and base with heating. Under these conditions, chemical β-elimination takes place, resulting in a LMWH that contains an unsaturated urinate residue in the nonreducing end. Cleavage occurs specifically at iduronic acid, without preference for the presence or absence of a 2-O-sulfo group. This is the method used to prepare enoxaparin (Linhardt and Gunay, 1999).

LMWH products differ substantially from one another in their physical structure, biologic properties, and pharmacologic properties (Table 1). Although the average MW of LMWHs is similar, the proportion of the different saccharide chain lengths differs within each LMWH, and the end-residue alterations of the heparin fragments are specific to the depolymerization method (Table 4).

Efforts are underway to develop a scalable chemoenzymatic approach to synthetize ULMWHs (Xu et al., 2011, Linhardt and Liu, 2012, Masuko and Linhardt, 2012, Xu et al., 2012). Newer depolymerized heparin-based agents have been developed, which mimic the anticoagulant and anti-thrombotic effects of heparin. These include the ULMWHs such as AVE-5026 (semuloparin) and RO-14 (Viskov et al., 2009, Lima et al., 2013, Rico et al., 2011). The mean MW of ULMWHs ranges between 1,000 and 3,000 Da (Fareed, 2008).

b) Differentiation of LMWHs

The differentiation of LMWH molecules based on chemical structure may be characterized as primary, relating to molecular structure; secondary, relating to the “chemical fingerprint,” which differs with each depolymerization process; and tertiary, the “pharmacologic fingerprint” manifested in AT–binding sequences and biologic and pharmacologic effects that may be different in various populations. Modification of molecular structure and the chemical/pharmacologic/clinical effects (using β-elimination as the

example) are shown in Figure 5, Table 4, and Table 5. Structural differences in LMWHs result in differentiating tertiary effects (Fareed, 2008).

Several LMWHs have been developed (e.g. enoxaparin, dalteparin, tinzaparin, bemiparin, etc.). Manufacturers use anti-FXa data and AT activities to differentiate their LMWH products. These data also have clinical significance (e.g. the plasma anti-FXa activity at approved doses for DVT prevention). However, the clinical differentiation of LMWH agents has not been compared on an agent-versus-agent basis. Comparisons are based on available data from individual studies and data clearly indicate that LMWHs are not interchangeable one-for-one (Fareed, 2008). Each LMWH is a unique chemical entity with a particular MW distribution profile, specific anti-FXa: anti-FIIa activities, rate of plasma clearance, and recommended dosage regimen (Hirsh and Levine, 1992). All have slightly different properties and licenses for different risk situations (Blann and Khoo, 2009). Studies comparing different LMWHs using standard analytical methods have demonstrated considerable biochemical differences between the different products. The anti-FXa activities ranged from 80 to 160 international units (IU)/mg, and the anti-FXa: anti–FIIa ratios ranged from 2:1 to 80:1. The heparin cofactor II (HCII) affinity, thrombin generation inhibition, PF4 and protamine neutralization, cellular interactions, and release of mediators from the vascular endothelium also vary from product to product (see Mechanism of action of heparin and LMWHs and pharmacological effects) (Samama et al., 2012). Consequently, the results of clinical trials or pharmacokinetic studies cannot be extrapolated from one product to another (Fareed and Walenga, 2007, Racine, 2001, van der Heijden et al., 2000).

Table 3. Characteristics of LMWHs

Active Product (brand name)	Methods of heparin depolymerization	Average molecular weight (Da)	Anti-FXa (IU/mg)	Anti-FIIa (IU/mg)	Anti-FXa/ anti-FIIa ratio
Enoxaparin (Clexane®)	Alkaline β-eliminative cleavage of the benzyl ester of heparin	4,034	105	27	3.9
Tinzaparin (Innohep®)	β-eliminative cleavage by the heparinase enzyme	4,500	83	45	1.8
Nadroparin (Fraxiparin®)	Deaminative cleavage with nitrous acid	4,279	95	27	3.5
Reviparin (Clivarin®)	Deaminative cleavage with nitrous acid	4,395	130	40	3.3
Dalteparin (Fragmin®)	Deaminative cleavage with nitrous acid	5,663	130	58	2.2
Certoparin (Sandoparin®)	Deaminative cleavage with isoamyl nitrite	4,959	88	32	2.8
Parnaparin (Fluxum®)	Oxidative depolymerization with Cu2+ and H_2O_2	3,190	88	30	2.9
Ardeparin (Normiflo®)	Oxidative depolymerization with H_2O_2	6,000	100	30	2
Bemiparin (Hibor®)	Alkaline β-eliminative cleavage	3,600	80-120	5-20	8
Semuloparin (Mulsevo®)	Highly selective depolymerization by a phosphazene base	2,400	~160	~2	~80
RO-14	Selective depolymerization by β-elimination	2,200	80-140	~7	~20

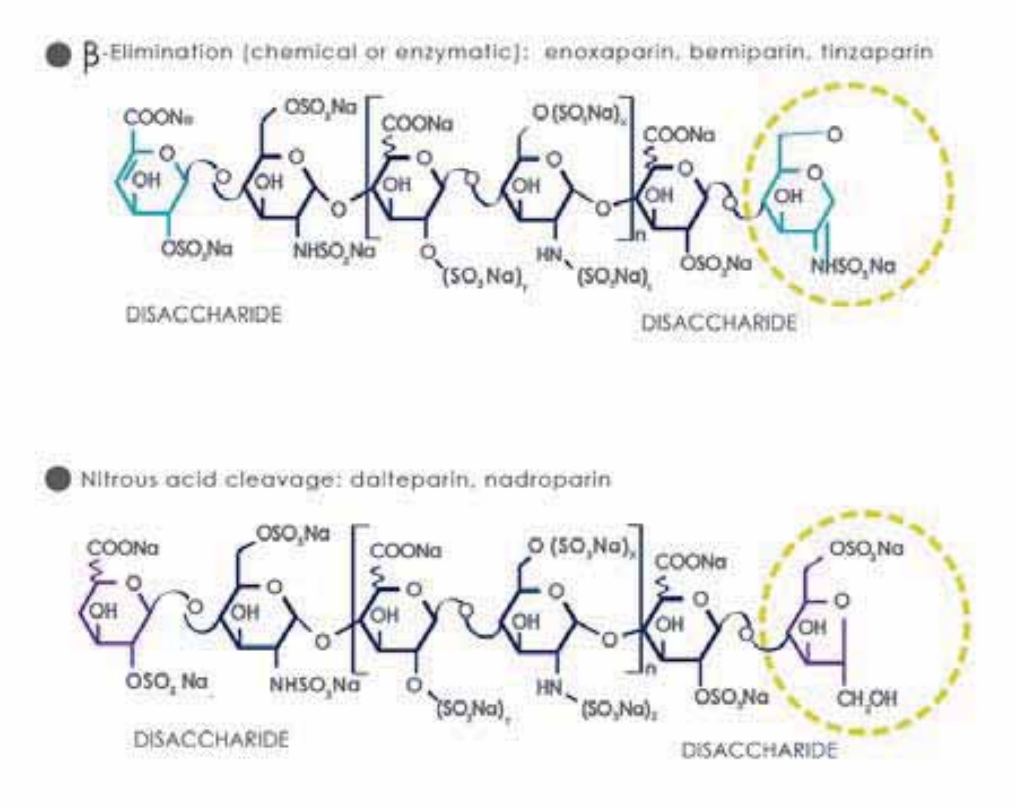

Figure 5. Primary differentiation of LMWHs: Structural modification at the cleavage point.
*Modified from Fareed J. Differentiation Among the Low-Molecular-Weight Heparins. Published in Low-Molecular-Weight Heparins: Patient Safety and Clinical Data Requirements for Follow-On "Generic" Biologic Compounds. CHEST Physician supplement 2008:1-15. Copyright © **(2008)** Elsevier Society News Group. All rights reserved.*

Table 4. Secondary differentiation of LMWHs
Depolymerization modifies the endogenous backbone*

Example: the β-elimination chemical fingerprints in the manufacturing of enoxaparin, bemiparin, tinzaparin			
	Enoxaparin	**Bemiparin**	**Tinzaparin**
Condition	Chemical β-elimination: basic media	Chemical β-elimination: basic media	Enzymatic β-elimination: neutral media
Reaction	Depolymerization of heparin benzyl ester by base	Depolymerization of heparin benzethonium salt by CTA^+, OH^-	Depolymerization of heparin by heparinase I
Main side reactions	➔ 1,6-anhydro ring, odd-numbered oligosaccharides ➔ 2-0 desulfation ➔ Epimerization in mannosamine	➔ 2-0 desulfation ➔ Epimerization in mannosamine	No side reactions
• **The 1,6 anhydro ring and odd-numbered oligosaccharides are both characteristic fingerprints of enoxaparin**			
** Modified from Fareed J. Differentiation Among the Low-Molecular-Weight Heparins. Published in Low-Molecular-Weight Heparins: Patient Safety and Clinical Data Requirements for Follow-On "Generic" Biologic Compounds. CHEST Physician supplement 2008:1-15. Copyright © (2008) Elsevier Society News Group. All rights reserved.*			

Table 5. LMWH tertiary effects

• **Anticoagulant effects**	• **Anti-inflammatory effect**
o Interaction with AT (anti-FXa, anti-FIIa activities), heparin cofactor II, platelet factor 4	• **Antiproliferative effects**
o Inhibition of factor VIIa generation	• **Immunologic effects**
o Release of mediators from endothelial cells (tissue factor pathway inhibitor, tissue plasminogen activator, plasminogen activator inhibitor)	• **Elimination half-life**
o Modulation of activated protein C	• **Renal clearance**
• **Antithrombotic effects**	• **Safety**
o Cellular interaction	• **Efficacy**
o Down regulation and release of cellular adhesion molecules	

** Modified from Fareed J. Differentiation Among the Low-Molecular-Weight Heparins. Published in Low-Molecular-Weight Heparins: Patient Safety and Clinical Data Requirements for Follow-On "Generic" Biologic Compounds. CHEST Physician supplement 2008:1-15. Copyright © (2008) Elsevier Society News Group. All rights reserved.*

4. Related compounds

a) Synthetic heparin pentasaccharide and its derivatives

(1) Fondaparinux

Fondaparinux (Arixtra®, MW 1,727 Da) was the first of a new class of antithrombotic agents distinct from LMWHs and UFH (Walenga et al., 2002). It is a chemically synthetic pentasaccharide that requires a 55-step block synthesis process. The pentasaccharide mimics the active site of heparin that binds to AT, exhibiting only FXa inhibitory activity leading to an inhibition of thrombin generation (Walenga et al., 1997). In contrast to UFH and LMWH, plasma anti-FXa activity corresponds directly to levels of fondaparinux. There is nearly complete bioavailability by the SC route, rapid onset of action, a prolonged half-life in both IV and SC dosing regimens (14-20 h), and it is renally excreted (Samama and Gerotziafas, 2003). It does not affect activated partial thromboplastin time (aPTT) or TFPI release. While fondaparinux was more effective than enoxaparin in reducing asymptomatic DVT in orthopedic surgery patients, hemorrhagic complications were either comparable to or more frequent than those for the LMWH (Samama and Gerotziafas, 2003). Fondaparinux has been used to treat HIT, since it has a low cross-reactivity with heparin antibodies (Warkentin, 2010), and its reported off-label use is up to 50% by some estimates (Schindewolf et al., 2014). However, there have also been very rare cases of HIT

reported where fondaparinux was implicated (Warkentin et al., 2007, Samama et al., 2012). Efficacy and safety of fondaparinux for HIT-treatment requires further evaluation (Schindewolf et al., 2014).

(2) Idraparinux and idrabiotaparinux

Chemical modifications of the original synthetic pentasaccharide increase the affinity to AT resulting in a more potent inhibition of FXa and longer half-life. Idraparinux (1,729 Da) is the first of these new oligosaccharides named ‘‘meta-pentasaccharides.’’ Idraparinux is obtained by incorporating an additional 3-O-sulfate group in the glucose moiety at the reducing end of the fondaparinux molecule, and additional methyl groups (Petitou et al., 2009, Petitou et al., 1997). This alteration in the chemical structure causes a significantly stronger binding to AT.

After SC injection, the half-life of idraparinux is about 80 h allowing a single injection per week. A dose-finding study has established the optimal dose given once a week to be comparable with warfarin for the treatment of DVT (Samama and Gerotziafas, 2003). The complete PK profile is complex and not well understood. After weekly SC administration of 2.5 mg idraparinux over 12 weeks, the elimination half-life increased to about 600 hours (h), and in clinical trials the elimination half-life was about 60 days after reaching steady state (Harenberg et al., 2008). In patients with DVT, once-weekly SC idraparinux for 3 or 6 months had an efficacy similar to that of UFH plus a VKA. However, in patients with PE, idraparinux was less efficacious than standard therapy. A 6-month extension study of thromboprophylaxis with idraparinux vs. VKAs showed comparable efficacy but idraparinux caused more bleeding (Buller et al., 2007b, Buller et al., 2007a). The same was true in patients with atrial fibrillation (Bousser et al., 2008).

Idrabiotaparinux (1,853 Da), a biotinylated form of idraparinux, can be specifically neutralized by the IV administration of avidin (Paty et al., 2010). The biotin arm does not interfere with the AT-dependent inhibition of FXa (Samama et al., 2012). A recent randomized, double-blind, non-inferiority study assessed the efficacy of idrabiotaparinux vs. warfarin in patients with atrial fibrillation at risk of stroke and systemic embolism. The efficacy outcome was the composite of all fatal or non-fatal strokes and systemic embolism. The study was terminated prematurely by the sponsor for strategic/commercial reasons, with 39% of the planned number of patients included and an average duration of treatment of 240 days. A similar incidence of stroke or systemic embolism was observed (idrabiotaparinux 1.5% per year vs. warfarin 1.6% per year; HR 0.98; 95% CI 0.49-1.66). The annual incidence of bleeding was 6.1% in the idrabiotaparinux and 10.0% in the warfarin group (HR 0.61; 95% CI 0.46-0.81) (Buller et al., 2014).

b) Danaparoid and related heparinoids

(1) Danaparoid

Danaparoid (mean MW 5,500 Da) has been in clinical use for more than 20 years for the prevention and treatment of thrombosis during pregnancy (does not cross the placenta and is not excreted in breast milk) and hemodialysis (Magnani and Gallus, 2006). Danaparoid is a mixture of low MW GAG components that result from the manufacture of UFH, mainly comprising heparan sulfate (84%), dermatan sulfate (12%), and chondroitin sulfate (4%), obtained without further depolymerization. Danaparoid acts as an anticoagulant primarily by catalyzing the inhibition of factor Xa in an AT-dependent fashion (Garcia et al., 2012). Danaparoid has a weaker anti-FXa activity than LMWH, but multiple other biologic targets of danaparoid exert profound effects on cellular responses (anti-inflammatory and anti-ulcer) and protease regulation by mechanisms other than those mediated by AT and HCII. Danaparoid has a mean half-life of approximately 18 h, and biologic and PK profiles distinct from UFH and LMWH. Although danaparoid was shown to be effective for the prevention of VTE in high-risk patients, it is no longer marketed for this indication (Garcia et al., 2012). Despite the sustained anticoagulant activity, the risk of bleeding with danaparoid is low (except in cardiopulmonary bypass where major bleeding can occur). Due to low cross-reactivity with heparin antibodies, danaparoid has been used to treat patients with HIT and it is the only agent that had been evaluated for HIT in a randomized clinical trial (Magnani and Gallus, 2006). Potential other clinical uses include anticoagulation for percutaneous coronary intervention and treatment of ACS, leg ulcers, cancer, sepsis, and acute ischemic stroke (TOAST-Investigators, 1998, Samama et al., 2012).

(2) Other heparinoids

Dermatan sulfate has been developed for the prophylaxis of VTE. Other heparinoids include heparan sulfate and sulfaminoheparosan derivatives derived from bacterial cell wall, but none of these drugs are in clinical use (Walenga and Fareed, 1991, Samama et al., 2012).

5. Mechanism of action of heparin and LMWHs and pharmacological effects

a) Anticoagulant effects

Heparin and LMWHs have no intrinsic anticoagulant activity. Heparin has a polycomponent anticoagulant mechanism inhibiting numerous coagulation factors, inhibiting platelet function, and enhancing the antithrombotic functions of vascular endothelium as well as fibrinolysis (Samama et al., 2012). To express its anticoagulant activity, heparin requires a plasma cofactor, the serine protease inhibitor, AT (Abildgaard, 1968, Brinkhous et al., 1939). Heparin inhibits coagulation, both *in vivo* and *in vitro*, by activating AT and accelerating the rate at which it inhibits various coagulation proteases. The most important anticoagulant activities are the inhibition of the coagulation factors Xa (Stuart-Prower factor), and thrombin (factor IIa) by heparin bound to AT (Hirsh and Raschke, 2004, Weitz, 1997).

By inhibiting FXa, heparin affects a critical juncture in the coagulation cascade (Figure 6). Heparin also affects thrombin-mediated coagulation mechanisms involving factor V, factor VIII, protein C, and thrombin activatable fibrinolytic inhibitor (TAFI). Moreover, heparin releases tissue factor pathway inhibitor (TFPI) (Fareed et al., 2000, Sandset et al., 1988), inhibits the release of P-selectin (a leukocyte cellular adhesion molecule that in the presence of tissue factor (TF) and microparticles, modulates the interaction between thrombosis and inflammation (Ramacciotti et al., 2009, Ramacciotti et al., 2010), impairs vascular barrier properties, and attenuates nitric oxide-mediated vasodilatation during dynamic changes in blood flow (Samama et al., 2012, VanTeeffelen et al., 2007). LMWHs also activate AT but have a reduced ability to inactivate thrombin because the smaller fragments cannot bind simultaneously to AT and thrombin. They also have lower binding properties to other proteins and cells that differentiate them from UFH. The effects of heparin on AT, HCII, and TFPI are explored below.

(1) Effects on antithrombin (AT)

AT (mean MW 55,000 Da) is a glycosylated, single-chain polypeptide composed of 432 amino acid residues. It is synthesized in the liver and circulates in plasma at an approximate concentration of 2.6 µM (Goodman et al., 2011). AT is one of the better known and studied members of the serpin superfamily that regulate the serine proteinases of the blood clotting cascade. AT plays a key anticoagulant role by preventing the activation of procoagulant proteinases except at a site of injury. Importantly, the activity of the serpin is itself regulated by the GAG cofactors heparin and heparan sulfate (Olson et al., 2010).

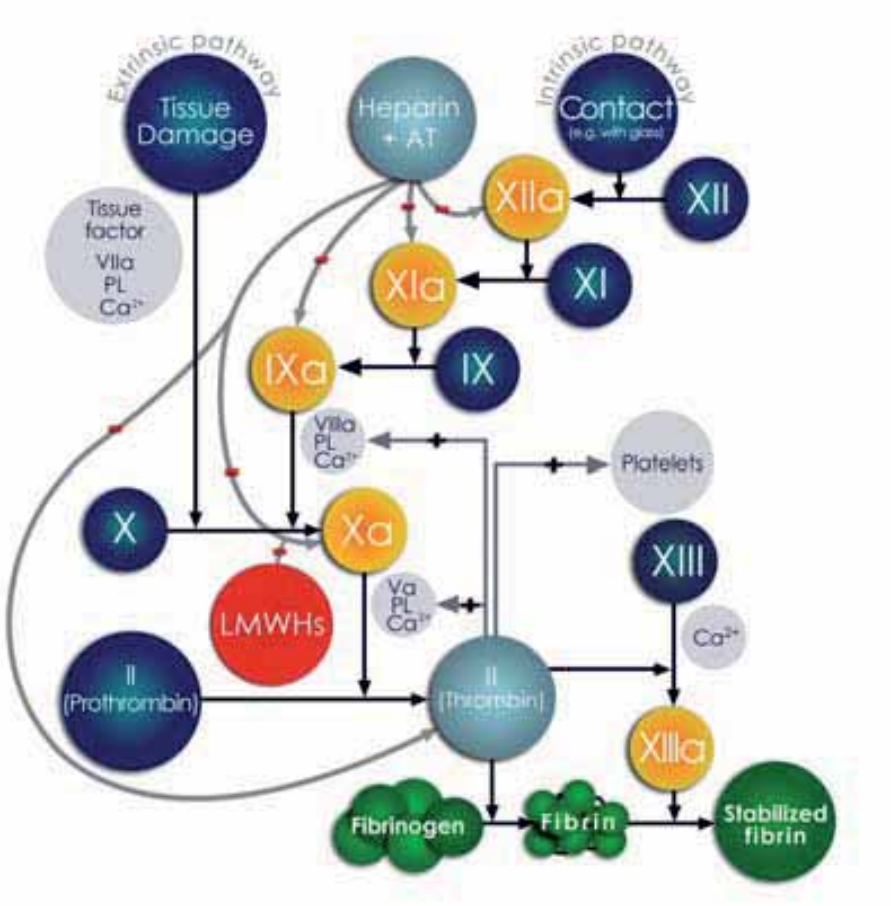

Figure 6. The coagulation cascade: sites of action of anticoagulant drugs. Heparins activate AT.

AT circulates in the bloodstream in a repressed reactivity state as a result of the serpin reactive center loop (RCL) providing only the minimal specificity determinants for recognizing the three main target proteinases, thrombin, FXa and factor IXa, and because favorable exosite interactions with the latter two proteinases are down-regulated by unfavorable interactions. Binding to heparin or heparin sulfate molecules of the luminal and subluminal blood vessel walls activate AT reactivity with its three target proteinases through allosteric changes that alleviate the unfavorable interactions with FXa and IXa, and strengthen exosite interactions with these proteinases. The GAGs also act as bridging cofactors to provide additional exosites for binding proteinases next to bound AT so as to augment AT-proteinase interactions (Olson et al., 2010).

AT regulates the activity of multiple blood clotting cascade proteinases, with only a minimal P1 Arg RCL determinant as bait, by acquiring proteinase binding exosite determinants on the serpin and by exploiting proteinase binding exosite determinants on heparin. This complex design serves to repress AT reac-

tivity until it is needed either at the blood vessel wall to prevent surface-mediated activation of blood clotting proteinases or at an injury site to control and localize blood clotting proteinase activity (Olson et al., 2010).

The primary mechanism of heparin and LMWHs is through association with AT. In the presence of heparin, inhibition of coagulation is accelerated 300 to 2,000-fold more than by AT alone (Samama et al., 2012). Heparin and heparan sulfate allosterically activate AT by binding the serpin through a common sequence-specific pentasaccharide that is only present in about one-third of the heparin molecules and is responsible for most of the anticoagulant effect of heparin (Andersson et al., 1976, Lam et al., 1976). The non-reducing end trisaccharide unit (3-0-sulfated glucosamine) of the pentasaccharide binds to lysine (Lys125 in helix D and Lys 114 in the P helix) and arginine (Arg129 in helix D) residues on AT and triggers a series of conformational changes in the protein (Atha et al., 1984, Lindahl and Pejler, 1987, Olson et al., 2010, Hirsh, 2007, Rosenberg and Lam, 1979). The binding is of high affinity, with a dissociation constant of ~50 nM at physiologic pH and ionic strength. The pentasaccharide is responsible for the bulk of the binding energy of full-length heparin and heparan sulfate chains, the longer GAGs binding AT with ~3-fold higher affinity than the pentasaccharide. The full-length GAGs may additionally activate AT as bridging cofactors, by binding both the serpin and proteinase, and promoting their interaction in a ternary complex. While allosteric activation by the sequence-specific heparin pentasaccharide selectively enhances AT reactivity with factors Xa and IXa, the bridging effect of full-length GAG chain augments AT reactivity with factors Xa and IXa and is solely responsible for upregulating AT reactivity with thrombin (Olson et al., 2010).

The interaction between heparin and AT produces an allosteric activation of AT. This binding causes a first set of induced-fit conformational changes in the heparin binding site and in the proteinase binding region, which increase heparin affinity and lock the serpin in the activated state. The conformation changes partially activate AT reactivity with factors Xa and IXa by causing a rearrangement of the allosteric core. The changes in the allosteric core alter the electrostatics of the surface around the RCL, so as to partly alleviate unfavorable interactions with bound factors Xa or IXa and strengthen favorable exosite interactions. A second set of induced-fit conformational changes further enhances the affinity of these interactions and increases the stability of the activated state. This causes further changes in the serpin surface electrostatics around the RCL, fully alleviates negative interactions with the serpin body, and further enhances positive exosite interactions. In summary, allosteric activation of AT results in stable complexes, increasing its affinity and allowing for interaction with the serine-active site of thrombin

and other coagulation enzymes, thereby irreversibly inhibiting their procoagulant activity (Figure 7) (Hirsh, 2007, Olson et al., 2010).

The conformational change triggered by the heparin-AT complex accelerates the rate of FXa inhibition by at least two orders of magnitude but has no effect on the rate of thrombin inhibition. To enhance the rate of thrombin inhibition by AT, heparin serves as a catalytic template to which both the inhibitor and the protease bind to form a ternary heparin/AT/thrombin complex (Figure 8) (Garcia et al., 2012). By inactivating thrombin, heparin not only prevents fibrin formation but also inhibits thrombin-induced activation of platelets and factors V,VIII, and XI (Beguin et al., 1988, Blajchman et al., 1989, Ofosu et al., 1987). Heparin-bound AT also weakly inhibits coagulation factors IXa, Xia, and XIIa, as well as the enzymes trypsin, plasmin, and kallikrein, and requires high concentrations of AT (~5 units) (Merlini et al., 1994). A weak inhibitory activity of this complex toward factor VIIa has been described, but the mechanism is unknown (Samama et al., 2012).

Inactivation of thrombin and other activated coagulation factors by heparin molecules is chain length dependent, whereas the inactivation of FXa only requires the presence of the high affinity pentasaccharide. Only heparin molecules composed of 18 or more saccharide units (MW >5400 Da) are of sufficient length for the binding and inhibition of thrombin (anti-FIIa activity). Thrombin is 10-fold more sensitive to inhibition than FXa, both because AT inhibits thrombin more rapidly than FXa, and because FXa is protected from inhibition by the AT/heparin when it is bound to phospholipid in the prothrombinase complex (Hirsh and Levine, 1992). Moreover, to inhibit thrombin, it is necessary for heparin to bind to the enzyme as well as to AT; to inhibit FXa, it is necessary only for heparin to bind to AT (Rang and Dale, 2012). With a mean MW of 15,000 Da, most of the chains of heparin are long enough to inhibit thrombin and FXa. The LMWHs increase the action of AT on FXa but not its action on thrombin, because the molecules are too small to bind to both the enzyme and inhibitor (Figure 9) (Goodman et al., 2011).

Thus, by definition, whereas heparin has an anti-FXa to anti-FIIa (thrombin) ratio of 1:1, the ratio for LMWHs ranges from 80:1 to 2:1 depending on the preparation (Hirsh and Levine, 1992, Martinez-Gonzalez et al., 2008, Viskov et al., 2009). This is because at least half of the LMWH molecules (mean MW of 5,000 Da, ~17 saccharide units) are too short to provide this bridging function and have no effect on the rate of thrombin inhibition by AT (Goodman et al., 2011). Importantly, these anti-FXa:anti-FIIa ratios are based on assays performed *in vitro* using platelet-poor plasma and may not reflect the anticoagulant profiles of these heparins in whole blood *in vivo* (Hirsh and Levine, 1992).

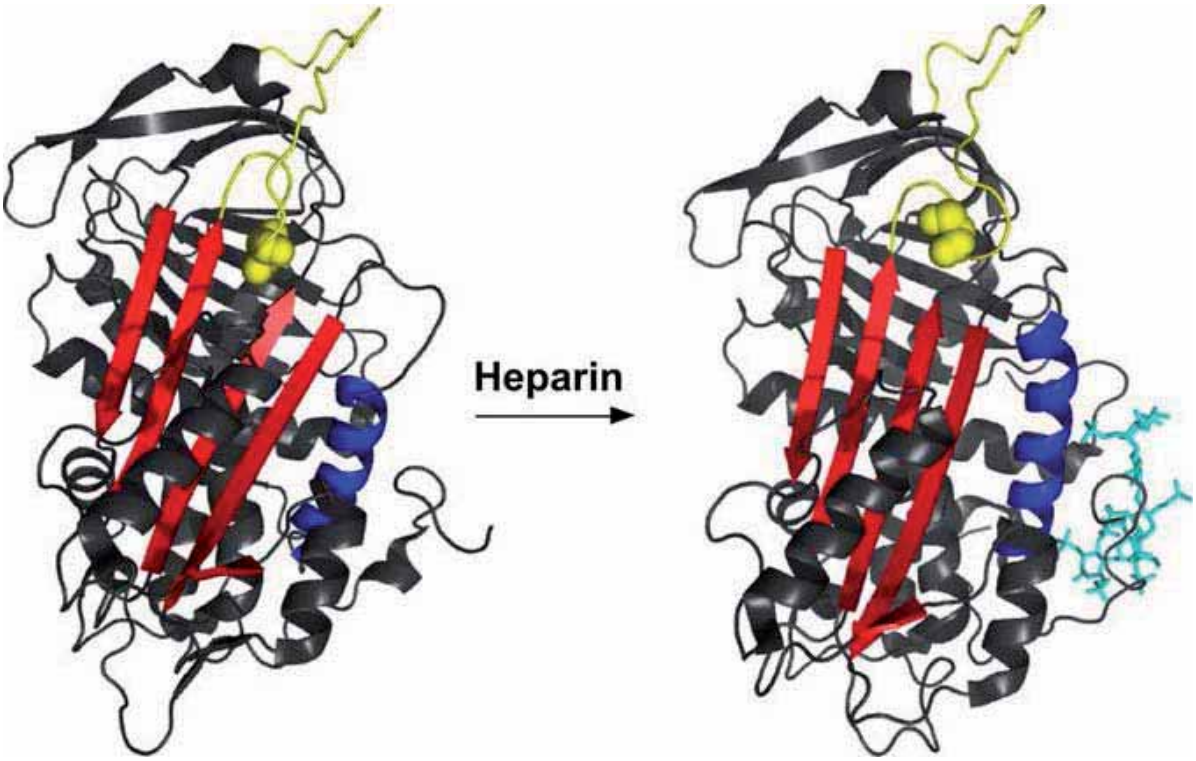

Figure 7. X-Ray structures of free and heparin pentasaccharide-complexed AT.
Ribbon representations of free AT on the left (pdb 1E05) and heparin pentasaccharide-complexed AT on the right (1E03) reveal the activating conformational changes induces in AT by the binding of the pentasaccharide (a mimetic shown in cyan stick representation). These include an extension of helix D and formation of a new P helix in the heparin binding site and an expulsion of the P14 serine residue (space-filling) of the serpin RCL (yellow), initially buried in β-sheet A (red) in free AT, from the A sheet and closing of the gap in the A sheet. *Reproduced from Olson et al. Molecular mechanisms of antithrombin-heparin regulation of blood clotting proteinases. A paradigm for understanding proteinase regulation by serpin family protein proteinase inhibitors. Biochimie 2010; 92(11):1587-1596. Copyright © (2010) Elsevier Masson SAS. All rights reserved.*

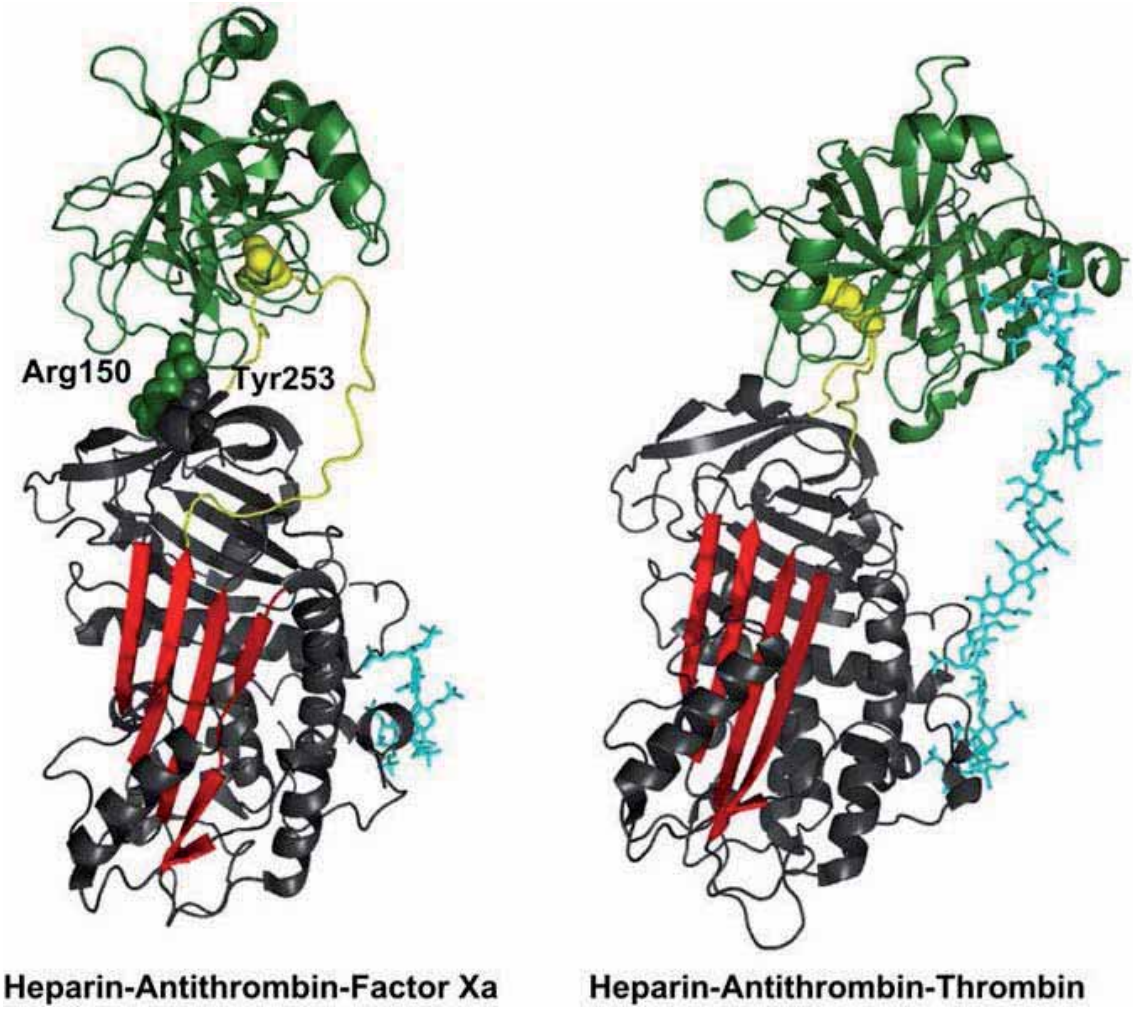

Figure 8. X-ray structures of heparin-AT-proteinase Michaelis complexes.
Shown in ribbon representation are the structures of ternary Michaelis complexes of heparin pentasaccharide-AT with S195A FXa on the left (pdb 2GD4) and heparin hexadecasaccharide-AT with S195A thrombin on the right (1TB6). The hexadecasaccharide contains a pentasaccharide mimetic, an uncharged saccharide linker and five terminal sulfated glucose saccharides. Heparin molecules are depicted in cyan (stick), proteinases in green and AT in gray. The A sheet is highlighted in red and the RCL in yellow. The critical Tyr253 exosite residue in strand 3C of AT and the complementary Arg 150 exosite residue of FXa as well as the P1 Arg are shown in space-filling representation. The structures reveal distinct orientations of the proteinase bound to the serpin RCL, with FXa bending downward toward the serpin body to form the critical exosite-exosite interaction and thrombin extending away from the serpin surface and bending in the opposite direction to interact with a heparin exosite on the extended polysaccharide chain. *Reproduced from Olson et al. Molecular mechanisms of antithrombin-heparin regulation of blood clotting proteinases. A paradigm for understanding proteinase regulation by serpin family protein proteinase inhibitors. Biochimie 2010; 92(11):1587-1596. Copyright © (2010) Elsevier Masson SAS. All rights reserved.*

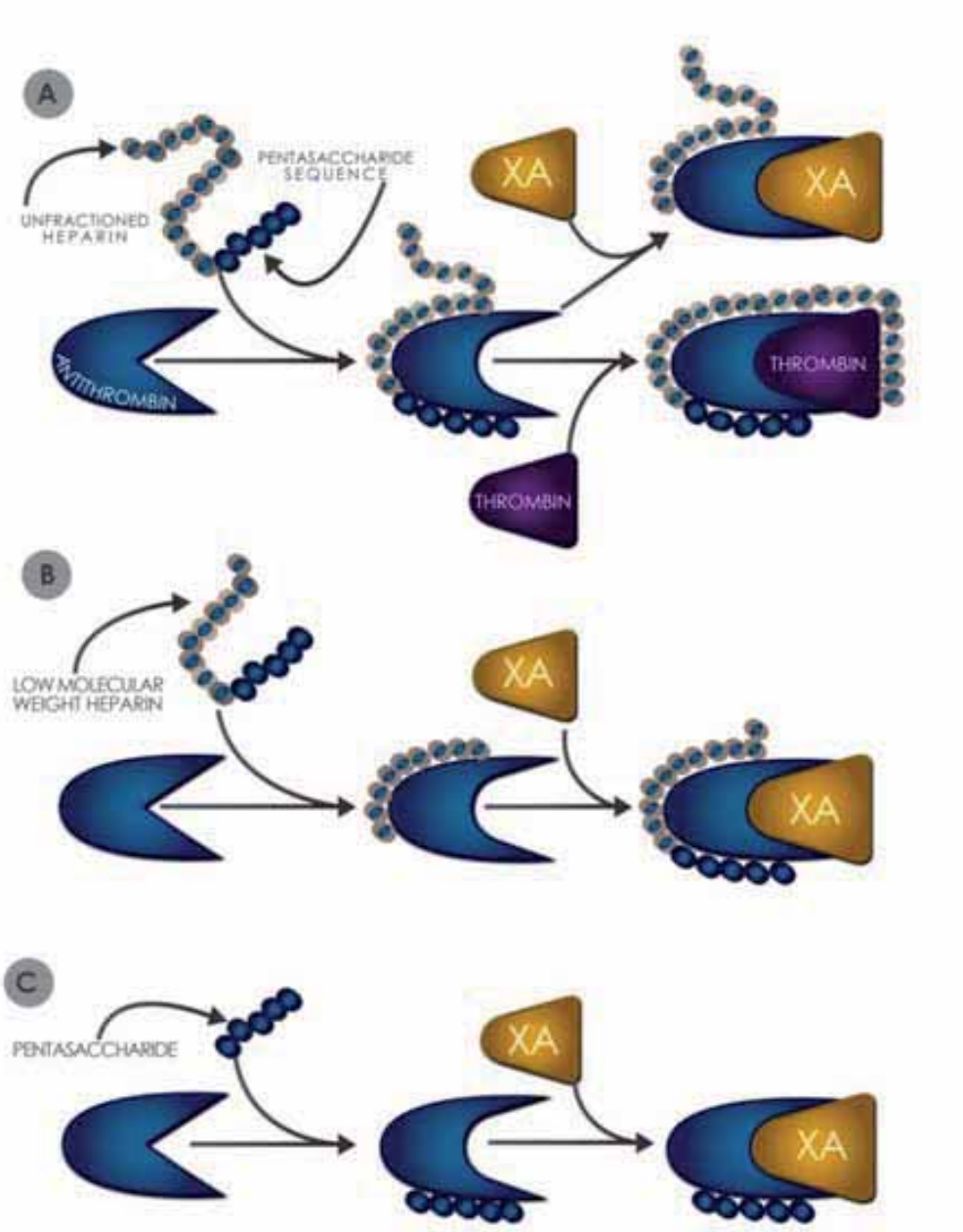

Figure 9. Mechanism of action of heparin, LMWH and fondaparinux, a synthetic pentasaccharide.
A. Heparin binds to AT via its pentasaccharide sequence. This induces a conformational change in the reactive center loop of AT that accelerates its interaction with FXa. To potentiate thrombin inhibition, heparin must simultaneously bind to AT and thrombin. Only heparin chains composed of at least 18 saccharide units (MW ~5,400 Da) are of sufficient length to perform this bridging function. With a mean MW of 15,000 Da, virtually all of the heparin chains are long enough to do this. B. LMWH has greater capacity to potentiate FXa inhibition by AT than thrombin because at least half of the LMWH chains (mean MW 4,500-5,000 Da) are too short to bridge AT to thrombin. C. The synthetic pentasaccharide, fondaparinux, accelerates only FXa inhibition by AT; the pentasaccharide is too short to bridge AT to thrombin.

(2) Effects on heparin cofactor II (HCII)

HCII (mean MW 60,000 Da), another serpin, is secreted from hepatocytes and circulates systemically at a concentration of ~1.0 µM (Ikeda et al., 2012). At concentrations higher than those usually administered clinically, the anticoagulant activity of heparin is also mediated by HCII (Hirsh and Levine, 1992, Garcia et al., 2012). HCII binds heparin and inhibits thrombin by forming a 1:1 stoichiometric complex with the enzyme. Thrombin interacts with the active site of HCII on the C-terminus and forms a covalent bond (Travis and Salvesen, 1983). This anticoagulant effect is specific to thrombin. Catalysis of HCII requires a higher concentration of heparin than that needed to promote thrombin inhibition by AT. The reaction is charge and chain length dependent, but pentasaccharide independent, and requires a minimum chain length of 24 monosaccharide units (MW ~7,200 Da) (Hurst et al., 1983, Maimone and Tollefsen, 1988, Petitou et al., 1988, Sie et al., 1988, Walenga et al., 1997). Pentasaccharide only promotes small increases in the HCII mediated AT activity at a relatively high concentration compared to that required for AT mediated FXa inhibition (Walenga et al., 1997).

HCII can be activated by a wide variety of agents including heparin, heparan sulfate, dermatan sulfate, pentosan polysulfate, and dextran sulfate (Scully et al., 1986, Scully and Kakkar, 1984, Yamagishi et al., 1984). Agents with relatively little sulfation, such as chondroitin 4-O- or 6-O-sulfate, keratan sulfate, and hyaluronic acid, do not activate HCII. High and low AT affinity fractions of heparin equally activate HCII if charge density is equal (Hurst et al., 1983, Samama et al., 2012). The rate of inhibition of thrombin by HCII is increased by three to four orders of magnitude by GAGs such as heparin, heparan sulfate, or dermatan sulfate. The predominant mechanism of GAG acceleration of thrombin inhibition by HCII appears to be allosteric, although longer GAG chains may have a higher affinity for thrombin than for HCII, introducing some template effects. GAG binding elicits a conformation change in HCII that enables a region of this serpin inhibitor to bind thrombin anion-binding exosite 1, a cluster of charged residues which also engages fibrinogen, thrombomodulin, factor V, and the carboxy-terminal portion of the leech thrombin inhibitor hirudin (Boyle et al., 2013).

HCII exerts various protective actions in the development of vascular and cardiac remodeling. It appears to be an independent inhibitory factor against peripheral arterial disease in elderly patients with cardiovascular risk factors and against coronary in-stent restenosis and carotid atherosclerosis (Aihara et al., 2009). More recently HCII has been proven to promote vascular endothelial function via an AMP-activated protein kinase-eNOS (endothelial nitric oxide synthase)-mediated pathway, leading to enhance-

ment of angiogenesis after ischemia. HCII might be a novel therapeutic target for patients with insufficient peripheral circulation (Ikeda et al., 2012).

(3) Effects on tissue factor pathway inhibitor (TFPI)

TFPI (MW 43,000 Da) is an endogenous coagulation proteinase inhibitor synthesized in the vascular endothelium. Three isoforms of TFPI are transcribed through alternative mRNA splicing: TFPIα, which contains an acidic amino-terminus followed by three tandem Kunitz-type protease inhibitor domains and a basic carboxy-terminus; TFPIβ, in which the Kunitz-3 and carboxy-terminus of TFPIα are replaced with a different carboxy-terminus containing a glycosyl phosphatidyl inositol (GPI) anchor; and TFPIδ, which is truncated following the Kunitz-2 domain (Girard et al., 1989, Holroyd et al., 2012, Broze and Girard, 2012). The plasma concentration of this 276 amino acid protein, is normally about 100 ng/mL (Lindahl et al., 1992). The microvascular endothelium is thought to be the principal source of TFPI and TFPIα is the predominant isoform expressed in humans. TFPIα, apparently attached to the surface of the endothelium in an indirect GPI-anchor-dependent fashion, represents the greatest *in vivo* reservoir of TFPI (Broze and Girard, 2012, Novotny et al., 1989). The plasma TFPI contains mostly 34,000 and 40,000-Da forms and the concentration is approximately 50 to 100 ng/mL (Sandset et al., 1988, Lindahl et al., 1990, Lindahl et al., 1992, Broze et al., 1994, Sandset and Abildgaard, 1991). Studies of normal tissues have detected TFPI protein in the endothelium of the microvasculature, smooth muscle cells, monocytes/macrophages, megakaryocytes/platelets, mesangial cells, fibroblasts, microglia, cardiomyocytes, and mesothelial cells (Broze and Girard, 2012). It has been reported recently that in healthy individuals, TF is exclusively associated with and expressed in circulating monocytes (Osterud, 2012).

Vascular injury-induced access of blood to TF leads to the formation of a TF-FVII/FVIIa complex and the triggering of blood coagulation (Osterud, 2012). TF and particularly the TF-FVII/VIIa complex, promote angiogenesis, both directly and indirectly, through regulation of thrombin generation and activation of intracellular signaling mediated by protease-activated receptors. TFPI regulates the initiation of the extrinsic coagulation pathway by producing FXa-mediated feedback inhibition of the TF/FVIIa catalytic complex. As such, TFPI is ideally situated to modulate the proangiogenic biological actions of TF/VIIa (Holroyd et al., 2012). The Kunitz-2 domain of TFPI is responsible for FXa inhibition and the Kunitz-1 domain is responsible for FXa-dependent inhibition of the factor VIIa/tissue factor catalytic complex (Broze and Girard, 2012). The third Kunitz-type domain and the carboxy-terminus of TFPI mediate its binding to heparin and cell surfaces including the endothelium (Wesselschmidt et al., 1993). The mechanism of

TFPI action is complex and involves the formation of a final quaternary inhibitory complex that contains TF/FVII, TFPI, and FXa (Broze, 2003). It is the formation of the TF-VIIa-TFPI-FXa complex that dampens ongoing coagulation (Holroyd et al., 2012). TFPI may also regulate angiogenesis independently of TF, through sequences within its polybasic carboxyl terminus (TFPI C), by directly blocking vascular endothelial growth factor (VEGF) receptor 2 activation and attenuating the migratory capacity of endothelial cells (Holroyd et al., 2012).

Heparin displaces TFPI bound to endogenous GAGs on the surface of the endothelium (Ariens et al., 1994, Warn-Cramer et al., 1993, Bara et al., 1993, Holst et al., 1993, Novotny et al., 1991, Brodin et al., 2004). Repeated heparin administration releases TFPI, showing no diminishing releasability with TFPI levels reaching 2- to 10-fold over baseline (Ariens et al., 1994). The release of TFPI induced acutely by heparin appears to involve the redistribution of TFPI from stores located near the plasma membrane (perhaps caveolae) to the cell surface, with the subsequent release into the media of a portion of the total cellular TFPI. During this process, the TFPI available at the surface of the cells remains unchanged or increases (Broze and Girard, 2012). UFH has been shown to deplete both circulating and endothelial-associated TFPI (Hansen et al., 1996), whereas LMWHs are responsible for a more selective decrease of TFPI (Hansen and Sandset, 1998). The main anti-FXa antithrombotic effect of heparin and LMWHs may be modulated by the release of TFPI from endothelium, among other secondary effects (Broze, 2003). Heparin increases the rate of inactivation of FXa and of TF/VIIa by TFPI (Abildgaard, 1993). Heparin-dependent inhibition of factor X activation requires both AT and TFPI, when unactivated factor TF/FVII is the stimulus (Walenga et al., 1997). LMWHs have a variable effect on TFPI release (Vogel et al., 1989, Mousa and Mohamed, 2004). Lower MW fractions of LMWHs stimulate a lower release of TFPI, leading to reduced antiangiogenesis (Mousa, 2013).

Neutralization of heparin by protamine sulfate results in a dramatic decrease in the plasma TFPI level (Harenberg et al., 1993, Hoppensteadt et al., 1995a). The anticoagulant activity of TFPI can be detected by the prothrombin time (PT), the aPTT, and clot-based anti-FXa assays (Samama et al., 2012, Kristensen et al., 1992, Lindahl et al., 1991a, Lindahl et al., 1991b).

Table 6. Anticoagulant effects of heparin.

Effect	Comment
Binds to AT and catalyzes the inactivation of thrombin and factors IIa, Xa, IXa, XIa and XIIa	Major mechanism for anticoagulant effect, produced by only one-third of heparin molecules (those containing the unique AT-binding pentasaccharide)
Binds to HCII and catalyzes inactivation of factor IIa	Requires high concentrations of heparin and is independent of the pentasaccharide
Binds to factor IXa and inhibits factor X activation	Requires very high concentration of heparin and is AT- and HCII-independent
Stimulates TFPI release and binds to AT and TFPI to inhibit factor A activation	Heparin-dependent inhibition of factor A activation requires both, AT and TFPI, when unactivated factor VII-TF is the stimulus

** Adapted from Garcia et al., (2012).*

(4) Other anticoagulant effects of heparin

(a) Effects on platelets

In vitro, heparin binds to platelets and, depending on the experimental conditions, can either induce or inhibit platelet aggregation (Eika, 1971, Kelton and Hirsh, 1980). Heparin inhibits serotonin-release induced by collagen in platelet rich plasma, whereas it increases adenosine diphosphate (ADP) -induced release (Fabris et al., 1983). *In vivo*, the efficacy of heparin is limited in part by its effect on platelets. Patients receiving intravenous heparin commonly experience an immediate, transient but mild non-immune-mediated thrombocytopenia, associated with biochemical evidence of platelet activation (McMahon et al., 2013).

Heparin has been shown to bind to platelet integrin αIIbβ3, a major platelet surface receptor for fibrinogen and other RGD (arg-gly-asp) -containing proteins. Like other integrins, αIIbβ3 is a bidirectional receptor that undergoes conformational changes and induces intracellular signaling upon ligand engagement (outside-in signaling), as well as upon cell activation by soluble proteins such as thrombin or ADP (inside-out signaling). Both processes contribute to a signaling cascade that ultimately results in profound morphological and biochemical changes in the platelet. Binding to αIIbβ3 induces platelet activation and aggregation, although the relationship between binding and activation is unclear. It has been recently described that binding of heparin to clusters of basic amino acids in the headpiece and/or leg domains of αIIb may stabilize the transition of αIIbβ3 to an open conformation with enhanced affinity for ligands, facilitating outside-in signaling and platelet activation (McMahon et al., 2013).

High MW heparin fractions with low affinity for AT have a greater effect on platelet function than low MW fractions with high AT affinity (Salzman et al., 1980). Both *in vivo* and *in vitro*, UFH appears to be a stronger stimulant of platelet activation than LMWHs. LMWHs have less effect on aggregation than UFH when the platelets are stimulated with ADP (McMahon et al., 2013), and they have almost no reactivity on collagen, epinephrine, and thrombin-induced platelet aggregation (Dunn et al., 1984, Samama et al., 2012).

Heparin also binds to and inhibits von Willebrand factor (vWF) (Sobel et al., 1991), resulting in a reduction of thrombotic risk in general and control of platelet–endothelium interactions in specific (Montalescot et al., 2000a). The weaker anti-platelet effect of LMWHs is associated with a lower bleeding risk, along with a lesser anti-thrombotic effect than for heparin (Samama et al., 2012).

The interaction of heparin with platelets (Fernandez et al., 1986) and endothelial cells (Blajchman et al., 1989) may contribute to heparin-induced bleeding by mechanisms independent of its anticoagulant effect (Garcia et al., 2012).

(b) Effects on fibrinolysis

A weak profibrinolytic effect has been described, but this was not clearly related to the MW of heparin (Vairel et al., 1983). Heparin regulates tissue plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI) release from endothelial cells, and the anti–FIIa activity of heparin induces a reduced activation of TAFI, a carboxypeptidase that inhibits fibrinolysis (Ammollo et al., 2009, Colucci et al., 2002). An increase in the porosity of the fibrin network has been demonstrated. These actions combined result in accelerated fibrinolysis (Lisman et al., 2003). At comparable anti-FXa *in vitro* concentrations, LMWHs exhibit different profibrinolytic activities (Collen et al., 2000). The clinical significance of these results remains to be established (Samama et al., 2012).

(i) In vitro measurement of anticoagulant effects

Measurement of coagulation factor activity using absolute physicochemical techniques is not possible (Raut and Hubbard, 2010). The measurement methods of anticoagulant activities of heparin and LMWHs were established over many years for UFH and are all based on its ability to delay the clotting time of animal or human plasma, except for the US Pharmacopoiea (USP) method in which the strength of the clot in sheep plasma is assessed. The European Pharmacopoeia (EP) method is based on measurement of the aPTT in sheep plasma, and the method used at the National Institute for Biological Standards &

Control (NIBSC) is similar, but using human plasma instead of sheep (Gray et al., 2008). The inherent variability of locally prepared and calibrated reference standards can give rise to poor agreement between laboratories and methods (Raut and Hubbard, 2010). Therefore, in 1999 the World Health Organization (WHO) initiated a harmonization program of measurement of anticoagulant activity of UFH and introduced a "global" method, which is based on a chromogenic assay measuring potentiation of inhibition of thrombin by purified AT (Gray et al., 2008). These International Standards define the International Unit for the analyte (Raut and Hubbard, 2010).

In all these methods, the activity of samples of heparin are measured by comparison with a reference standard with a known or assigned potency (Raut and Hubbard, 2010). The 1st International Standard for UFH was established by WHO in 1942, and this has been replaced at regular intervals – the current WHO Standard is the 6th (Control, 2013a). The EP and the USP both issue working standards. Despite the considerable technical differences, when different methods have been compared in international collaborative studies of UFH, the potencies given by the various methods have agreed to within a few percent (Gray et al., 2000). This is a corroboration of one of the basic principles of biological assays, i.e. that when standard and test are similar in composition ("like vs. like"), the potencies are largely independent of the method used. However, when the first samples of LMWH were assayed against the UFH Standard this was clearly not the case – there was large variability between laboratories, even when ostensibly using the same method. For instance, the coefficient of variation (CV) among seven laboratories carrying out a chromogenic method on the same LMWH sample was 43% (Barrowcliffe et al., 1985). There was also a tendency for non-parallelism between the log dose- response lines of the LMWH and UFH Standard, rendering many of the assays statistically invalid. In addition, as expected from the known properties of LMWH, there was a large difference in potency between methods based on inhibition of FXa, and those based on thrombin inhibition or delay of clotting times. The anti-FXa:anti-FIIa ratio differed widely among the various LMWH products, and continues to do so; the ratio ranges from 1.8 to 80 (Table 3) (Gray et al., 2008).

Because of all these problems, it became clear that the UFH Standard was unsuitable for measurement of the anticoagulant activities of LMWHs. It was therefore decided to establish a separate standard for LMWH, on the basis that "like vs. like" would give better reproducibility. It was recognized that LMWHs as a group were not identical to each other, and so the appropriate material for a standard had to be carefully chosen to be "in the middle" of the group with regard to its MW and anticoagulant properties. Following a preliminary study, two of eight LMWHs were identified as giving the least inter-laboratory

variability when used as a standard for assay of the other preparations, with CVs in the range of 4–14% (Barrowcliffe et al., 1985). These two preparations were then subjected to a large international collaborative study, and one of the materials was established by WHO as the 1st International Standard for LMWHs in 1986 (Barrowcliffe et al., 1988). Although WHO Standards are traditionally assigned a single potency, this would have been inappropriate in the case of LMWH, because of the large difference between potencies by anti-FXa and anti-FIIa assays (around 2.5 fold). Accordingly, the LMWH Standard was assigned two values, one for anti-FXa assays and another for thrombin inhibition assays (including APTT and anti-FIIa chromogenic methods) (Gray et al., 2008).

The aim of the LMWH standard is to allow reproducible and consistent measurements of in-vitro anticoagulant activities of the various products (Gray et al., 2008). The 1st, the 2nd, and the 3rd International Standard (Control, 2013b), which has been recently issued, have been used by manufacturers of all LMWHs to calibrate their products, with the exception of the synthetic pentasaccharide, fondaparinux, which is measured and dosed in mg. It should be noted that the anti-FXa activity of fondaparinux can be validly estimated against the International Standard for LMWH and it is found to be in the range of 800–900 IU/mg. In addition, the EP issues a working standard for LMWH, calibrated against the WHO Standard (Gray et al., 2008).

b) Non-anticoagulant effects of heparin

In addition to its well described anticoagulant effect, heparin and some polysaccharides derived from heparin have been found to interact with a wide variety of biological pathways and systems, raising the possibility that such drugs may have wider therapeutic uses than inhibiting coagulation. Heparin is a highly sulphated molecule, and due to this property has a very high negative charge that allows it to bind to a very wide array of positively charged biological materials (Page, 2013, Lever and Page, 2012). Heparin also contains three functional groups that occupy multiple sites on its individual saccharide units: –OSO3−, –NHSO3−, and –COO−. This structural diversity induces a diverse range of non-anticoagulant properties. Only 20% to 30% of GAG components produce anticoagulant activity by binding AT, the other 70% to 80% exhibit multiple biologic actions that are only partially understood (Nugent, 2000, Turnbull et al., 2001, Lane and Adams, 1993).

In mammals, heparin is present together with histamine in the granules of mast cells, which reside within mucosal and connective tissues suggesting that physiologically heparin may be involved in the regulation of inflammatory responses. Mast cells contain an array of inflammatory mediators packed

into their granules which are released on stimulation, and heparin has been found packed in conjunction with a range of cationic molecules, for example, chymase and tryptase. Many different proteins involved in the inflammatory cascade, including cytokines, growth factors, adhesion molecules, cytotoxic peptides, and tissue-degrading enzymes, are functionally dependent on heparan sulphate and have heparin-binding domains in their structure. This allows them to recognize and bind to heparin, in many cases limiting cellular activation and subsequent tissue damage and remodeling (Page, 2013, Lever and Page, 2012).

In addition to endogenous heparin being an anti-inflammatory agent, there are now many experimental and clinical studies demonstrating positive anti-inflammatory activities of heparin, suggesting that such activities could be exploited for therapeutic use (Page, 2013). Heparin's effects on angiogenesis, viral infectivity, cell function, wound healing and embryo implantation are also being actively being researched (Samama et al., 2012).

(1) Interactions with heparin-binding proteins

The list of proteins that bind heparin has grown to well over 100 (Nugent, 2000). Heparin-binding proteins can be divided into: 1) proteins that enhance the anticoagulant activity such as AT and HCII found in blood and released by heparin such as TFPI and lipase, and 2) proteins that inhibit the anticoagulant activity of heparin such as PF4, histidine-rich glycoprotein (HRG), protamine, and vitronectin. Some of these interactions are dependent on the MW of the heparin molecules, while others require calcium ions (Lijnen et al., 1983).

There are different types of heparin binding (Niewiarowski et al., 1979). The first type relates to a specific saccharide sequence of heparin, present on only one-third of the heparin chains; the second is covalent bonding, and the third depends on charge density and chain length (Samama et al., 2012).

Many well-known heparin-binding proteins are critically involved in the process of inflammation and include cytokines, growth factors, adhesion molecules, cytotoxic peptides, and tissue-degrading enzymes (Lever and Page, 2012).

(a) Histidine-rich glycoprotein

HRG is a single polypeptide chain α_2-plasma glycoprotein of approximately 75,000 Da, and has a plasma concentration of approximately 100-150 mg/L. It is synthesized in liver parenchymal cells, although

some studies suggest that HRG may also be produced by immune cells such as monocytes and macrophages, and by megakaryocytes (Jones et al., 2005). Second to AT, HRG is one of the most abundant heparin-binding proteins in human plasma (Poon et al., 2011).

HRG belongs to the group of proteins that neutralize heparin anti-coagulant activity, by preventing the formation of heparin-AT complexes that inhibit activated coagulation factors such as thrombin (Burch et al., 1987, Poon et al., 2011, Samama et al., 2012). HRG binds to UFH and LMWHs with high affinity, however, only the long-chain fragments of heparin have both anti-FXa and anti–FIIa activities completely neutralized (Lijnen et al., 1983, Lane et al., 1986). HRG requires interaction with saccharide sequences, in addition to the AT-binding pentasaccharide of heparin, in order to efficiently express its anti-heparin activity (Lane et al., 1986, Samama et al., 2012).

HRG, in the presence of physiologically attainable concentrations of zinc (Mori et al., 2003) and calcium (Burch et al., 1987), has the ability to reverse heparin-induced inhibition of smooth muscle cell proliferation by binding heparin *in vivo*. Thus, it is possible that HRG may be one of the unidentified factors that reverse the inhibitory effect of heparin on smooth muscle cell proliferation in arterial microenvironments *in vivo* (Hajjar et al., 1987, Jones et al., 2005). In fact, the high affinity of HRG for heparin has resulted in HRG being investigated as an alternative antidote for heparin overdose (Jones et al., 2005).

HRG can also regulate the AT activity of HCII (Tollefsen and Pestka, 1985) and can interact with fibrinogen and be incorporated into fibrin clots. Although it has been observed that HRG has no effect on the extent of fibrinogen conversion into fibrin by thrombin during the formation of fibrin clots, the presence of HRG did retard the rate of conversion of fibrinogen to fibrin (Leung, 1986, Poon et al., 2011).

(b) Vitronectin

Vitronectin, also called complement S protein, is a multi-functional protein found predominantly in the plasma (about 400 mg/L) and in the extracellular matrix (Chillakuri et al., 2010). Binding of vitronectin to UFH or LMWHs is associated with neutralization of the anticoagulant activity and, unlike with PF4 and HRG, is not chain-size dependent. However, this anti-heparin effect is of minor importance (Samama et al., 2012, Lane et al., 1987).

(c) Vascular cells

Heparin binding to vascular endothelium has been considered as evidence of an additional vascular mechanism of action of heparins (Barzu et al., 1986). The direct interaction of endothelial cells with heparin depends on the MW of the heparin (Tobelem, 1989). Affinity for endothelial cells increases as a function of charge density (degree of sulphation). Binding sites are not specific receptors for heparin (Barzu et al., 1986). Once attached, heparin molecules lose their anticoagulant effect and are internalized. Heparin also binds to smooth muscle cells, which could play a role in atherosclerosis (Clowes and Karnowsky, 1977, Karnovsky et al., 1989). LMWHs and pentasaccharide have the same beneficial activity against atherosclerosis, but shorter saccharide chains are inactive against smooth muscle cell growth in the vasculature (Castellot et al., 1986). Heparin interacts with endothelial cell growth factors (ECGFs), which results in attenuation of proliferation of vascular smooth muscle cells, a product of mitogenic activity (Castellot et al., 1982, Clowes and Karnowsky, 1977, Garcia et al., 2012, Samama et al., 2012, Schreiber et al., 1985).

(d) Platelet factor 4

PF4, also known as chemokine CXCL4, is a cationic 7,800 Da protein which form tetramers at physiological pH and ionic strength. PF4 is released from the alpha-granules of activated platelets as a complex with a chondroitin sulfate proteoglycan carrier. It disappears rapidly from plasma as it transfers to higher affinity heparan sulfate on endothelial cells, inhibiting local AT activity and thus promoting coagulation (Prechel and Walenga, 2013). PF4 is a potent inhibitor of UFH, but not of LMWHs (Hirsh and Levine, 1992). PF4 also prevents activation of HCII by both heparin and dermatan sulfate (Tollefsen and Pestka, 1985). PF4, which is released from blood platelets at sites of vascular injury, binds with high affinity to UFH. The ability of PF4 to neutralize the anticoagulant activities of heparin oligosaccharides is inversely related to the molecular size of the fragments (Lane et al., 1986). Heparin bound to PF4 produces an immunogenic response characterized by synthesis of IgG antibodies specific to the heparin-PF4 complex that can lead to the potential life-threatening disorder of HIT (Samama et al., 2012, Warkentin et al., 2003, Gogstad et al., 1983, Amiral et al., 1992, Lane et al., 1984, Gruel et al., 2013). Reduced binding of LMWHs to platelets and PF4 may explain the lower incidence of HIT (Warkentin et al., 1995).

It has also been recently demonstrated that PF4 stimulates vascular smooth muscle cell injury responses both *in vitro* and *in vivo*, in a mouse carotid ligation model. PF4 drives a vascular smooth muscle cell inflammatory phenotype including a decline in differentiation markers, increased cytokine production,

and cell proliferation. These effects are mediated, in part, through increased expression of the transcription factor Krüppel-like factor 4 (Shi et al., 2013).

(i) Lipase release

Lipoprotein lipase (LPL) and hepatic lipase (HL) are two critical enzymes in lipid and lipoprotein metabolism (Levy, 1958). LPL catalyzes hydrolysis of triglycerides (TGs) in chylomicrons and very low density lipoprotein (VLDL) particles. HL is synthesized by hepatocytes and bound to heparin sulfate proteoglycans at the surface of liver sinusoidal capillaries, which hydrolyzes TGs and phospholipids in chylomicron remnants, intermediate density lipoproteins (IDLs), and high density lipoproteins (HDLs) (Imamura et al., 2008).

LPL and HL are released after an IV injection of heparin or LMWH. Repeated injections of heparin or LMWH do not exhaust the release (Yu and Hill, 2006). Elevated levels cause a lipid-clearing lipolytic effect on the blood which is less for LMWHs than for heparin (Persson et al., 1985). Lipase release is associated with an anti-FXa activity that is not neutralized by protamine or PF4 (Samama et al., 2012, Imamura et al., 2008, Millot et al., 1987).

(2) Non-anticoagulant effects of heparin relevant to inflammation

(a) Effects on inflammatory mediators

Heparin can inhibit the activation of a range of inflammatory cells. Likewise, certain enzymes and cytotoxic mediators released from these cells, involved in propagation of the inflammatory response and subsequent tissue damage and remodeling, have also been shown to be inhibited by heparin. These include elastase (Walsh et al., 1991, Redini et al., 1988), cathepsin G (Redini et al., 1988), eosinophil peroxidase (Pegorier et al., 2006), eosinophil cationic protein (Fredens et al., 1991), major basic protein (Swaminathan et al., 2005), certain cytokines (Muramatsu and Muramatsu, 2008), and chemokines (Page, 2013, Lever and Page, 2012, Shute, 2012).

Many growth factors, including basic fibroblast growth factor (FGF) (Bono et al., 1997), and transforming growth factor-beta (TGF-β) (McCaffrey et al., 1989), both of which are involved in the regulation of smooth muscle proliferation (a feature of the tissue remodeling seen in diseases including asthma, atherosclerosis, and coronary stenosis), are bound by heparin. A long established property of heparin is that of inhibition of vascular smooth muscle cell proliferation (Clowes and Karnowsky, 1977), an effect which

is known to be independent of the anticoagulant actions of heparin and which extends to airway smooth muscle (Page, 2013, Lever and Page, 2012).

Heparin is also known to inhibit the degranulation of isolated human mast cells in response to a variety of stimuli, and hence inhibit the release of histamine (Inase et al., 1993). This effect is considered to be due to inhibition of inositol 1,4,5-triphosphate (IP_3) -dependent calcium release by heparin (Ghosh et al., 1988). The cytotoxic effects of the proinflammatory cytokine tumor necrosis factor (TNF)-α -activated eosinophils on endothelial cells are also markedly inhibited by heparin (Slungaard et al., 1990), as is the homotypic aggregation and chemotaxis of eosinophils in response to complement factor C5a, another inflammatory mediator bound by heparin (Teixeira et al., 1996, Matzner et al., 1984). Furthermore, UFH inhibits lipopolysaccharide-induced activation of endothelial cells via inhibition of p38 mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB) (Page, 2013, Lever and Page, 2012, Li et al., 2012).

Heparin has also been shown to bind to the surface of neutrophils (Leculier et al., 1992) and can inhibit their degranulation (Brown et al., 2003, Lever et al., 2007), homotypic aggregation (Brown et al., 2003, Bazzoni et al., 1993, Freischlag et al., 1992, Laghi Pasini et al., 1984), the production of superoxide anions, the activity of lysosomal enzymes, and the ability of neutrophils to activate platelets (Evangelista et al., 1992, Bazzoni et al., 1993). Furthermore, heparin is able to inhibit neutrophil activation in response to thrombin-stimulated platelet products, in addition to inhibiting thrombin-induced platelet aggregation (Piccardoni et al., 1996), and at high concentrations, platelet α-granule secretion is inhibited (Page, 2013, Lever and Page, 2012, Rohrer et al., 1992).

(b) Effects on cellular adhesion

Heparin has been shown to inhibit each of the different stages involved in inflammatory cell recruitment into tissues (Lever and Page, 2012). Heparin inhibits leukocyte-endothelial adhesion, both *in vitro* (Bazzoni et al., 1993, Silvestro et al., 1994, Smailbegovic et al., 2001) and *in vivo* (Lever et al., 2010), and limits the ultimate accumulation of cells in inflamed tissues, in response to both allergic and nonallergic stimuli (Page, 2013, Lever and Page, 2012).

Heparin is known to bind directly to several adhesion molecules expressed during inflammation, such as the selectins (P and L-selectins) that are predominantly concerned with the rolling stages of adhesion, without which firm adhesion and transmigration cannot proceed (Koenig et al., 1998, Fritzsche et al.,

2006, Giuffre et al., 1997). Heparin inhibits the release of P-selectin from platelets and endothelial cells and also binds to P-selectin and L-selectin, and to CD11b/CD18 (β-integrin adhesion molecule mac-1; macrophage 1), an important molecule for the firm adhesion of leukocytes to the endothelium (Samama et al., 2012, Lever and Page, 2012, Peter et al., 1999, Diamond et al., 1995). Indeed, the anti-metastatic effects of heparin can be ascribed (see effects of heparin in cancer), at least in part, to inhibition of P- and L-selectin function (Page, 2013, Lever and Page, 2012, Stevenson et al., 2005, Stevenson et al., 2007a, Borsig, 2007).

Heparin also binds to the platelet endothelial cell adhesion molecule-1 (PECAM-1), an IgSF-adhesion molecule thought to be involved in leukocyte transmigration due to its location at intercellular junctions on the endothelium (Watt et al., 1993). Similarly, heparin is able to bind directly to neuronal cell adhesion molecule (NCAM) (Cole et al., 1986). The resulting interaction is important for the physiological functioning of this protein in neuronal development (Page, 2013, Lever and Page, 2012).

In summary, heparin has the potential to interfere with each of the events involved in inflammatory cell recruitment, namely rolling, triggering, adhesion, and transmigration (Lever and Page, 2012).

(c) Inhibition of heparanase

Heparan sulphate proteoglycans (HSPGs) possess roles in growth and development, are key structural components of extracellular matrices, and are involved in the localization and bioactivity of a wide array of mediators, including enzymes, growth factors, cytokines, and chemokines (Powell et al., 2004, Turnbull et al., 2001). The endo-β-glucuronidase heparanase (HPSE1) is responsible for the site-selective cleavage of heparan sulphate chains, thus regulating the activity of the wide range of proteins that are functionally dependent upon HSPG. HPSE1 activity has been demonstrated in spleen, lymph nodes, leukocytes, and platelets, as well as in endothelial and smooth muscle cells. Moreover, HPSE1 activity in tumor cells has been found to correlate positively with metastatic potential (McKenzie, 2007, McKenzie et al., 2000). In the contexts of both inflammatory diseases and cancer, release of HPSE1 by tumor or inflammatory cells facilitates their diapedesis and migration to tissue sites, and promotes angiogenesis and tissue remodeling through release or activation of growth factors. Thus it is not surprising that this enzyme has also been reported as a potential target for novel anti-inflammatory drugs (Page, 2013, Lever and Page, 2012).

Heparin has long been known to be an inhibitor of HPSE1 activity (Bar-Ner et al., 1987), and it is also well established that heparan-degrading enzymes are released by certain leukocytes during the process of diapedesis (Lider et al., 1990, Matzner et al., 1992). When heparin is used at low doses in lymphocyte-driven inflammatory processes such as allergic encephalomyelitis (Lider et al., 1989, Willenborg and Parish, 1988), delayed-type hypersensitivity (DTH), and graft-versus-host reactions (Gorski et al., 1991, Naparstek et al., 1993), leukocyte infiltration into tissues is markedly inhibited and it has been suggested that this effect is via inhibition of HPSE1 by heparin. It has further been demonstrated that vascular endothelial cells also secrete heparanase, and that exposure of endothelial cells to proinflammatory cytokines upregulates this secretion, further suggesting an important role for this enzyme in inflammation (Page, 2013, Lever and Page, 2012, Chen et al., 2004, Edovitsky et al., 2006).

(3) Non-anticoagulant effects of heparin: Preclinical and clinical studies

(a) Effects on acute inflammatory reactions

In animal studies, pretreatment with heparin has been shown to inhibit eosinophil infiltration into the inflamed lung (Sasaki et al., 1993, Seeds et al., 1995, Seeds et al., 1993) and skin (Teixeira and Hellewell, 1993), neutrophil accumulation in the inflamed peritoneal cavity (Lever et al., 2010, Nelson et al., 1993), independently of anticoagulant activity (Seeds and Page, 2001, Lever et al., 2010), and to inhibit vascular permeability induced by certain autacoids or the bacterial formyl peptide (Jones et al., 2002). Additionally, platelet-activating factor-induced bronchial hyperresponsiveness was inhibited by heparin administration in rabbits, and in an allergic sheep model, inhaled heparin was found to inhibit the acute airway responses to inhaled allergen (Page, 2013, Lever and Page, 2012).

in a number of preclinical models, heparin has been found , to protect against ischemia-reperfusion injury. For instance, in a hamster dorsal skin chamber model, leukocyte-endothelial adhesion induced by ischemia-reperfusion is inhibited by heparin pretreatment, as is cardiac muscle damage (Kilgore et al., 1999, Becker et al., 1994). Furthermore, administration of heparin subsequent to transient focal cerebral ischemia in rats was found to reduce the degree of brain injury by inhibiting reperfusion-induced leukocyte accumulation (Yanaka and Nose, 1996). Heparin has also recently been suggested as a plausible agent for limitation of the delayed neurological injury that follows subarachnoid hemorrhage (Page, 2013, Lever and Page, 2012, Simard et al., 2010).

(b) Effects on human inflammatory diseases

Recently, heparin has shown potential in the management of clinical asthma (Diamant et al., 1996, Ahmed et al., 1993) and chronic obstructive pulmonary disease (COPD) (Venge et al., 1996, Brown et al., 2006). In patients with allergic rhinitis, topical heparin has been observed to reduce eosinophil recruitment into the nose following allergen exposure (Vancheri et al., 2001) and to be of potential value in the treatment of inflammatory bowel disease (Page, 2013, Petaja, 2011, Gaffney and Gaffney, 1996).

Importantly, in none of the above mentioned clinical studies, was heparin treatment found to elicit significant hemorrhagic side effects, either when administered systemically or locally. However, given that the anticoagulant actions of heparin appear not to be necessary for the majority of beneficial effects seen in models of inflammation, it seems likely that novel drugs which retain the anti-inflammatory effects of the parent heparin molecule, without the anticoagulant effects, will be useful in the management of inflammatory diseases that have been found to respond positively to the administration of heparin or low-molecular-weight heparin; for example, selectively 2,3-O-desulphated heparin, which is currently in clinical trials for COPD (Page, 2013, Lever and Page, 2012, Fryer et al., 1997).

(c) Effects of heparin in cancer

In the nearly 130 years since Trousseau first described migratory thrombophlebitis in cancer patients, thromboembolism has become a well-established presenting sign and complication of cancer. The coagulation system is activated in cancer and is further amplified by treatment with chemotherapy, radiation, or surgery. Hypercoagulation is documented in virtually all cancer types, albeit at different rates, and is the second leading cause of death in cancer patients. The relationship between clotting activation and carcinogenesis supports the view of cancer as a hypercoagulable state and holds implications for the development of thrombosis, enhancement of tumor growth, and risk of poor clinical outcomes. Although it is well recognized that cancer can activate the coagulation cascade, it is less well known that activation of the coagulation system may also support tumor progression. Additionally, platelet activation in cancer patients and its impact on tumor progression and metastasis further expand the role of the hemostatic system in malignancy. The problem of thrombosis in patients with metastatic diseases is a serious concern for clinicians (Mousa, 2006).

A link between improved survival and heparin treatment was suggested by Lebeau (1994) in small cell lung cancer patients receiving heparin or LMWH for the prevention or treatment of VTE, an observation

that launched fundamental research and clinical trials (Kakkar et al., 2010, Stevenson et al., 2005, Ludwig et al., 2006, Stevenson et al., 2007b, Goodger et al., 2008, Altinbas et al., 2004). In both animal studies and clinical trials, heparin has an antiproliferative property, whereby it inhibits cell growth, cell adhesion, cellular microparticle formation, smooth muscle cell proliferation, and tumor growth (Mousa, 2006, Lee et al., 2003, Zacharski and Ornstein, 1998, Folkman, 1985, Ferretti et al., 2006, Kakkar, 2005, Kakkar et al., 2004). Fondaparinux is inactive in these respects (Ludwig et al., 2006, Stevenson et al., 2005). Heparin has been postulated to have an anti-metastatic effect due to a decrease in neoangiogenesis, possibly mediated by binding to vascular endothelial growth factors, cytokines, and adhesion molecules (Collen et al., 2000, Mousa, 2013). Heparin could inhibit metastasis by interference with P- and L-selectin–moderated cell–cell interactions, and by inhibition of extracellular-matrix protease heparanase and angiogenesis (Borsig, 2007, Li et al., 2010, Ludwig et al., 2006, Nelson et al., 1993, Stevenson et al., 2005, Stevenson et al., 2007b, Akl and Schunemann, 2012). Other mechanisms have been attributed to the modulation of the angiogenic activity of fibroblast growth factor by heparin (Li et al., 2010, Closse and Hauser, 1978, Ferretti et al., 2006, Goodger et al., 2008, Kakkar, 2005, Zacharski and Ornstein, 1998) or by non-anticoagulant components that inhibit cancer growth (Samama et al., 2012, Casu et al., 2008).

The accumulation of metastatic tumor cells into tissues, like leukocytes, is dependent upon adhesion to the vascular endothelium and subsequent diapedesis, and many similarities exist between the processes utilized by inflammatory cells and tumor cells in this respect, including a dependency on platelet activation (Borsig et al., 2001, Vlodavsky and Friedmann, 2001, Pitchford et al., 2003). Heparin has been demonstrated repeatedly to reduce metastasis of carcinoma cells in animal models (Parish et al., 2001, Mousa et al., 2006, Sciumbata et al., 1996, Alonso et al., 1996, Nakajima et al., 1988). It has been suggested that the basis of the anti-metastatic effects of UFH lies in the inhibition of fibrin deposition around tumor cells, a factor considered to protect the cells from immune attack (Alonso et al., 1996). Nonetheless, many studies have found that fractions of heparin with much reduced anticoagulant activity, or none at all, also inhibit metastasis (Mousa et al., 2006, Sciumbata et al., 1996). Specific mechanisms thought to be involved in this effect include inhibition of heparanase activity (McKenzie, 2007), selectin function (Stevenson et al., 2007b, Borsig, 2007), and the TF pathway (Amirkhosravi et al., 2007). TF can promote angiogenesis and metastasis via mechanisms related and unrelated to plasma coagulation (Mousa, 2010). It has been suggested that the effects of heparin and related molecules in models of tumor growth and metastasis rely, at least in part, on the promotion of TFPI release from endothelial cells (Amirkhosravi et al., 2007). Regarding selectin function, clinically relevant levels of LMWH, with

respect to anticoagulation, have been shown to inhibit experimental metastasis in a manner that correlates with the ability to inhibit P- and L-selectin function; the pentasaccharide fondaparinux, which lacks this ability, was found to be without effect in the same assays, at levels normalized for anticoagulant activity (Stevenson et al., 2005), suggesting that it is not the anticoagulant effects *per se* of heparin that contribute most significantly to effects on tumor cell metastasis (Stevenson et al., 2007b). Moreover, mice deficient in both P- and L-selectin were found to be protected against experimental metastasis and, importantly, in these mice treatment with heparin conferred no further protection (Stevenson et al., 2007b) in contrast to the marked effects seen in wild-type animals in a range of studies (Page, 2013)

Protective effects of heparin in cancer models extend beyond the inhibition of metastasis to include those on tumor growth and angiogenesis. Heparin has long been known to be antiangiogenic and its inhibitory effects on heparanase are again well established (Vlodavsky et al., 2012). Growth-factor-induced endothelial cell proliferation is inhibited by UFH and LMWHs (Marchetti et al., 2008, Khorana et al., 2003, Takahashi et al., 2005). While standard LMWHs in this respect were found to be more potent than UFH, ULMWHs species, including the anticoagulant pentasaccharide fondaparinux, were without effect (Marchetti et al., 2008, Khorana et al., 2003). Moreover, antiangiogenic and anti-metastatic effects may further be mediated through interference with the chemokine system, which is known to be involved in these phenomena (Mehrad et al., 2007). Therefore, it is likely that heparins inhibit angiogenesis and metastasis via an array of mechanisms, including but by no means limited to heparanase inhibition (Page, 2013, Lever and Page, 2012).

Research on the clinical translation of the pre-clinical evidence presented above has yielded important but, at times, inconclusive evidence. Does antithrombotic therapy improve survival in cancer patients? This question has been addressed from different perspectives. Thromboprophylaxis with LMWH is well established in patients undergoing cancer surgery and hospitalized cancer patients, while outpatient prophylaxis remains contentious. LMWH are recommended over UFH and vitamin K antagonists for initial treatment and secondary prophylaxis (3-6 months) after cancer-related VTE (Kreher and Riess, 2014). Although only the CLOT[1] study (Lee et al., 2003) has demonstrated statistically significant reduction in symptomatic VTE using dalteparin compared with VKA, all major LMWHs are recommended in guidelines (Lyman et al., 2013, Lee et al., 2013).

[1] Randomized Comparison of Low-Molecular-Weight Heparin ver¬sus Oral Anticoagulant Therapy for the Prevention of Recurrent Venous Thromboembolism in Patients with Cancer

As it relates to anticoagulant therapy (oral or parenteral) in cancer patients without VTE there have been several studies, but no conclusive evidence to date. Three randomized trials have been performed assessing the influence of warfarin in survival in cancer patients who do not have overt VTE (Chahinian et al., 1989, Zacharski et al., 1984, Maurer et al., 1997). No significant increase in median survival was demonstrated in any of them, whereas major bleeding rates were higher than in the control groups (Cunningham et al., 2009).

Several clinical trials performed over the past decade evaluated the impact of LMWHs on survival and safety in cancer patients without VTE. One of these trials examined the role of UFH in small cell lung cancer (Lebeau et al., 1994), whilst other trials have focused on the potential benefits of LMWH in a heterogeneous variety of different malignancies (Klerk et al., 2005, Sideras et al., 2006, Altinbas et al., 2004, Kakkar et al., 2004). Although the FAMOUS[2] (Kakkar et al., 2004) and MALT[3] studies suggested that LMWHs may significantly increase median survival in patients with advanced solid tumor types and a favorable prognosis, definitive conclusions on the basis of these studies was not possible because of their small size, and the marked heterogeneity in the patients cohorts enrolled (Cunningham et al., 2009). Altinbas et al.(2004) further investigated whether LMWH may also influence survival in small cell lung cancer patients. They found a marked difference in overall tumor response rate (69.2% vs. 42.5%; P = 0.07) and median survival (13 months vs. 8.0 months; P = 0.01) favoring dalteparin vs. placebo; the beneficial effect on survival was observed in patients with either limited or extensive disease stages.

A meta-analysis of 11 clinical trials (Kuderer et al., 2007) evaluating the impact of anticoagulants on survival and safety in cancer patients without VTE showed that anticoagulation significantly decreased 1-year overall mortality (RR = 0.905; 95% CI 0.847-0.967). Relative risk (RR) for mortality was 0.877 (95% CI 0.789-0.975) for LMWH and 0.942 for warfarin (95% CI 0.854-1.040). Major bleeding episodes occurred less frequently in patients who received LMWH (Absolute risk difference (ARD) = 1%) compared with patients who received warfarin (ARD = 11.5%; P < .0001). A more recent meta-analysis (Akl et al., 2011a) evaluated nine RCTs (n = 2857) assessing the benefits and harms of parenteral anticoagulation (UFH or LMWH) in patients with cancer but no therapeutic or prophylactic indication for anticoagulation. The effect of heparin therapy on mortality was not statistically significant at 12 months (RR = 0.93; 95% CI 0.85-1.02) but it was statistically significant at 24 months (RR = 0.92; 95% CI 0.88-0.97). Heparin therapy was associated with a statistically and clinically important reduction in VTE (RR = 0.55; 95% CI 0.37-0.82).

[2] Fragmin Advanced Malignancy OUT-come Study
[3] Malignancy and Low Molecular Weight Heparin Therapy study

There were no statistically significant effects on major bleeding (RR = 1.30; 95% CI 0.59-2.88), minor bleeding (RR = 1.05; 95% 0.75-1.46) or quality of life.

One of the problems of many trials conducted in the past is that they have been small and underpowered. This problem was tackled in one of the most ambitious trials to date in this area, the SAVE-ONCO clinical study (Agnelli et al., 2012). This study enrolled over 3,200 patients with metastatic or locally advanced solid tumors, half of whom received semuloparin 20 mg QD. Clinically, semuloparin significantly reduced the incidence of VTE but had no significant effect on major bleeding or mortality. The investigators did not identify a subgroup effect by type or stage of cancer (Akl and Schunemann, 2012). Akl and Schunemann (2012) performed a pooled analysis -using data from the most recent Cochrane review (Akl et al., 2011a), the SAVE-ONCO trial, and another recent study that included 503 patients (van Doormaal et al., 2011). According to their results if 1,000 patients with cancer were to use a prophylactic dose of LMWH, over a period of 12 months death would be averted in approximately 30 patients, VTE would be averted in 20, and 1 would have a major bleeding episode. In summary, it does seem that some cancer patients could benefit from the use of a long-term prophylactic dose of LMWHs. Future research should further investigate the survival benefit of different types of anticoagulants in patients with different types and stages of cancer. The decision for a patient with cancer to start heparin therapy for survival benefit should balance the benefits and downsides and integrate the patient's values and preferences (Akl et al., 2011a).

(d) Effects of heparin on wound healing and tissue repair

Administration of heparin by inhalation, both alone and in combination with N-acetylcysteine, has been found to reduce the acute lung injury in the management of smoke inhalation injury in survivors of fire (Cancio, 2009, Toon et al., 2010, Miller et al., 2009). Application of heparin-binding epidermal growth factor-like growth factor (HB-EGF), a potent epithelial cell mitogen which is known to be up-regulated both in human burn tissue and during healing of experimental burn tissue (Cribbs et al., 2002), has been shown to promote and accelerate the reepithelialization of partial-thickness burn injuries specifically through potentiation of the expression of transforming growth factor-α, another member of the EGF family of growth factors involved in wound repair (Cribbs et al., 1998). Application of HB-EGF also promotes healing of ileal tissue following experimental reanastomosis surgery (Page, 2013, Lever and Page, 2012, Radulescu et al., 2011).

Moreover, topically applied heparin has been found to promote effective tissue repair in rabbit trachea, in a model of tissue healing following airway surgery, further suggesting that the immunomodulatory effects of heparin may be useful in the specific situation of tissue repair following localized injury (Page, 2013, Lever and Page, 2012, Sen et al., 2009).

(e) Effects on embryo implantation and trophoblast development

Pregnancy is a hypercoagulable state. Successful pregnancy outcome is highly dependent on satisfactory placental development and sustained placental function. Over the last decade, evidence has accumulated to suggest that some cases of recurrent pregnancy loss and later pregnancy complications are due to an exaggerated hemostatic response during pregnancy leading to placental thrombosis and infarction. Compromised placental perfusion caused by thrombosis may lead to placental infarctions and maternal complications of pregnancy. Reports published during the recent past suggest that recurrent pregnancy loss is associated with an increased risk of thrombosis (Chakraborty et al., 2013). Heparin seems to be effective in ameliorating pregnancy outcome in thrombophilic women with previous recurrent pregnancy loss, preeclampsia, intrauterine growth restriction, and sudden fetal death. A prophylactic effect of heparin treatment has also been proposed in terms of prevention of adverse pregnancy outcomes recurrence in women with a history of recurrent miscarriage, severe preeclampsia, placental abruption, low neonatal birth weight, and intrauterine fetal death not related to thrombophilia (Kupferminc et al., 2011), although literature in this field is quite controversial (Tersigni et al., 2012, Berker et al., 2011).

The molecular mechanisms by which heparin might exert its potential therapeutic effects on human reproduction are still not fully understood (Tersigni et al., 2012). The theory of placental thrombosis and infarction as a cause for early pregnancy loss was the original rationale for this thromboprophylaxis application of heparins. However, intravascular or intervillous blood clots are rarely found in first trimester placenta and decidua samples from patients suffering from early miscarriage. Moreover, heparins have also been shown to be effective in the treatment of women with recurrent miscarriage without apparent causes or inherited thrombophilia. In addition, LMWHs given in the luteal phase of the menstrual cycle seem to be beneficial to improve the implantation rate as well as the live birth rate in women with repeated implantation failure treated in an *in vitro* fertilization program. Taken together, these clinical observations suggest molecular effects of heparin beyond its classical anticoagulatory action (Fluhr et al., 2011a). Recent research indicates that both UFH and LMWHs inhibit inflammatory effects in the hu-

man endometrium (Spratte et al., 2013) and exert a cytoprotective effect by regulating trophoblast proliferation, invasion, and differentiation (Chen et al., 2012).

Fluhr et al. (2010) have shown that UFH and LMWHs modulate the decidualization of human endometrial stromal cells (ESCs) *in vitro*. Heparin dose- and time-dependently delayed the production of insulin-like growth factor-binding protein (IGFBP)-1 and amplified the levels of prolactin (PRL) and IGF-1. IGFBP-1 and PRL are the major products of the decidualized endometrium and are known to play an important role in endometrial differentiation and implantation. They act locally and show typical expression patterns during the second half of the menstrual cycle determining the narrow time frame of endometrial receptivity, called 'window of implantation'. The effects of heparin on the decidualization of human ESCs seem to be independent of its anticoagulatory function, but rather depend on the charge and the size of this polysulfated GAG. Therefore, highly sulfated polysaccharides with a MW >17,000 Da might be an interesting pharmacological approach for the therapy of endometrial pathologies, e.g. the treatment of women suffering from recurrent miscarriage or repeated implantation failure (Fluhr et al., 2011a).

LMWHs may also exert their therapeutic effects in human reproduction by inducing the expression of HB-EGF, reducing TNF-α induced decidual apoptosis, suppressing NF-κB-mediated secretion and expression of IL-8 and -6, and by inhibiting inhibit interferon gamma (IFN-γ) signaling in human ESCs (Di Simone et al., 2010, Fluhr et al., 2011b, Di Simone et al., 2012, D'Ippolito et al., 2012).

HB-EGF plays a role in blastocyst implantation and is down-regulated in preeclampsia and in hypertensive pregnancy disorders associated with defective extravillous trophoblast (EVTC) invasion. Defective placentation and severe preeclampsia are also features of the antiphospholipid syndrome (APS). Abnormal HB-EGF expression plays a pathogenic role in antiphospholipid antibody (aPL)-mediated defective placentation and placental APS tissue displays reduced expression of HB-EGF. Reduction of aPL-mediated HB-EGF is partly responsible for the defective placentation associated with APS. It has been shown that heparin inhibits aPL binding and restores HB-EGF expression in a dose-dependent manner, thus offering protection from aPL-induced damage (Di Simone et al., 2010). LMWHs are able to promote EVTC invasiveness by enhancing matrix metalloprotease-2 (MMP-2) activity and inducing the expression/secretion of HB-EGF and cysteine-rich angiogenic inducer 61 (Cyr61) in EVTC (Di Simone et al., 2012). This effect seems to be mediated by an increased DNA binding activity of activator protein 1 (AP-1) (D'Ippolito et al., 2012).

The pro-inflammatory cytokine, TNF-α, also plays a role in the pathogenesis of early pregnancy complications. UFH and LMWHs reduce TNF-α induced decidual apoptosis (Di Simone et al., 2012), and suppress the NF-κB-mediated secretion and expression of IL-8 and -6, as well as other molecules in decidualized and undifferentiated human ESCs. Heparins are able to inhibit TNF-α/NF-κB-mediated inflammatory effects in the human endometrium independently of its classical function as an anticoagulant (Spratte et al., 2013).

Uterine Natural Killer cells (NK) secrete high levels of chemoattractants, such as IFN-γ. IFN-γ dilates and thins the walls of maternal spiral arteries to enhance blood flow to the implantation site. This remodeling aids in the development of the placenta as it invades the uterus in its quest for nutrients. UFH, as well as LMWHs, are able to inhibit IFN-γ signaling in human ESCs and therefore might be clinically interesting agents to modulate the actions of this pro-inflammatory cytokine at the implantation site (Fluhr et al., 2011b).

The specific patient subpopulations that may benefit the most from LWMH therapy to prevent recurrent miscarriages have not yet been identified completely. Neither has the ideal scheme been described. It has been recently observed that combined therapy with aspirin-LMWH in hyperhomocysteinemic women with polycystic ovarian syndrome confers an added benefit in terms of prevention of recurrent pregnancy loss, compared to monotherapy in non-hyperhomocysteinemic patients (Chakraborty et al., 2013).

(f) Effects on bone metabolism

Many studies have verified that UFH can induce bone loss in subjects with normal bone (Meng et al., 2014). Heparin inhibits osteoblast formation and activates osteoclasts, promoting bone loss. The reduced binding of LMWHs to osteoblasts results in a lower incidence of activation of osteoclasts, and lower levels of bone loss (Bhandari et al., 1998, Shaughnessy et al., 1995).

In recent years, it has become clear that heparin's effects on bone metabolism are mediated via complex interactions with BMPs. BMPs are recognized for their ability to induce bone formation *in vivo* and *in vitro*. Heparin inhibits bone morphogenic protein-2 and -6 (BMP-2 and BMP-6) osteogenic bioactivities. Heparin inhibits BMP-2 osteogenic bioactivities by binding to both BMP-2 and the BMP receptor (BMPR) (Kanzaki et al., 2008), and it also dose-dependently inhibits BMP6-induced new bone and cartilage formation (Brkljacic et al., 2013). Paradoxically, heparin also enhances the biological

activities of BMP-2 by protecting BMP-2 from degradation and inhibition by BMP antagonists, and heparin alone enhances osteoblast growth, differentiation, and mineralization. It has also been shown that prolonged culture with heparin stimulated BMP-2 induced osteogenic activity via down-regulation of BMP-2 antagonists and inhibitory SMADs (intracellular proteins that transduce extracellular signals from transforming growth factor beta ligands to the nucleus where they activate downstream gene transcription). Therefore, it could also be expected that the appropriate timing of heparin administration could promote bone healing mediated by BMP-2 (Kanzaki et al., 2011). Heparin's effects on BMPs are complex and this is an area under active research.

(g) Other conditions that could potentially benefit from heparin treatment

Heparin, and the related molecule pentosan polysulfate, have been shown to have beneficial activity in the treatment of interstitial cystitis (Lilly and Parsons, 1990) and indeed the latter drug has been approved for such use in a number of countries (Page, 2013).

Another area is the potential use of heparin(s) to treat and prevent protracted labor. It has been suggested that the administration of LMWHs in pregnant women for the prevention of thrombosis, is associated with a shorter induction time to labor (Ekman-Ordeberg et al., 2010, Ekman-Ordeberg et al., 2009). This effect may be related to inhibition of IL-8 (Osman et al., 2003). Recent phase 2 clinical studies conducted with tafoxiparin, sponsored by Dilafor, have confirmed that this low-anticoagulant LMWH is effective in reducing the incidence of extended labor (http://www.dilafor.com)(Page, 2013).

Due to the ability of heparin to act as a mucolytic agent and/or via its effect on neutrophil activations, inhaled heparin could be used in the treatment of cystic fibrosis or in patients with COPD (Page, 2013, Serisier et al., 2006, King and Rubin, 2002).

6. Pharmacokinetic/pharmacodynamic profile of UFH and LMWHs

In clinical studies, pharmacokinetic data are necessary to obtain a reasonable pharmacodynamic analysis and therefore define the relationship between drug, patient, and pathology. Pharmacokinetic parameters rely on an accurate assessment of the drug concentration in the blood or target tissues. This is difficult to achieve with UFH and LMWHs because their main components, GAGs, are normally present in biological fluids and tissues. Radiolabelling with tritium (^{3}H), sulfur (^{35}S), or technetium (^{99m}Tc) to some extent obviates the problem (Laforest et al., 1991, de Swart et al., 1984, Psuja, 1988). However, as the UFH or LMWH is metabolized, breakdown products diffuse the radiolabel throughout the body, confus-

ing the pharmacokinetic profile. Moreover, a metabolite such as a tetra- or pentasaccharide may initiate, compete with, or synergize with some of the therapeutic effects of some UFH/LMWHs. Investigators are therefore forced to substitute the direct measurement of UFH or LMWHs with some of the characteristic anticoagulant activities, such as anti-FXa, anti-FIIa, aPTT, and TFPI activity. When measured through time, these biological markers are used to define the disposition profile of UFH and LMWHs, but do not necessarily reflect the clinical therapeutic effects of these drugs (Cornelli and Fareed, 1999, Walenga, 1993).

a) Biological markers to assess the pharmacokinetic / pharmacodynamic profile of UFH and LMWHs

(1) Anti-FXa assay

The most commonly used methodology to measure anti-FXa activity is the chromogenic assay. The heparin anti-FXa assay is a two-step chromogenic method based on the inhibition of a constant amount of FXa by the tested heparin in the presence of exogenous AT (stage 1), and hydrolysis of a FXa specific chromogenic substrate (e.g.CS11(65)) by the FXa in excess (stage 2) (ANIARA, 2010).

The anti-FXa chromogenic assay uses an FXa substrate onto which a chromophore has been linked (Figure 10). FXa cleaves the chromogenic substrate, releasing a colored compound that can be detected with a spectrophotometer and is directly proportional to the amount of FXa present (Walenga and Hoppensteadt, 2004). When a known amount of FXa is added to plasma containing UFH or LMWH, the heparin catalyzes FXa inhibition by forming an inhibitory complex with AT, rendering less FXa available to cleave the substrate. Therefore, the residual amount of FXa remaining in the sample is inversely proportional to the original amount of LMWH or UFH. Consequently, higher levels of LMWH or UFH in the sample lead to lower chromogenic intensity. By correlating this result with a standard curve produced with known amounts of heparin, we can calculate the heparin concentration in the plasma. The results are provided in concentration of anti–FXa (IU/mL). Some reagents for the anti-FXa assay use a patient's own AT, and other reagents add AT exogenously to make a complex of heparin and AT (Bates and Weitz, 2005, Szigeti, 2012, Barras, 2013, ANIARA, 2010).

A low level of anti-FXa may be seen if the specimen is not collected at the right time or if there was a delay in separation of the plasma from the cellular component of the blood. A high level of anti-FXa may

be seen if the specimen is contaminated with heparin (specimen drawn from lines containing heparin) (Szigeti, 2012).

The anti-FXa assay can also be used to guide the determination of therapeutic APTT ranges in the clinical management of UFH (Newall, 2013). The anti-FXa assay is suitable to monitor fondaparinux and danaparoid if the appropriate standard curve is used (Szigeti, 2012).

There are some pitfalls with the use of biological assays such as the anti-FXa assay. For instance, biological assays are subject to interference from clotting factors and inhibitors of coagulation, and there is considerable variation between the values obtained with different biological assays (Dawes and Pepper, 1982). Furthermore, when using anti-FXa assays for monitoring not only UFH, but also LMWH and fondaparinux, there is a lack of assay standardization and poor comparability between commercially available kits, with differences of up to 30% in UFH levels demonstrated (Funk, 2012, Kitchen et al., 2000). Some authors have also warned that the measurements of anti-FXa activity are overestimated since calcium is often omitted in the test systems, not considering that *in vivo* FXa is inactivated in the presence of calcium (Andrassy and Eschenfelder, 1996). AT deficiency affects the assay, however this is rare (Barras, 2013).

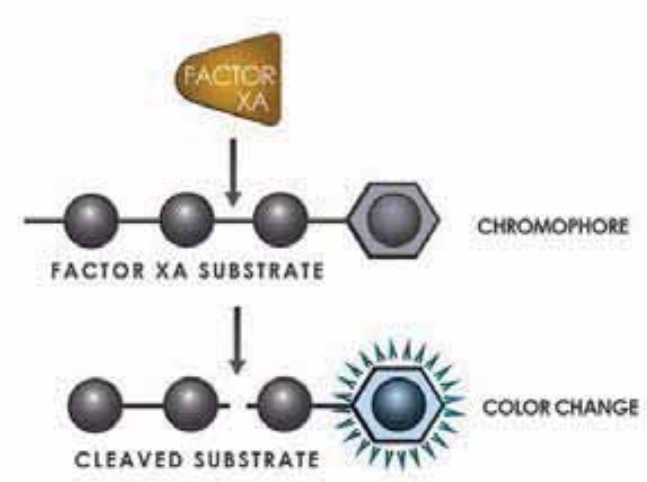

Figure 10. FXa heparin assay.
FXa is added to plasma containing synthetic FXa substrate that has chromophore attached to 1 end. When substrate is cleaved by FXa, chromophore undergoes color change, which can be quantified. Extent of color change is directly proportional to enzyme activity. If heparin or LMWH is present in plasma sample, it will promote FXa inhibition by AT rendering less FXa available to cleave substrate. By comparing result to extent of substrate hydrolysis in samples containing known amounts of heparin, we can calculate heparin concentration in plasma. *Adapted with permission from Lippincott Williams and Wilkins/Wolters Kluwer Health: Circulation (Bates and Weitz, 2005), © 2005*

(2) Anti-FIIa assay

A chromogenic assay is used to test UFH or LMWHs for their AT activity. The standard chromogenic anti-FIIa assay is performed in an artificial system consisting of highly diluted plasma to which AT is added (Iorio et al., 1994). The heparin anti-FIIa assay is a kinetics/competitive assay based on the inhibition of a constant amount of thrombin (FIIa) by the tested heparin in the presence of exogenous AT, and the simultaneous hydrolysis of a thrombin specific chromogenic substrate by remaining active thrombin. Para-Nitroaniline (pNA) is then released from the substrate. The amount of pNA released is then a relation of the residual thrombin activity. There is an inverse relationship between the concentration of heparin and color development.

(3) aPTT

aPTT is performed by first adding a surface activator (e.g. kaolin, celite, ellagic acid, or silica) and diluted phospholipid (e.g. cephalin) to citrated plasma. The phospholipid in this assay is called partial thromboplastin because TF is absent. After incubation to allow optimal activation of contact factors (factor XII, factor XI, prekallikrein, and high-molecular-weight kininogen), calcium is then added, and the clotting time is measured (Bates and Weitz, 2005).

Although the clotting time varies according to the reagent and coagulometer used, the aPTT typically ranges between 22 and 40 seconds. aPTT may be prolonged with deficiencies of contact factors; factors IX, VIII, X, or V; prothrombin; or fibrinogen. Specific factor inhibitors, as well as non-specific inhibitors, may also prolong the aPTT. Fibrin degradation products and anticoagulants (e.g. UFH, LMWHs, DTIs, or warfarin) also prolong the aPTT, although the aPTT is less sensitive to warfarin than is the PT (Bates and Weitz, 2005).

(4) TFPI assay

TFPI is being assayed with increasing frequency by researchers attempting to further understand the complexities of the coagulation system. There are a number of methods available for measurement of TFPI, however immunological measurement by an enzyme linked immunosorbent assay (ELISA) is the most common assay used (Summerhayes, 2013). The assay is a classical two-antibody sandwich assay with a monoclonal capture antibody directed against the third Kunitz-type domain of human TFPI, and a polyclonal rabbit peroxidase-labelled anti-human TFPI detecting antibody.

The assay is sensitive to full-length and carboxy-terminus truncated TFPI with intact third Kunitz-type domain, but not to two-domain TFPI. TFPI associated with lipoproteins is not or only sparsely detected, and TFPI in complex with FXa only partially measured (Ostergaard et al., 1997).

TFPI in standards and samples is sandwiched by the immobilized polyclonal antibody and biotinylated polyclonal antibody specific for TFPI, which is recognized by a streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added creating a blue colored solution. The reaction is stopped by the addition of a citrate stop solution, changing the color of the reaction solution to yellow. TFPI levels are determined by measuring sample solution absorbance and comparing against those of a standard curve (Nordic-BioSite).

b) Pharmacokinetic/pharmacodynamic profile of UFH

UFH is not absorbed through the gastrointestinal mucosa and therefore, must be given parenterally. The two preferred routes of administration for heparin are by continuous IV infusion or SC injection (Berkowitz, 1995). After a bolus IV injection of low doses of UFH, anti-FXa activity disappears according to a slightly convex curve, when the logarithm of the heparin anticoagulant activity is plotted as a function of time. This curve is almost always preceded by a rapid initial loss of heparin anticoagulant activity (de Swart et al., 1982). At higher doses, UFH disappears with a concave-convex pattern. Under continuous IV infusion, there is a non-linear relationship between the dose of UFH injected and the steady-state plasma concentration (Boneu et al., 1990). UFH anticoagulant activity estimated by the aPTT disappears faster than when estimated by the anti-FXa activity in the first phase. Higher anticoagulant levels with the anti-FXa assay than with the aPTT were also found on continuous infusion in healthy volunteers, as well as in patients treated for DVT or PE (de Swart et al., 1982).

After IV injection of 23 mg (3750 IU), mean peak values were 0.73 ± 0.12 IU/mL of anti-FXa activity and 0.62 ± 0.11 IU/mL of anti-FIIa activity. Mean area under the curve (AUC) values of anti-FXa activity were 36 IU*min/mL and 32 IU*min/mL of anti-FIIa activity (Bara et al., 1985). Half-life of UFH increased with increasing dose (Boneu et al., 1990, Olsson et al., 1963, Estes et al., 1969). The half-life of UFH after IV injection of 23 mg (3750 IU) was 35 min (Bara et al., 1985). It increased to 60 min with an IV bolus of 100 IU/kg, and to 150 min with a bolus of 400 IU/kg (Bjornsson et al., 1982, de Swart et al., 1982, Olsson et al., 1963).

UFH is cleared through a combination of a rapid saturable phase and a much slower first-order mechanism (Bjornsson et al., 1982, de Swart et al., 1982, Olsson et al., 1963). The saturable phase of heparin clearance is believed to be due to binding to endothelial cell receptors (Barzu et al., 1985, Mahadoo et al., 1978), plasma proteins, and macrophages (Friedman and Arsenis, 1974). Bound heparin is internalized and depolymerized (Dawes and Papper, 1979, McAllister and Demis, 1966). Binding of UFH to plasma proteins reduces its anticoagulant activity, because less is available to interact with AT, and the unpredictable anticoagulant response reflects the wide variability in plasma concentrations of HBPs (Chan et al., 2004). Some of these HBPs are acute phase reactants, the concentrations of which increase in ill patients, whereas others like PF-4 and vWF factor, are released during the clotting process (Weitz, 1997). The slower non-saturable mechanism of clearance is largely renal. The contribution of the two mechanisms to the clearance of heparin varies according to the dose delivered and the MW of the heparin preparation. At low doses, UFH is removed mainly via the saturable mechanism, while at higher doses the contribution of the non-saturable mechanism to its clearance becomes pre-eminent. This model accounts for the major pharmacokinetic properties of UFH (Olsson et al., 1963, Garcia et al., 2012, Boneu et al., 1990). The complex kinetics of clearance renders the anticoagulant response to heparin nonlinear at therapeutic doses, with both the intensity and duration of effect rising disproportionately with increasing dose (Garcia et al., 2012, Bjornsson et al., 1982, de Swart et al., 1982, Olsson et al., 1963).

After IV administration of small doses (10-5,000 IU) of UFH, the drug is excreted in urine as more or less desulphated molecules, part of them smaller than the injected heparin molecules. After larger doses of heparin, intact (not desulphated) heparin molecules with full preservation of their anticoagulant activity are excreted in urine (Dawes and Papper, 1979).

When the SC route is selected for delivery of treatment doses of UFH, the dose of UFH should be higher than the usual IV dose (Berkowitz, 1995), as absolute bioavailability of anti-FXa activity of UFH administered SC has been reported to be 28.6% (Bara et al., 1985). However, the absolute bioavailability of the anti-FXa activity increases with the dose delivered and tends toward 100% at high doses (Boneu et al., 1990).

After SC administration of 29 mg (4,850 IU) mean peak values were 0.039 IU/mL of anti-FXa activity and 0.035 IU/mL of anti-FIIa activity. Mean AUC values of anti-FXa activity were 12.9 IU*min/mL and 6 IU*min/mL of anti-FIIa activity. After SC administration of 29 mg (4,850 IU) half-life of UFH was 177 min (Bara et al., 1985).

Importantly, regardless of the route of administration, there are large inter-individual variations in the pharmacodynamics of UFH (de Swart et al., 1982, Bjornsson and Wolfram, 1982). For instance, men show more rapid clearance than women, and age, height, weight, and smoking also have an effect on heparin distribution and kinetics (Cipolle et al., 1981, Rosborough and Shepherd, 2004). It is likely that the sex difference is predominantly related to the differences in lean mass and blood volume between men and women of the same height and weight, as there is a clear relation between body weight and UFH requirements. A body weight based dose adjustment in obese and non-obese patients is generally recommended, but models including age, height and weight have also been proposed (Rosborough and Shepherd, 2004, Smith and Wheeler, 2010).

c) Pharmacokinetic/pharmacodynamic profile of LMWHs

It was first shown by Johnson et al.(1976) that the SC injection of a LMW fraction of heparin gave much higher and more prolonged blood levels by anti-FXa assay that either UFH or a high MW fraction, and this observation has been repeated many times (Barrowcliffe, 1995).

As with UFH, pharmacokinetic parameters of LMWHs are commonly determined by quantifying the biological activity. LMWHs have pharmacokinetic properties superior to those of UFH (Bara and Samama, 1990, Hirsh, 2007, Weitz, 1997, Bara and Samama, 1988, Bradbrook et al., 1987). They produce a more predictable anticoagulant response than UFH, show a higher absolute bioavailability than UFH, and a longer and dose independent half-life (Bara and Samama, 1990). The inhibitory activity of LMWHs against FXa persists longer than their inhibitory activity against FIIa, reflecting the more rapid clearance of longer heparin chains (Weitz, 1997, Boneu et al., 1988). LMWHs possess a decreased propensity to bind to the vascular endothelium and to plasma proteins, endothelial cells, and macrophages (Chan et al., 2004). Their reduced binding to macrophages explains why they are not cleared by hepatic mechanisms to the same extent as UFH, and why renal clearance is slower than hepatic uptake, thereby accounting for the longer plasma half-life of LMWHs (Weitz, 1997). LMWHs are mainly removed by non-saturable renal excretion (Boneu et al., 1990).

The half-lives of the various LMWHs and their bioavailability, as measured by their anti-FXa or anti-FIIa activities differ to a certain extent (Andrassy and Eschenfelder, 1996). Pharmacodynamic data cannot establish which LMWH is better for a particular clinical use, and the suggestion is to rely on those products that have the widest range of pharmacodynamic data and are supported by clear clinical evidence.

Direct comparisons should only be drawn between LMWHs given at the same dose, determined by the same test (Cornelli and Fareed, 1999).

Data on the time-course of anti-FXa and anti-FIIa activities, and effects on aPTT, TT, and TFPI are presented below. Given the mechanism of action of LMWHs, the most relevant biological marker is the anti-FXa activity and thus, the main focus of this section.

(1) Anti-FXa activity

A one-compartment model, while an oversimplification, can approximate the time-course of anti-FXa activity of LMWHs after SC administration (Cornelli and Fareed, 1999). The most relevant pharmacokinetic parameters obtained after administration of SC prophylactic and therapeutic doses of different LMWHs in healthy volunteers are summarized in Table 7.

The absolute bioavailability of the anti-FXa activity varies from 86-87% for SC dalteparin (Bratt et al., 1986) to 98% for nadroparin (Mismetti et al., 1998). Other LMWHs have a SC bioavailability in the range of 90 to 98% (91% for enoxaparin, 90% for tinzaparin, >90% for parnaparin and ardeparin) (Troy et al., 1997).

Thanks to the high proportion of shorter heparin chains, subcutaneously (SC) injected LMWHs are easily absorbed from SC tissue and have lower tendency to bind to endothelial cells than UFH (Samama and Gerotziafas, 2000, Bara et al., 1985). After SC administration, peak anti-FXa levels are present within 3 to 5 h after administration (Bara and Samama, 1990, Bradbrook et al., 1987, Handeland et al., 1990). Elimination half-life following SC administration is between 3 to 6 h and is dose independent (Barrowcliffe, 1995, Boneu et al., 1990). The similarity between T_{max} and elimination half-lives (t ½) demonstrates that the absorption constant (K_a) is important in determining the time course of blood concentrations, and indicates that the injection site may be a determinant for activity or that the excipients (e.g. buffers, preservatives) may influence the absorption rate (Cornelli and Fareed, 1999).

Peak plasma activity and AUCs of anti-FXa activities are positively correlated with the injected doses of LMWHs in a linear fashion (Samama and Gerotziafas, 2000), and repeated doses of LMWHs also increase the AUC (Andrassy and Eschenfelder, 1996). After repeated SC administration, steady-state activity levels are well predicted by single-dose pharmacokinetics (Sanofi-Aventis, 2011).

Following SC injection, the volume of distribution (Vd) of anti-FXa activity of most LMWHs is close to the plasma or blood volume (Samama and Gerotziafas, 2000, Andrassy and Eschenfelder, 1996). This parameter, determined by the measurement of the anti-FXa activity after SC injection, varies for the same LMWH between different authors. The apparent Vd for dalteparin is 40-60 mL/kg (about 3.6 L for a normal individual of 60 kg), and about 200 mL/kg (11.8 ± 6.5 L) for a dose of 2,500-5,000 anti-FXa IU. These variations are probably due to underestimation of the AUC, which arises from lower measurements of the anti-FXa activity. In contrast, the same authors demonstrated that after SC injections of 10,000 IU anti-FXa the Vd is 7.3 ± 2.0 L. After IV injection of 120 IU/kg anti-FXa of dalteparin, the apparent Vd is 3.4 ± 0.5 L (Samama et al., 2001).

Metabolism of heparin involves depolymerization and desulfation. LMWHs are eliminated by a nonsaturable renal mechanism following first-order kinetics (Samama and Gerotziafas, 2000). Clearance (Cl) and t ½ of LMWHs do not change as a function of the administered dose, as opposed to UFH. Following SC administration, elimination rate is the combined effect of elimination and diffusion into the blood stream from the injection site (Andrassy and Eschenfelder, 1996). After IV dosing of enoxaparin labeled with the gamma-emitter ^{99m}Tc, 40% of radioactivity and 8 to 20% of anti-FXa activity were recovered in urine in 24 h (Laforest et al., 1991).

(2) Anti-FIIa activity

Potentiation of thrombin inhibition (anti-FIIa activity and prolongation of aPTT) requires chains of minimum length of 18 saccharides (MW ~5,400 Da) in order for the same heparin chain to bind both AT and thrombin (Barrowcliffe, 1995, Lane et al., 1984). Due to their lower mean MW distribution, LMWHs exhibit very low anti-FIIa activity and monitoring the plasma kinetics of the anti-FIIa activity is rather difficult. Anti-FIIa activity has been shown to be about three to eight times lower than the anti-FIIa activity of UFH (Samama and Gerotziafas, 2000). The bioavailability of LMWHs, as determined by their anti-FIIa activity, is around 60-70% (Andrassy and Eschenfelder, 1996).

The SC bioavailability of LMWHs, as determined by their anti-FIIa activity, is around 60-70%. Following SC administration plasma anti-FIIa levels increase in a dose-dependent fashion (Andrassy and Eschenfelder, 1996). Anti-FIIa levels disappear faster than anti-FXa levels; depending on the dose they are detectable for 2-4 h following IV injection. This might be due to the fact that, in contrast to anti-FXa, anti-FIIa is neutralized by PF4. Therefore, the ratio of anti-FXa:anti-FIIa activity increased with time,

which indicates different clearances for both activities. The Vd for anti-FIIa activity is larger than for anti-FXa, which is due to increased protein binding (Andrassy and Eschenfelder, 1996).

After a single SC injection of the LMWH doses used for prevention of DVT, plasma anti-FII activities are weaker, exhibit higher inter-individual variability and are cleared more rapidly from blood than the respective anti-FXa activities. No measurable anti-FIIa activity was detected after injection of enoxaparin 2,000 IU, in contrast to dalteparin 2,500 IU, nadroparin 3,075 IU, and enoxaparin 4,000 IU (Collignon et al., 1995). A_{max} of anti-FIIa activity has been reported to be higher for dalteparin than for enoxaparin. Reviparin is comparable with enoxaparin in terms of its anti-FIIa activity. Tinzaparin has both a lower A_{max} for its anti-FXa and anti-FIIa levels (Andrassy and Eschenfelder, 1996). SC injection of bemiparin exerts only minimal anti-FIIa activity (Depasse et al., 2003, Falkon et al., 1995b).

(3) Effects on aPTT and TT

LMWHs modestly prolong the aPTT or thrombin time (TT), compared with UFH (Samama and Gerotziafas, 2000, Bara et al., 1985). Among the different LMWHs, dalteparin induces the greatest prolongation of aPTT, and the obtained A_{max} and AUC values are twice as high as those obtained after administration of equivalent doses of tinzaparin or enoxaparin (Friedel and Balfour, 1994, Frydman, 1996). Bemiparin exerts a significantly lower prolongation of aPTT than tinzaparin (Depasse et al., 2003).

When administered to heatlhy volunteers at a dose of 1.5 mg/kg SC, enoxaparin increased the TT and the aPTT up to 1.8 times the control values.. Enoxaparin, at a 1 mg/kg dose (100 mg/mL concentration) administered SC every 12 h to patients in a large clinical trial, resulted in aPTT values of 45 seconds or less in the majority of patients (n = 1607). A 30 mg IV bolus immediately followed by a 1 mg/kg SC administration resulted in aPTT post-injection values of 50 seconds. The average aPTT prolongation value on Day 1 was about 16% higher than on Day 4 (Sanofi-Aventis, 2011).

With nadroparin, maximal prolongation of aPTT and TT occurs at approximately 4 h. After SC administration of prophylactic nadroparin 2,850 IU in healthy volunteers, maximum aPTT and TT were increased by a negligible 2 seconds at 4 h, and aPTT returned to baseline by 8 h. After administration of treatment doses (nadroparin 171 IU/kg SC), aPTT was only slightly prolonged (GlaxoSmithKline_Inc., 2011).

Dalteparin 5,000 IU/day was compared with nadroparin 3,750 IU/day and enoxaparin 4,000 IU/day in a crossover study in 12 healthy volunteers. Mean aPTT was prolonged by dalteparin to a significantly

greater extent than by the other two LMWHs, although anti-FXa activities were not reported (Dunn and Sorkin, 1996, Stiekema et al., 1993).

Bemiparin (2,500–12,500 IU anti-FXa SC) significantly increased the aPTT ratio by 0.21–0.58 and the TT ratio by 0.11–1.54; however, these increases were not thought to be clinically relevant (Falkon et al., 1995b, Falkon et al., 1997). Bemiparin exerts a significantly lower prolongation of aPTT than tinzaparin (Depasse et al., 2003).

(4) Effects on TFPI

TFPI is released from the endothelium by heparin, thus potentiating the inhibitory effect on the plasma coagulation of heparin (Lindahl et al., 1991b). The form of TFPI that is released appears to be full-length TFPIα (Novotny et al., 1989). *In vitro*, LMWHs, have been shown to be more efficient than UFH in increasing the functional activity of TFPI in endothelial cells. In a study performed by Westmuckett et al.,(2001) it was shown that the regulation of the expression and secretion of TFPI by UFH, bemiparin, and dalteparin occurs differently when endothelial cells are grown under shear stress (0.27, 4.1 and 19 dyne/cm^2). All three preparations increased the expression of TFPI by 60 to 120% in EC under minimal flow, but only bemiparin enhanced TFPI mRNA in EC under the arterial flow. For all three levels of flow tested, bemiparin induced the highest secretion and increase of both cellular TFPI and cell surface activity of the inhibitor.

Establishing the pharmacodynamic profile of the TFPI effect is important because potent anticoagulant synergism results *in vitro* when both the anti-FXa amidolytic and TFPI effects occur simultaneously (Falkon et al., 1998a, Falkon et al., 1995a). *In vivo*, TFPI release seems to be dependent on the MW of heparin and its derivatives. It appears that high MW components may contribute more to the TFPI release than the low MW ones (Ma et al., 2007, Mousa et al., 2003). The parenteral administration of UFH and LMWHs, but not fondaparinux, rapidly increases the circulating levels of total TFPI in plasma 1.5–3 fold (Novotny et al., 1991, Sandset et al., 1988, Naumnik et al., 2011, Walenga et al., 2002, Bara et al., 1993) A clear dose—response relationship has been demonstrated with different LMWHs (Bendz et al., 2000, Bara et al., 1993). Both, protamine chloride and protamine sulfate have been shown to neutralize the TFPI functional activity of UFH and LMWHs (Hoppensteadt et al., 1995b).

As LMWHs differ in their MW distribution, there are differences in TFPI release between currently available LMWHs. For instance, while bemiparin and enoxaparin had a similar effect on the release of TFPI

(Antonijoan et al., 2009); the A_{max} of the TFPI effect was significantly higher after administration of tinzaparin than after bemiparin in healthy volunteers (Depasse et al., 2003). The clinical significance of a greater effect on TFPI has not yet been elucidated (Chapman and Goa, 2003, Depasse et al., 2003).

UFH, but not LMWHs, given in therapeutic doses is associated with a progressive depletion of TFPI, which is associated with a strong rebound activation of coagulation after cessation of treatment (Sandset et al., 2000, Hansen and Sandset, 1998, Bendz et al., 1999, Gouin-Thibault et al., 2003). The progressive depletion of TFPI may explain the apparent superior efficacy of LMWHs observed in clinical trials (Sandset et al., 2000, Hansen and Sandset, 1998).

(5) Head-to-head comparisons of the pharmacokinetic/pharmacodynamic profiles of LMWHs

Few head-to-head comparisons of the PD profiles of the different LMWHs are currently available (Azizi et al., 1995, Bara et al., 1999, Collignon et al., 1995, Depasse et al., 2003, Antonijoan et al., 2009, Eriksson et al., 1995, Stiekema et al., 1993).

The anticoagulant effects after IV administration (over 1 min) of dalteparin (5,000 IU anti-FXa), nadroparin (7,500 Institute Choay units = ~3,075 IU anti-FXa), enoxaparin (4,000 IU anti-FXa), and danaparoid (3,750 IU anti-FXa) were compared in a randomized cross-over study in 12 healthy male volunteers. The time courses of anti-FXa activity of dalteparin, nadroparin, enoxaparin (n = 5) were best fitted by a monoexponential function and had comparable half-lives of 1.9 h, 2.3 h and 2.8 h, respectively. The time course of anti-FXa activity of danaparoid and enoxaparin (n = 4) were described by a biexponential function with terminal half-lives of 56.8 h and 27.7 h, respectively. Danaparoid injection was associated with a lower clearance (0.8 ± 0.2 L/h) of the plasma anti-FXa activity compared with dalteparin (2.0 ± 0.5 L/h), nadroparin (1.7 ± 0.5 L/h), and enoxaparin (1.6 ± 0.5 L/h). In comparison with the three LMWHs, the terminal half-life of plasma anti-FIIa activity after danaparoid (2.0 ± 0.9 h) was longer and the clearance of danaparoid (2.5 ± 1.5 h) was lower than that after enoxaparin (t ½ = 1.3 ± 0.5; Cl = 4.1 ± 0.1), dalteparin (t ½ = 1.4 ± 0.3; Cl = 3.1 ± 0.9), and nadroparin (t ½ = 1.5 ± 0.2; Cl = 2.9 ± 0.9). The AUC of the plasma anti-FIIa activity after administration of danaparoid was negligible compared with that obtained after injection of the LMWHs. The administration of the nadroparin, enoxaparin, and danaparoid was associated with similar mean prolongations of the aPTT after 1 h = 7.5 s, 7.7 s and 5.8 s, respectively. However, dalteparin prolonged the aPTT by 18.5 s, a significantly longer time. The aPTT had returned to baseline or near baseline values six h after administration of each of the four drugs. The mean prolongation of the thrombin clotting time (TCT) 1 h after danaparoid administration was significantly

less than after the LMWHs (danaparoid 2.9 ± 1.7 s, dalteparin 47.8 ± 0.9 s, nadroparin 17.8 ± 13.2 s, enoxaparin 24.7 ± 11.9 s) (Stiekema et al., 1993).

When dalteparin, enoxaparin, and tinzaparin were administered to healthy volunteers at doses recommended by the manufacturers for orthopedic surgery, the anti-FXa activity was considered bioequivalent (Cornelli and Fareed, 1999, Eriksson et al., 1995). The peak anti-FXa activity (A_{max}) was highest for enoxaparin (0.42 ± 0.11 IU/mL) and dalteparin (0.48 ± 0.13 IU/mL), and lower for tinzaparin (0.18 ± 0.04 IU/mL); the corresponding AUC values also followed this pattern.

Normalized to the same injected dose (1,000 IU), the relative actual amount of plasma anti-FXa activity generated by enoxaparin was 1.48 times greater than that of nadroparin and 2.28 times greater than that of dalteparin, while the plasma amount induced by nadroparin was 1.54 times greater than that of dalteparin. The apparent total body clearance of enoxaparin doses (Cl = 16.7 ± 5.5 and 13.8 ± 3.2 mL/min) was significantly smaller than those of nadroparin (Cl = 21.4 ± 7.0 mL/min) and dalteparin (Cl = 33.3 ± 11.8 ml/min), while dalteparin apparent clearance is about 1.5-fold greater than that of nadroparin. These LMMHs also differed by their renal excretion pattern; more fragments exhibiting an anti-FXa activity were recovered in urine following enoxaparin doses (6.4 and 8.7% of the dose, respectively) than following nadroparin (3.9%) and dalteparin (3.4%) injection. These differences in the disposition profiles explain why the t ½ values of these LMMHs are different: dalteparin 2.8 h, nadroparin 3.7 h, enoxaparin 4.1 h, and bemiparin 5.2 h (Collignon et al., 1995, Falkon et al., 1995b).

In a single dose, randomized, cross-over study in 10 healthy volunteers comparing the pharmacokinetic/pharmacodynamic profiles of reviparin (4,250 IU anti-FXa) and enoxaparin (40 mg; 4,000 IU anti-FXa), the overall 24 h profiles of plasma anti-FXa and anti-FIIa activities were shown to be similar. A_{max} and the $AUC_{0\text{-}24}$ of plasma anti-FXa activity after reviparin administration were both slightly, but significantly lower than those observed after enoxaparin administration (difference between treatments of 0.03 IU/mL (95% CI 0.01-0.05) and 0.56 IU/mL*h (95% CI 0.22-0.90) for A_{max} and $AUC_{0\text{-}24}$ respectively). After adjustment for *in vitro* anti-FXa activity, the statistical difference between the two LMWHs persisted for the $AUC_{0\text{-}24,}$ but not for the A_{max} of plasma anti-FXa activity. T_{max} and the MRT values for plasma anti-FXa activity did not significantly differ between the two drugs. The t ½ for reviparin did not significantly differ from that of enoxaparin (2.7 ± 0.7 h vs 3.5 ± 0.9 h respectively, not significant (NS)). A_{max} of the plasma anti-FIIa activity after reviparin administration was also slightly, but significantly, lower than that observed after enoxaparin administration (difference between treatments of 0.018 IU/mL (95% CI 0.01-0.025), whereas the $AUC_{0\text{-}24}$ of anti-FIIa activity vs time was not. A slight but significant increase of

the aPTT of a similar magnitude was observed after both reviparin and enoxaparin injections (Azizi et al., 1995).

The relationship between bleeding or prevention of thrombosis and *ex vivo* anti-FXa and anti-FIIa activities, aPTT, and D-dimers were evaluated in a multicenter double-blind randomized study (Bara et al., 1999). Patients undergoing total hip replacement (THR) (n = 440) and given prophylaxis once daily with tinzaparin 4,500 IU anti-FXa (n = 221) or enoxaparin 4,000 anti-FXa IU (n = 219). Both regimens were administered SC once daily. Blood samples for anti-FIIa, anti-FXa, D-dimers levels, and aPTT were taken at baseline, on day 1, day 5, and on the day of discharge (days 8-14), and clinical assessments were performed daily until day 14. A significant correlation was observed between anti-FIIa activity and anti-FXa activity and the dose of each LMWH injected. The anti-FXa activity was significantly higher with enoxaparin and the anti-FIIa activity was significantly higher with tinzaparin. No clear relationship between these two activities and the clinical outcomes was observed. This was also true with regards to aPTT. Before and after surgery, D-dimers were significantly higher in patients with DVT than in those without DVT but had no predictive value (Bara et al., 1999).

Pharmacokinetic/pharmacodynamic profiles of bemiparin (3,500 IU anti-FXa) and tinzaparin (4,500 IU anti-FXa) administered SC to 12 healthy male volunteers were compared in a single-center, randomized, crossover study. Bemiparin (mean ± SEM T_{max} = 2.47 ± 0.14 h, A_{max} = 0.34 ± 0.02 IU/mL, AUC_{0-24}= 2.80 ± 0.30 IU/mL*h and t ½= 4.41 ± 0.58 h) exerted a significantly more rapid, more potent, and more prolonged anti-FXa activity than tinzaparin (mean ± SEM T_{max} = 3.17 ± 0.17 h, A_{max} = 0.25 ± 0.02 IU/mL, AUC_{0-24} = 1.71 ± 0.15 IU/mL*h and t ½ = 3.35 ± 0.70 h). The plasma level increase for free and total TFPI was significantly lower with bemiparin (mean ± SEM = 94.05 ± 5.67 ng/mL) than with tinzaparin (mean ± SEM = 128.92 ± 9.34 ng/mL). Free and total TFPI peak levels occurred earlier than anti-FXa activity peak levels for both LMWH preparations, but no statistical difference appeared between the two preparations for TFPI T_{max} (bemiparin T_{max} = 1.75 ± 1 h, tinzaparin T_{max} = 1.33 ± 0.14 h). Bemiparin exerted only minimal anti-FIIa activity and did not prolong TT, whereas tinzaparin elicited significant anti-FIIa activity and prolonged TT. Bemiparin (maximum aPTT ratio = 1.33 ± 0.04) exerted a significantly lower prolongation of aPTT than tinzaparin (maximum aPTT ratio = 1.50 ± 0.05). No difference was observed for APTT prolongation T_{max} between the two preparations. Globally, the overall tolerability of both formulations revealed no relevant adverse effects (Depasse et al., 2003).

It is to this day unknown whether the pharmacokinetic and pharmacodynamic differences of LMWHs are related to different risk:benefit ratios for the respective products. Therefore, the clinical findings associ-

ated with a given LMWH preparation cannot be extrapolated to a congener or generalized to the entire class of LMWHs. Clinical trials comparing the risk:benefit ratios of the different LMWHs are still warranted (Samama and Gerotziafas, 2000).

7. Safety profile

The most common side effect of heparin therapy is hemorrhage, which can range from minor to life threatening, and is related to the total administered dose and the degree of prolongation of the aPTT, rather than the route of administration. Fondaparinux and LMWHs have a lower bleeding risk. Although bleeding can be attributed to thrombin inhibition, the effect of these drugs on vascular endothelium and platelets also contributes to the bleeding (Samama et al., 2012).

The main non-hemorrhagic side effects of heparin are HIT and osteoporosis. HIT occurs in 0.1% to 5% of patients treated with UFH (Warkentin et al., 2003). HIT is caused by IgG subclass, heparin-dependent antibodies. These antibodies bind to a conformationally modified epitope on PF4. Simultaneous binding of these antibodies to Fc receptors on the platelet surface causes platelet activation. Activated platelets shed highly prothrombotic microparticles and are then removed from the circulation causing thrombocytopenia. In addition, these activated platelets and microparticles provide a surface onto which coagulation factor complexes can assemble to promote thrombin generation. This phenomenon can then trigger venous or arterial thrombosis, with venous thrombosis being more common (Linkins et al., 2012, Warkentin and Kelton, 1996). During HIT, typically the platelet counts are only moderately reduced. Occasionally patients do not have thrombocytopenia, but their platelet counts decrease by 50% from pretreatment levels. The risk of HIT is related to characteristics of the patient, the type of heparin used, and the clinical setting. Older patients and women are at increased risk. Surgical patients have a higher risk than medical patients, possibly because of the release of cytokines during tissue injury, and orthopedic surgery may pose a particularly high risk (Kelton et al., 2013).

The risk of HIT is also related to the duration of heparin exposure and characteristics of the heparin molecule. When administered to patients after surgery, UFH carries a higher risk (1.0 to 5.0%) than LMWHs, which are associated with a risk of 0.1 to 1.0% (Kelton et al., 2013, Alban, 2012). This underscores the fact that the interaction between heparin with PF4 is chain-length dependent. Although binding to PF4 is reduced, LMWHs can form complexes with PF4, so in patients with HIT antibodies there is cross-reactivity (Linkins et al., 2012, Bakchoul and Greinacher, 2012, Prechel and Walenga, 2013).

Table 7. Mean (SD) anti-FXa activity-derived pharmacokinetic parameters of different LMWHs after SC injection

Active Product (brand name) (IU Anti-FXa activity)	A_{max} (IU/mL)	T_{max} (h)	AUC_{0-t} (IU/mL*h)	t ½ (h)	Cl (mL/min)	Absolute bioavailability (%)	References
Enoxaparin (Clexane®)							
2,000	0.28 ± 0.06	2.35 ± 0.56	1.96 ± 0.55	3.95 ± 0.65	16.7	-	(Eriksson et al., 1995, Collignon et al., 1995, Azizi et al., 1995)
4,000	0.42 ± 0.11	3.17 ± 0.06	3.47 ± 0.6	4.28 ± 1.06	15.6	91	
4,000	0.57 ± 0.14	2.91 ± 0.5	4.57 ± 1.04	4.37 ± 0.47	13.8	-	
4,000	0.45 ± 0.05	3.1 ± 0.4	3.00 ± 0.68	-	-	-	
4,000	0.45 ± 0.08	3.00 (1.00-4.00)*	3.33 ± 0.80	4.71 ± 1.77	-	-	(Antonijoan et al., 2009)
Nadroparin (Fraxiparin®)							
3,075	0.32 ± 0.09	3.62 ± 0.73	2.35 ± 0.63	3.74 ± 0.68	21.5	89-98	(Collignon et al., 1995)
41 IU/kg (~2870 IU/70 kg)	0.61 ± 0.15	3.42 ± 1.17	5.08 ± 1.22	3.79 ± 1.49	-	-	(GlaxoSmithKline_Inc., 2011)
166 IU/kg (~11,620 IU/70 kg)	1.34 ± 0.15	4.67 ± 1.10	15.10 ± 2.30	11.20 ± 8.00	-	-	(GlaxoSmithKline_Inc., 2011)
Dalteparin (Fragmin®)							
2,500	0.22 ± 0.07	2.82 ± 0.92	1.26 ± 0.40	2.81 ± 0.84	33.3	86	(Collignon et al., 1995)
2,500	0.20 ± 0.08	2.30 ± 0.60	1.10 ± 0.30^{+}	3.40 ± 1.30	38.9 ± 8.80	-	(Simoneau et al., 1992)
5,000	0.49 ± 0.30	3.00 ± 0.06	3.20 ± 0.80	2.30 ± 0.06	-	-	(Eriksson et al., 1995, Friedel and Balfour, 1994)
10,000	0.98 ± 0.30	3.40 ± 0.80	8.70 ± 2.60^{+}	4.10 ± 0.80	20.70 ± 5.80	-	(Simoneau et al., 1992)
120 IU/kg (~8,400 IU/70 kg)	0.6 ± 0.10	228 ± 40 min	-	-	-	87 ± 6	(Bratt et al., 1986)

Active Product (brand name) (IU Anti-FXa activity)	A_{max} (IU/mL)	T_{max} (h)	AUC_{0-t} (IU/mL*h)	t ½ (h)	Cl (mL/min)	Absolute bioavailability (%)	References
Reviparin (Clivarin®)							
4,250	0.42 ± 0.06	3.1 ± 0.60	2.44 ± 0.59	3.30 ± 1.00	19	>90%	(Azizi et al., 1995)
40 IU/kg (~2,800 IU/70 kg)	0.23 ± 0.09	2.30 ± 1.03	0.60 ± 0.27	-	-	-	(Andrassy et al., 1994, Del Bono et al., 2011)
60 IU/kg (~4,200 IU/70 kg)	0.39 ± 0.10	1.80 ± 0.40	1.09 ± 0.02	-	-	-	
80 IU/kg (~5,600 IU/70 kg)	0.52 ± 0.11	2.80 ± 0.40	2.57 ± 0.34	-	-	-	
Tinzaparin (Innohep®)							
4,000	0.25 ± 0.02	3.17 ± 0.17	1.71 ± 0.15	3.35 ± 0.70	2,659.42 ± 193.69**	-	(Depasse et al., 2003)
4,500	0.18 ± 0.04	3.08 ± 0.79	1.35 ± 0.90	2.97 ± 1.01	22	85	(Eriksson et al., 1995)
175 IU/kg (~12,250 IU/ 70 kg)	0.87 ± 0.15	4.40 ± 0.70	9.00 ± 1.10+	3.30 ± 0.80	-	-	(LEO-Pharmaceutical-Products, 2008)
Certoparin (Sandoparin®)							
8,000	0.61 ± 0.13	246.66 ± 87.04	5.76 ± 1.17	4.9 ± 1.10	1,396 ± 312**	99	(Hoffmann et al., 2002)
Bemiparin (Hibor®)							
~2,500	0.34 ± 0.08	2.00 – 3.00	2.02 ± 0.53	5.31 ± 1.59	-	81	(Falkon et al., 1995b)
3,500	0.45 ± 0.07	3.00 (2.00 – 4.00)*	3.69 ± 0.88	5.44 ± 1.60	-	-	(Antonijoan et al., 2009)
3,500	0.35 ± 0.06	3.00 (2.00 – 4.00)*	2.42 ± 0.76	4.20 ± 1.48	1,190.80 ± 381.40**	-	(Rico et al., 2014)
3,500	0.34 ± 0.02	2.47 ± 0.14	2.80 ± 0.30	4.41 ± 0.58	1,291.23 ± 133.25**	-	(Depasse et al., 2003)

Active Product (brand name) (IU Anti-FXa activity)	A_{max} (IU/mL)	T_{max} (h)	AUC_{0-t} (IU/mL*h)	t ½ (h)	Cl (mL/min)	Absolute bioavailability (%)	References
~5,000	0.54 ± 0.06	3.00 – 4.00	4.70 ± 0.58	5.29 ± 1.12	-	95.59	(Falkon et al., 1995b)
7,500	1.22 ± 0.27	3.00 – 6.00	12.82 ± 2.27	5.19 ± 1.32	-	-	(Falkon et al., 1997)
9,000	1.42 ± 0.19	3.00 – 6.00	16.55 ± 2.18	5.44 ± 0.49	-	-	(Falkon et al., 1997)
12,500	2.03 ± 0.25	3.00 – 6.00	23.53 ± 4.05	5.41 ± 0.94	-	-	(Falkon et al., 1997)
115 IU/kg (~8,000 IU/70 kg)	0.90 ± 0.20	4.00 (2.00 – 6.00)*	8.24 ± 1.37	4.08 ± 0.54	838.40 ± 96.90**	-	(Rico et al., 2014)
Semuloparin							
0.1 – 1.4 mg/kg	0.41 – 10.90 μgEq/mL	-	11.00 – 173 μgEq*h/mL	9.80 – 11.4	0.60 ± 0.17 L/h	97.7	(Dubruc et al., 2009)
RO-14							
2,450	0.24 ± 0.05	2.33 ± 0.82	1.88 ± 0.98	4.97 ± 3.30	1,588.37 ± 985.02**	-	(Rico et al., 2011)
4,550	0.49 ± 0.09	2.67 ± 0.82	4.35 ± 1.68	6.10 ± 2.82	8,670.96 ± 3343.74**	-	(Rico et al., 2011)
10,150	0.97 ± 0.30	3.00	9.64 ± 3.35	8.24 ± 5.04	10,595.76 ± 4735.40**	-	(Rico et al., 2011)

*Median (range)
** mL/h
***min
$^{+}$ $AUC_{0-\infty}$

DTI's and danaparoid are currently favored in published guidelines for the management of HIT. Due to the lack of familiarity of physicians with these compounds, fondaparinux and argatroban (in patients with renal impairment) have been proposed by some as potential alternatives. Further clinical studies are warranted (Kelton et al., 2013).

Long-term use of UFH data has been associated with a 2.2-5% incidence of heparin-induced osteoporotic fracture (Lefkou et al., 2010). Osteoporosis is caused by binding of heparin to osteoblasts, which then release factors that activate osteoclasts in an interleukin 11-dependent fashion (Rajgopal et al., 2006). The risk of osteoporosis is lower with LMWH than with UFH, probably up to six fold less, but data is still scarce. This might be a reflection of the lower affinity of LMWH for osteoclasts and osteoblasts (Garcia et al., 2012). Until large clinical trials are designed to investigate pre- and post-treatment bone density, and to compare different dosages of LMWH effect on the bone density in different patient groups, no safe conclusions can be made (Lefkou et al., 2010).

A rare but potentially lethal acute "anaphylactic" reaction to heparin can also occur. Heparin therapy is also associated with transient elevations in serum transaminase levels, which is likely not of clinical importance. Prolonged exposure to heparin can result in osteoporosis, and skin reactions. Eosinophilia, alopecia, and hyperkalemia are rare complications. Multiple anticoagulants, antiplatelet drugs, and thrombolytic drugs have an additive, even synergistic effect, and non-anticoagulant agents can affect the anticoagulant effects of heparin (Samama et al., 2012, Alban, 2012).

Recently, the US Food and Drug Administration (FDA) issued a drug safety communication with recommendations to decrease risk of spinal column bleeding and paralysis when using LMWHs. The agency recommends placement or removal of a spinal catheter to be delayed for at least 12 h after administration of prophylactic doses of enoxaparin. Longer delays (24 h) are indicated as appropriate to consider for patients receiving higher therapeutic doses of enoxaparin (1 mg/kg twice daily or 1.5 mg/kg once daily) (FDA, 2013). Such delays may not be necessary for all LMWHs. For instance, bemiparin can be administered 6 h after surgery and it seems to be compatible with neuraxial anesthesia. No cases of spinal hematoma were reported In a large (n = 1,009), prospective, observational study in patients undergoing THR or total knee replacement (TKR) receiving bemiparin (3,500 IU/day started 6 h after surgery) for thromboprophylaxis. This occurred in spite of a high frequency of neuraxial anesthesia alone (87.4%), or in combination with general anesthesia (92.9%) (Martinez-Gonzalez et al., 2008, Abad et al., 2007).

8. Laboratory monitoring of UFH, LMWHs, and related compounds

a) Unfractioned heparin

Investigators have reported a relationship between the dose of UFH administered and both its efficacy and safety (Berkowitz, 1995, Dawes and Papper, 1979, Raschke et al., 1996). Because the anticoagulant response to heparin varies among patients, it is standard practice to monitor UFH and to adjust the dose based on the results of coagulation tests. When given in therapeutic doses, the anticoagulant effect of UFH is usually monitored using the aPTT (Eikelboom and Hirsh, 2006). The activated clotting time (ACT) is used to monitor the higher UFH doses given to patients undergoing percutaneous coronary interventions or cardiopulmonary bypass surgery (Bowers and Ferguson, 1994).

In an effort to standardize heparin monitoring, the therapeutic range of 1.5 to 2.5 times aPTT control was considered by the American College of Chest Physicians (ACCP) as equivalent to 0.2 to 0.4 U/mL heparin by protamine titration, or 0.35 to 0.70 IU/mL by anti-FXa assay (Kitchen et al., 1999, Hirsh et al., 2008a). However, monitoring aPTT poses several challenges. Firstly, the variability of aPTT to heparin is dependent on the reagent (Brandt and Triplett, 1981, Kitchen et al., 1996), which can be selected with more sensitivity to heparin based on their lipid composition and the concentration of phospholipids (Kitchen et al., 1999, Hirsh et al., 2008a, Kitchen et al., 1994, Toulon et al., 1998). Secondly, the evidence for adjusting the dose of heparin to maintain a "therapeutic range" is weak, and is based on results of a post hoc subgroup analysis of a descriptive study that suggested an aPTT ratio between 1.5 and 2.5 was associated with a reduced risk of recurrent VTE (Basu et al., 1972). The clinical relevance of this therapeutic range is uncertain because it has not been confirmed by randomized trials. The results of a randomized trial in patients with VTE showed that unmonitored weight-adjusted UFH SC given twice daily in high doses, was as safe and effective as unmonitored, weight-adjusted LMWH, challenging the requirement for aPTT monitoring of heparin administered SC (Kearon et al., 2006).

Moreover, since the aPTT ratio and the anti-FXa activity may be discrepant in a given patient, it is recommended by the ACCP that the dose in patients with heparin resistance, who require unusually high doses of heparin to achieve a therapeutic aPTT ratio, should be adjusted on the basis of anti-FXa levels rather than on the aPTT results (Hirsh et al., 2008a, Kitchen and Preston, 1996, Levine et al., 1994). In some groups of patients, often in intensive care units, an unusually low dose of UFH is sufficient to

achieve a therapeutic aPTT ratio. The increased response to heparin could be due to congenital or acquired contact factor deficiency (van Veen et al., 2009, Samama et al., 2012).

Given the several biologic factors can influence the aPTT independent of the effects of UFH, many institutions have transitioned to monitoring heparin with anti-FXa levels, rather than the aPTT. Clinical data from the last 10-20 years have begun to show that a conversion from aPTT to anti-FXa monitoring may offer a smoother dose-response curve, such that levels remain more stable, requiring fewer blood samples and dosage adjustments (Vandiver and Vondracek, 2012). For example, in a single-center, retrospective, observational, cohort study (n = 100) conducted in an 852-bed academic medical center, the performance of the aPTT with the anti-FXa activity for efficiency and safety of monitoring IV UFH infusions were compared. Mean (SD) time to achieve therapeutic anticoagulation was significantly less in the anti-FXa group compared with the aPTT group (28 ± 16 vs. 48 ± 26 h, $P < 0.001$). A greater percentage of anti-FXa patients compared to aPTT patients achieved therapeutic anticoagulation at 24 h (OR 3.5; 95% CI 1.5-8.7) and 48 h (OR 10.9; 95% CI 3.3-44.2), and patients in the anti-FXa group also had more test values within the therapeutic range (66% vs. 42%, $P < 0.0001$) (Guervil et al., 2011).

Yet another example was a retrospective, single-center, cohort study (n = 186) conducted at a community teaching hospital. A DVT/PE treatment protocol, in which patients' doses of IV UFH were adjusted based on blood plasma anti-FXa level monitoring, was compared with a protocol based on monitoring with the blood plasma aPTT. In patients undergoing IV UFH therapy whose blood plasma was monitored with anti-FXa assay levels, as opposed to the aPTT, there was a higher percentage of UFH test results within the goal range (69% vs. 41%; $P < 0.0001$), fewer monitoring tests were needed (2.08 vs. 2.73; $P = 0.001$), and fewer dose adjustments were required per 24-h period (0.62 vs. 1.47; $P < 0.0001$) (Vandiver and Vondracek, 2013).

These examples provide evidence that monitoring IV UFH infusions with the anti-FXa assay, compared to the aPTT, achieves therapeutic anticoagulation more rapidly, maintains the values within the goal range for a longer time, and requires fewer adjustments in dosage and repeated tests (Guervil et al., 2011). Given the minimal increased acquisition cost of the anti-FXa reagents, it can be argued that the anti-FXa is a cost-effective method for monitoring UFH (Vandiver and Vondracek, 2012).

b) Low molecular weight heparins

LMWHs are typically administered in fixed or weight-adjusted doses for thromboprophylaxis and in weight-adjusted doses for therapeutic purposes. At prophylactic doses, the PT and aPTT are not significantly prolonged by LMWHs. Therapeutic doses prolong the aPTT, although to variable degrees with different reagents (Samama et al., 2012, Walenga, 1993). While routine monitoring is not generally recommended, if necessary the chromogenic assay measuring anti-FXa activity is used (Laposata et al., 1998).

Monitoring anti-FXa activity could be challenging for practical reasons and may provide flawed information for patient management, due to the inherent characteristics of the test that neither measures the essential molecule nor the net effect (Hemker et al., 2005, Bounameaux and de Moerloose, 2004). The net anticoagulant effect is not only the result of the anti-FXa activity. It is co-determined by plasma characteristics such as the concentration of AT, the level of HBPs, and the thrombin-forming power of the hemostatic system (Al Dieri et al., 2006). Although anti-FXa activity is the closest we have gotten to a practical risk predictor of bleeding when using LMHW therapy, there is a rather poor correlation between the anti-FXa activity and the safety and/or efficacy of LMWHs (Gouin-Thibault et al., 2010). In some studies, high anti-FXa levels have been associated with an increased bleeding risk (Morabia, 1986, Nieuwenhuis et al., 1991, Garcia et al., 2012), however other studies have not demonstrated such a relationship (Bara et al., 1992, Prandoni et al., 1992, Walenga et al., 1991). Furthermore, the benefits of monitoring anti-FXa activity have not been clinically demonstrated. For instance, a randomized controlled trial comparing monitored and unmonitored dalteparin for treatment of VTE showed no benefit of monitoring (Alhenc-Gelas et al., 1994).

In spite of the above, and lacking a better alternative in view of the dearth of evidence, some authorities and independent authors have suggested monitoring anti-FXa activity of LMWHs to decrease the risk of bleeding in special situations, such as in obese patients, in those who are pregnant, or those who suffer renal insufficiency (Clark, 2008, Weitz, 1997, Hirsh, 2007, Garcia et al., 2012). Part of this work will explore the issue of dosing and monitoring the effect of LMWHs in the elderly and in the renally impaired, so it will not be covered in this section. As it relates to obese and pregnant patients, there is a developing consensus that monitoring is advisable in these patient populations.

(1) Monitoring LWMHs effects during pregnancy

Pregnancy changes renal function and the distribution of fluid, which affects the clearance and distribution of the drugs, and makes predicting a therapeutic dose more difficult (Bates et al., 2012). As warfarin is commonly contraindicated during early pregnancy, and LMWHs do not cross the placenta (Andrew et al., 1985; Doutremepuich et al., 1985; Forestier, Daffos, & Capella-Pavlovsky, 1984; Forestier, Daffos, Rainaut, & Toulemonde, 1987); LMWHs with accompanying anti-FXa monitoring are recommended for indications such as recurrent DVT and in pregnant women with mechanical heart valves. In high-risk patients, trough anti-FXa monitoring is often used to ensure constant anticoagulation, although there is no consensus on the target concentration. Monitoring is also indicated in patients who receive extended therapy or do not have the expected response, for example, those who thrombose or bleed during therapy. Anti-FXa monitoring should be considered in patients at high risk of bleeding as, unlike UFH, the anticoagulant effects of LMWHs are not so readily reversible (Barras, 2013).

(2) Monitoring LWMHs effects in obese patients

The dosing and monitoring of LMWHs in obese patients is contentious. The latest edition of the ACCP Evidence-Based Clinical Practice Guidelines state “the markedly obese patient and the patient with low body weight may require intermittent monitoring because of possible differences in the pharmacokinetics of LMWHs in such patients compared with patients closer to ideal body weight” (Kearon et al., 2012).

As LMWHs are hydrophilic, they are predominantly distributed in plasma and lean tissue and do not easily partition into adipose tissue. The clearance of LMWHs correlates with lean body mass, therefore the addition of adipose weight into the weight-based dose calculation is difficult to justify (Green and Duffull, 2003). Dosing based on total body weight may result in excessive concentrations so physicians often introduce an arbitrary dose adjustment that has never been formally evaluated. One method is to ‘cap’ the dose (for example 100 mg for enoxaparin), regardless of the patient’s total body weight, however capping is likely to result in sub-therapeutic concentrations. Despite suggestions that anti-FXa monitoring should only be considered in the morbidly obese (Nutescu et al., 2009), monitoring peak activity in adults with a total body weight more than 100 kg is justifiably common practice (Barras, 2013). Empirical dose-adjustments should be avoided.

(3) Dose modification of LMWHs in special populations

No strategies have been evaluated in large, randomized studies to assist in dose modification once anti-FXa activity is known. A small Australian study demonstrated that the risk of bleeding is reduced when doses are individualized using anti-FXa concentrations (Barras et al., 2008). Other dose reduction strategies for obesity and renal impairment have been proposed, but are yet to be tested against clinical outcomes (Nutescu et al., 2009). Drug monitoring principles suggest that a linear dose adjustment could be used if the clearance of the LMWHs is stable, or an extension in dosing frequency if clearance is significantly reduced (Barras, 2013).

c) Fondaparinux

Routine monitoring is not required due to predictable pharmacokinetics. A chromogenic anti-FXa measurement can be used, with fondaparinux as the assay calibrator, the results then reported as fondaparinux concentration (mg/mL) and not in IU as for heparin. The aPTT is not affected by fondaparinux (Depasse et al., 2004, Samama et al., 2012).

d) Danaparoid

Routine monitoring is not required; if desired, the chromogenic anti-FXa measurement can be used with danaparoid as the calibrator. The aPTT is not affected by danaparoid (Samama et al., 2012).

9. Neutralization of heparins

One advantage of UFH is that its anticoagulant and bleeding effects are reversed with IV protamine sulfate and protamine chloride. Protamine sulfate is a protein derived from fish sperm that binds to heparin to form a stable salt. One milligram of protamine sulfate will neutralize approximately 100 units of heparin (Hirsh, 2007). Protamine sulfate is cleared from the circulation with a half-life of about 7 min. Because the half-life of IV UFH is 60-90 min when administered as in infusion, heparin given during the preceding several hours needs to be considered when calculating the protamine dose. The aPTT can be used to evaluate the effectiveness of protamine treatment (Garcia et al., 2012).

Protamine is associated with adverse reactions such as severe hypotension if administered too rapidly (Ovrum et al., 1991), and may also activate the complement system causing pulmonary vasoconstriction, pulmonary hypertension, and peripheral vascular collapse (Procaccini et al., 1987, Garcia et al., 2012, Samama et al., 2012). There is also a risk of allergic reactions including anaphylaxis, especially in pa-

tients who have received protamine sulfate-containing insulin, have undergone vasectomy, or have known sensitivity to fish. Pretreatment with corticosteroids and antihistamines can be used for these cases (Garcia et al., 2012).

While protamine effectively neutralizes heparin, it has limited value in the neutralization of LMWHs (Racanelli et al., 1985), only partially reversing the anti-FXa activity (Weiler et al., 1990) and neutralizing the normally limited anti-FIIa activity (Garcia et al., 2012). For a LMWH, such as bemiparin, protamine sulphate neutralization has been calculated to be around 30% (Falkon et al., 1998b). It is likely that incomplete neutralization of anti-FXa activity reflects that protamine does not bind to LMWH fragments within the LMWH preparations that have low sulfate charge density (Crowther et al., 2002). Protamine does not reverse the anticoagulant effect of fondaparinux. Several approaches have been unsuccessful as an antagonist for heparin and LMWHs, including recombinant PF4 and heparinase-1; a salicylamide derivative, PMX-60056, is under development (Samama et al., 2012, Choi et al., 2005).

10. Heparin contamination

In January 2008, health authorities in the United States began receiving reports of clusters of acute hypersensitivity reactions in patients undergoing dialysis that had been occurring since November 2007. Symptoms included hypotension, facial swelling, tachycardia, urticaria, and nausea. Although initial investigations focused on dialysis equipment, an investigation by the Centers for Disease Control and Prevention identified the receipt of heparin sodium for injection (1,000 IU/mL, in 10-mL and 30-mL multidose vials), manufactured by Baxter Healthcare, as a common feature of the cases. This finding led Baxter Healthcare to recall, nine lots of heparin sodium for injection, on January 17, 2008. As of April 13, 2008, there were 81 reports of deaths that involved at least one sign or symptom of an allergic reaction or hypotension in patients receiving heparin since January 1, 2007. After this initial recall, there were continuing reports of allergic-type reactions after injection of bolus heparin, including some deaths, not only in patients undergoing dialysis but also in patients in other clinical settings, such as those undergoing cardiac procedures. On February 28, 2008, Baxter Healthcare recalled all remaining lots and doses of its multidose and single-dose vials of heparin sodium for injection and HEP-LOCK heparin flush products. On March 6, a heparin recall was announced in Germany because of a cluster of reactions in patients undergoing dialysis that were linked to a different manufacturer's heparin. Following a heparin screening process suggested by the FDA, it was revealed that there was a widespread contamination of the

heparin supply in at least 12 countries (Kishimoto et al., 2008) which led to over 149 deaths worldwide (Samama et al., 2012).

The contaminant was identified as an unusual oversulfated form of chondroitin sulfate (OSCS), representing up to approximately 30% wt/wt in suspect lots of heparin. In addition, dermatan sulfate, a known impurity of heparin, was found in selected samples. Analysis of the contaminant unexpectedly revealed an unusual type of sulfation not found in any natural sources of chondroitin sulfate and indicated that OSCS, containing four sulfates per disaccharide unit, is structurally similar to heparin (Kishimoto et al., 2008). This appeared to be the result of an intentional adulteration of therapeutic heparin. Because LMWHs are produced from UFH, OSCS was also found in various batches of LMWHs (Samama et al., 2012).

Using both contaminated heparin products and the synthetically produced derivative, it was shown that the OSCS can directly activate the contact system and induce the generation of C3a and C5a anaphylatoxins *in vitro*. Moreover, it was also shown that OSCS activates kallikrein *in vivo* and can induce a profound dose-dependent hypotensive response in pigs and rats; the response in rats can be abrogated with bradyzide, a rodent-selective B(2) bradykinin receptor antagonist (Kishimoto et al., 2008, McKee et al., 2010).

11. Current therapeutic role of LMWHs

DVT, encompassing VTE and PE is a highly prevalent condition. VTE is the third most common vascular disease after coronary heart disease and stroke, with approximately 300,000 new cases diagnosed annually in the United States (Go et al., 2013). PE accounts for 5-10% of hospital deaths and is, therefore, often quoted as the most preventable cause of death in hospital. Hospitalized patients are at a 100 times greater risk than primary care patients and between 25-30% of non-fatal VTEs occur in patients with prior hospitalization (Bateman et al., 2013).

The prevention of VTE has been identified as a major health need nationally and internationally to improve patient safety. A recent multinational, observational, cross-sectional study carried out in 358 hospitals from 32 different countries (the ENDORSE study[4]) (Cohen et al., 2008) showed that 51.8% of patients were at risk of VTE and only 50.2% of patients who were deemed to be at risk received prophy-

[4] Epidemiologic International Day for the Evaluation of Patients at Risk for Venous Thromboembolism in the Acute Hospital Care Setting

laxis. A retrospective review of patients with a diagnosis of VTE was performed in 2010 in New Zealand and supported these findings. It demonstrated that 25% of patients with a VTE had been admitted to hospital in the preceding three months. Of these patients, two thirds had not received appropriate prophylaxis (Bateman et al., 2013).

The positive contribution of LMWHs to the prophylaxis and treatment of VTE/PE is unquestioned. Since their development two decades ago, LMWH have addressed some of the disadvantages shown by UFH. LMWHs produce a more predictable anticoagulant response than UFH, reflecting their better bioavailability, longer half-life, and dose-independent clearance (Weitz, 1997). Moreover LMWHs require fewer injections and produce fewer adverse events. In most cases these characteristics have led to the elimination of the need for monitoring and have reduced the risk of bleeding (Gray et al., 2008, Weitz, 1997, Costantino et al., 2012). Due to their advantages, LMWHs have largely replaced UFH for many indications and are nowadays part of the standard care for the prevention and treatment of VTE (Gray et al., 2008, Hirsh and Raschke, 2004, Kearon et al., 2008, Garcia et al., 2012, Prescrire-International, 2013b).

For medically ill and post-operative patients requiring parenteral VTE prophylaxis, LMWHs have become a suitable replacement for UFH (Kahn et al., 2012, Gould et al., 2012). In hospitalized medical patients receiving thromboprophylaxis, LMWHs were associated with a lower risk of DVT, fewer injection site hematomas, and no differences in bleeding when compared to UFH (Wein et al., 2007, Alquwaizani et al., 2013).

In patients with DVT or PE, initial treatment with LMWHs is primarily aimed at preventing thrombus extension. After this initial phase, the goal of treatment is to prevent recurrences, which can be fatal. LMWHs have largely replaced IV UFH in patients with acute VTE who are able to continue therapy unmonitored in the ambulatory setting (Kearon et al., 2012, Alquwaizani et al., 2013).

In ACS, patients with ST-segment elevation myocardial infarction treated with fibrinolysis and LMWH had a lower incidence of death or non-fatal recurrent myocardial infarction, but a higher rate of major bleeding, than those treated with fibrinolysis and UFH (Antman et al., 2006). Similarly, in unstable angina/non-ST-segment elevation myocardial infarction, LMWHs therapy reduced the incidence of death, myocardial infarction, or urgent revascularization when compared to UFH (Alquwaizani et al., 2013, Antman et al., 1999).

There are now various new oral anticoagulants (NOACs), approved in several countries around the world: dabigatran (a DTI), and rivaroxaban and apixaban (direct FXa inhibitors). All three are now approved to reduce the risk of stroke and systemic embolism in patients with non-valvular atrial fibrillation. Rivaroxaban also is indicated for treatment of DVT and PE and thrombosis prophylaxis in THR or TKR surgery (Soff, 2012). Other NOACs currently in development are edoxaban (available in Japan since 2011) (Daiichi-Sankyo, 2014) and betrixaban (Portola-Pharmaceuticals, 2014). Although the development of these options is revolutionizing the antithrombotic landscape, much is needed to be learned about these agents. Before rivaroxaban or other NOACs may be safely and effectively used for treatment of thrombosis, additional guidance will be required for the circumstances, such as drug-interactions, monitoring plasma levels in high-risk situations, dosing in some patient populations (e.g. renal dysfunction, marked extremes of body weight), and safety in the presence of thrombocytopenia (Soff, 2013, Bauer, 2013).

More comparative clinical evidence is also needed. So far, the harm-benefit balance of rivaroxaban does not appear more favorable than that of an LMWH followed by an adjusted-dose vitamin K antagonist. Clinical practice guidelines largely agree on the use of LMWH or fondaparinux as initial therapy for most patients with DVT or PE (Prescrire-International, 2013b). Regarding, long term treatment of DVT, an independent literature review conducted by Prescrire International (2013c), concluded that there is no evidence that rivaroxaban or dabigatran had a better harm-benefit balance than warfarin. Therefore, despite the development of NOACs, LMWHs will continue to play a major role in the prevention and management of thrombotic and cardiovascular disorders (Fareed et al., 2008, Prescrire-International, 2009, Guyatt et al., 2012, Prescrire-International, 2012).

Interestingly, and thanks to the emerging data on the non-anticoagulant effects of LMWHs, their use is actively being researched for a myriad of indications. One of the areas that has caught more attention over the past decade is cancer, since VTE is one of the most common and serious complications of cancer. The incidence of VTE varies with cancer type and stage, but in general it is reported that ~20 % of cancer patients develop VTE at some point during their illness, and 20 % of VTE occurs in cancer patients. PE remains one of the most common causes of death in cancer patients. It has been estimated that one in every seven hospitalized cancer patients who die, do so from PE. When venous and arterial thromboses are considered in the aggregate, thrombosis is second only to the cancer itself as the cause of death. Importantly, it has been suggested that the thrombotic risk is not simply a reflection of late-

stage tumor burden, but there is likely a component of the inherent tumor biology contributing to the thrombosis (Soff, 2013).

Since active cancer and ongoing chemotherapy are associated with a significantly increased risk of VTE, there has been considerable focus on the potential benefit of primary thrombosis prophylaxis in ambulatory cancer patients. The calculus is a balance between potential reduction in morbidity and mortality from a VTE with the risk of hemorrhage, cost, and inconvenience of the anticoagulation. Although no human study has demonstrated a direct anti-tumor effect of anticoagulation, the recent SAVE-ONCO study has provided some support for primary prophylaxis (Agnelli et al., 2012). Semuloparin, an ULMWH, was compared with placebo for primary prophylaxis in patients with metastatic or locally advanced solid tumors, and undergoing chemotherapy. Semuloparin reduced the incidence of VTE from 3.4 to 1.2 %, a hazard ratio (HR) of 0.36 ($P < 0.001$), with no significant increase in major or clinically relevant bleeding. This study suggested that anticoagulant doses and clinical conditions may be titrated, allowing for reduced thrombosis without a concomitant significantly increased risk of bleeding. However, as with other similar trials in the past, the reduction in thrombosis events was not associated with an improvement in overall survival. If primary prophylaxis will ultimately have a place in oncology practice, it will probably be based on risk stratification (Khorana, 2012). In the meantime there are clinical trials assessing the potential benefits of LMWHs in breast, colorectal, lung, prostate, and veno-oclusive cancers (Page, 2013).

It is indeed tempting to consider the use of the newer anticoagulants for treatment of VTE in cancer. In the EINSTEIN-DVT and EINSTEIN-PE studies, rivaroxaban was shown to be non-inferior to a regimen of heparin or LMWH followed by warfarin (Bauersachs et al., 2010, Buller et al., 2012). However, in both EINSTEIN studies[5], cancer patients represented a small percent of the patients, not allowing for meaningful subgroup analysis. Most importantly, rivaroxaban was shown only to be not inferior to warfarin, and warfarin is no longer considered to be standard of care for treatment of cancer-associated thrombosis (Soff, 2013, Bauer, 2013).

Finally, the potential benefits of therapy with LMWHs are not restricted only to cancer. A number of new approaches are being investigated to exploit the non-anticoagulant actions of UFH/LMWHs. UFH and LMWHs are being investigated in a range of conditions, such as infertility, hemodialysis, inhalation

[5] The EINSTEIN clinical trial program consisted of three phase 3 clinical trials for the treatment of deep vein thrombosis (DVT) and pulmonary embolism (PE), and for reducing the risk of recurrence of DVT and PE in more than 9400 patients: EINSTEIN–DVT, EINSTEIN–PE, and EINSTEIN–Extension.

burns, inflammation in intraocular lens implantations and chronic glomerulonephritis, vulvodynia, ulcerative colitis, diabetic foot ulcers, ovarian cancer, metastatic pancreatic cancer, pregnancy complications, cystic fibrosis, COPD, prolonged labor, and microalbuminuria (Page, 2013).

12. LMWHs: Outstanding issues

In spite of the nearly 3 decades of clinical research experience with LMWHs, to date there are some pending assignments as it relates to their pharmacological characterization and the potential clinical implications. Firstly, as it has been stated before, there have been very few head to head comparisons between the different LMWHs, and inter-study comparisons are not appropriate mainly because the large variability in the methodology of the studies and the assays. Of particular interest is the role of a lower mean MW distribution of the heparin chains and their pharmacokinetic/pharmacodynamic impact. Depasse et al.(2003) compared the pharmacological profile of bemiparin, a second generation LMWH with a mean MW of 3,600 Da, a specific anti-FXa activity of 80-120 IU/mg, and a high anti-FXa:anti-FIIa ratio of 8; and tinzaparin with a mean MW of 4,500 Da, an specific anti-FXa activity of 83 IU/mg, and an anti-FXa:anti-FIIa activity ratio of less than 2. An important comparison still lacking until recently, was that of bemiparin, with the lowest mean MW and the highest anti-FXa:anti-FIIa ratio; and the prototype and most commercially successful LMWH, enoxaparin which possesses a similar mean MW as tinzaparin but is more potent and has a higher anti-FXa:anti-FIIa ratio.

Another area that sprouted a lot of interest in the last decade is the development of an ULMWH. An ULMWH would possess more heparin chains with the specific pentasaccharide sequence that is responsible for AT-binding, but unlike the synthetic pentasaccharide fondaparinux, it would continue showing the non-anticoagulant effects of the LMWHs.

A third area that is still contentious to this day is the issue of dosing in elderly patients or those that are renally impaired. These patient populations are at a higher risk of both bleeding and DVT, and lacking appropriate evidence the tendency over the past couple of decades has been to: a) avoid LMWHs in these patient populations, depriving them of a very useful therapeutic option, or b) empirically adjusting the dose, an approach that frequently leads to inappropriate antithrombotic levels and lack of efficacy.

These 3 issues are the subject of this work and a brief introduction to them is offered herein.

a) Comparative pharmacodynamic time-course of bemiparin and enoxaparin in healthy volunteers

Bemiparin sodium (Hibor®, Ivor®, Zibor®, Badyket®, Hepadren®, Ivorat® Heporax®, and Ivormax®, Laboratorios Farmacéuticos Rovi, S.A.) is a LMWH obtained by alkaline depolymerization of commercial-grade heparin sodium from porcine intestinal mucosa. It is considered a "second-generation LMWH" and has the lowest mean MW (3,600 Da), the longest half-life (5.3 h), and the highest *in vitro* anti-FXa:FIIa activity ratio (8:1) of all commercially available LMWHs (Table 3)(Chapman and Goa, 2003, Martinez-Gonzalez et al., 2008, Planes, 2003).

Bemiparin was developed in the 90s (Falkon et al., 1997, Falkon et al., 1998a, Falkon et al., 1998b, Falkon et al., 1995a, Falkon et al., 1995b, Kakkar et al., 2000, Navarro-Quilis et al., 2003), launched in Spain in 1998, and is now available in more than 33 countries worldwide (15 in the European Union). Bemiparin 2,500 II/day and 3,500 IU/day is indicated for the prevention of thromboembolic disease in patients (at moderate or high risk of VTE) undergoing general or orthopedic surgery, for prophylaxis in medical patients at moderate or high risk, for secondary prophylaxis to avoid recurrence of VTE in patients with DVT and transient risk factors, and for prevention of clotting in the extracorporeal circuit during hemodialysis. Bemiparin SC (115 IU kg/day, usually for 7 ± 2 days) is also licensed for the treatment of established DVT, with or without PE. Bemiparin is the only LMWH licensed in Europe for use in regimens that initiate therapy postoperatively (first dosage administered 6 h after surgery) for surgical thromboprophylaxis (Martinez-Gonzalez et al., 2008).

Bemiparin was developed with one basic idea in mind; a LMWH with a lower mean MW could have a higher anti-FXa/anti-FII activity ratio and would potentially have a better safety and efficacy profile than other existing LMWHs. A lower mean MW also determines changes in the pharmacodynamic profile of a LMWH, and although one can establish a comparison using data derived from different studies, no clinical trial had until recently assessed how the pharmacodynamic profiles of bemiparin and the reference LMWH, enoxaparin, differ from each other. We conducted a randomized, single-blind, cross-over study with single SC dose of bemiparin 3,500 IU or enoxaparin 4,000 IU administered to healthy volunteers (Antonijoan et al., 2009). The objectives of this study were to compare the pharmacodynamic time-course of the two LMWHs, bemiparin and enoxaparin, at high prophylactic doses as assessed by the anti-FXa activity, anti-FIIa activity, anti-FXa/FIIa activity ratio, free and total TFPI release, APTT, TT, and

thromboplastin-thrombomodulin-mediated time (TP-TmT). Chapter 4 of this work presents the study results and discussion of the findings.

b) Safety assessment and pharmacodynamics of a novel ULMWH (RO-14) in healthy volunteers-a first-time-in-human single ascending dose study

The rationale to target either thrombin or FXa has been discussed over the years. Inhibition of thrombin stops the propagation and amplification of coagulation by preventing the formation of fibrin and thrombin-mediated activation of factors V, VIII, XI, XIII, and platelets, and consequently halts further thrombin generation. Inhibition of FXa prevents the activation of prothrombin to thrombin and in turn prevents the burst of thrombin without affecting the existing level of thrombin. The residual level of thrombin should be able to ensure primary hemostasis and reduce bleeding risk. Because FXa is situated at the juncture of the intrinsic and extrinsic pathways and collectively controls the generation of thrombin, it is more effective to target FXa than individual coagulation proteases upstream in the cascade (Lee and Player, 2011).

A number of preclinical experiments have provided evidence to support the concept that inhibition of FXa is a highly effective antithrombotic approach. There appears to be a larger safety margin with respect to bleeding in comparison to inhibition of thrombin (Viskov et al., 2009, Lee and Player, 2011, Gould and Leadley, 2003). However, based on the assumption that even with very effective FXa-inhibition trace amounts of thrombin will be generated during thrombosis, a residual anti-thrombin activity could be highly beneficial for effective prevention of thromboembolism without having an influence on an increased bleeding liability (Viskov et al., 2009). With these 2 concepts in mind, there have been some attempts to develop LMWHs with better efficacy and safety ratios, by decreasing the mean MW and increasing the proportion of AT-binding sequence heparin fragments, thereby enhancing their anti-FXa:anti-FIIa activity ratio (Lima et al., 2013, Jeske et al., 2011). The first of such attempts was the development of bemiparin (Falkon et al., 1995b, Sanchez-Ferrer, 2010). Extensive depolymerization studies of the heparin backbone with various chemical agents, together with new analytical technologies, have made possible the development of ULMWHs with much lower mean MW and even higher anti-FXa:anti-FIIa activity ratios. Semuloparin (AVE5026, Mulsevo®, Sanofi-Aventis) (Gras, 2012) and RO-14 (Laboratorios Farmacéuticos Rovi, S.A.) are the first ULMWHs to be developed.

RO-14 is obtained by selective chemical depolymerization of heparin in a non-aqueous medium, following a β-elimination method. RO-14 has a mean MW of 2,200 Da, and has an anti-FXa *in vitro* activity be-

tween 80 and 140 IU/mg and anti-FII *in vitro* activity lower than or equal to 7 IU/mg. Its anti-FXa:anti-FIIa activity ratio is approximately 20. The main theoretical advantage of RO-14 is conferred by the lower mean MW, a potentially longer elimination half-life, and by the larger anti-FXa:FIIa activity ratio.

As part of the initial clinical development program, we performed an open-label, randomized, First-Time-in-Human (FTIH) ascending dose study with an alternating cross-over design (Rico et al., 2011). The objectives of this study were to evaluate the safety and pharmacodynamic profile of RO-14 in healthy males, as assessed by the anti-FXa activity and anti-FIIa activity. Chapter 5 includes a thorough presentation of the results of this study.

c) Pharmacodynamics and safety assessment of Bemiparin after multiple prophylactic and single therapeutic doses in young and elderly healthy volunteers and in subjects with varying degrees of renal impairment

LMWHs have a good safety profile but, unlike UFH, they are predominantly cleared by the kidneys (Frydman, 1996, Weitz, 1997). Clearance studies in animals, cellular binding studies and clinical studies all indicate that the balance between renal and non-renal clearance is dependent on the MW; the higher the MW of the LMWH, the more the balance is shifted towards non-renal clearance (Johansen and Balchen, 2013). Due to potential prolongation of the biologic half-life and bioaccumulation of anti-FXa activity, renal clearance is considered a liability when dealing with patient populations in whom renal function could be compromised, such as the elderly or patients with CKD (Gray et al., 2008, Weitz, 1997, Hirsh and Raschke, 2004, Wyatt et al., 2006). Aging is associated with a number of physiologic and pathophysiologic changes, such as differences in lean body mass and a reduction in hepatic and renal function. These changes provoke pharmacokinetic alterations that affect drug absorption, volume of distribution, drug metabolism, and renal clearance, and they may have a direct impact upon the safety profile, efficacy, and dosing in elderly patients. Of all the physiologic changes, the most relevant is age-related reduction in renal clearance (Wyatt et al., 2006). Given that renal excretion plays a significant role in the elimination of the anti-FXa activity of LMWHs, it has been postulated that a physiological reduction in renal function related to aging may have noticeable effect on the PD of LMWHs (Samama and Gerotziafas, 2000).

In patients with CKD, the progressive decline in glomerular filtration rate (GFR) and the associated uremic milieu leads to perturbations in hemostasis and thrombosis pathways, leading to the development of both a pro-thrombotic and hemorrhagic state. Fibrinogen, plasminogen activation

inhibitor (PAI), and TF are all increased in CKD, as is platelet dysfunction. Furthermore, CKD is often accompanied by proteinuria, which even in small amounts (> 30 mg/dL) may considerably increase the thrombosis risk (Sood et al., 2013).

Both elderly patients and patients with CKD have higher risk of VTE (Parikh et al., 2011, Go et al., 2013, Monreal et al., 2006), and they also have higher risk of bleeding (Spencer et al., 2009, Spencer et al., 2008, Shoeb and Fang, 2013). When treating these patients, physicians need to consider the patient's renal function, the general bleeding risk, and the LMWH that is intended to be used, among other factors (Schmid et al., 2009c). Dosing decisions are of paramount importance, as under-dosing LMWHs in these patients could result in lack of efficacy and an increased risk of VTE, whereas overdosing could lead to hemorrhage. Yet, these decisions are still made without robust scientific evidence supporting them. Randomized controlled trials evaluating LMWHs have generally excluded elderly patients and those with severe renal impairment (Garcia et al., 2012). Although pharmacodynamic studies in patients with CKD have, in general, demonstrated that clearance of the anti-FXa activity is highly correlated with creatinine clearance (Cl_{Cr}), the studies have not followed the same methodology; therefore, results between the different LMWHs cannot be directly compared, and recommendations should not be extrapolated from one LMWH to another (Schmid et al., 2009c, Brophy et al., 2001, Goudable et al., 1991, Fareed and Walenga, 2007, Netti et al., 2008).

To decrease the risk of bleeding in elderly or in patients with renal impairment, some authorities suggest monitoring of anti-FXa activity (Garcia et al., 2012). Empirical use of a lower dose of LMWHs, or the avoidance of LMWHs, have also been suggested (Harenberg, 2004, Hirsh et al., 2008a, Hirsh et al., 2008b). None of these options are particularly convenient and the supporting evidence for these recommendations is of low quality (Grade 2C, weak recommendation, low quality evidence) (Guyatt et al., 2012).

Prospective data evaluating LMWH use in elderly patients have been mostly limited to inpatient treatment (Clark, 2008), and most of the studies in the elderly have been in patients with some degree of renal impairment. With prophylactic doses for VTE, increases in exposure to anti-FXa activity have been reported with the use of enoxaparin (40 mg SC QD), for elderly medical inpatients at least 75 years of age with severe renal impairment ($Cl_{Cr} \leq 30$ mL/min) and those with lower body weight (Mahe et al., 2007). When therapeutic doses have been assessed, significant accumulation of anti-FXa activity has been observed with nadroparin in healthy elderly subjects with a Cl_{Cr} of 62±6 mL/min (Bauersachs, 2012,

Mismetti et al., 1998), but no correlation has been found between anti-FXa activity and Cl_{Cr}, age, or weight in elderly in-patients treated with tinzaparin (Pautas et al., 2002, Siguret et al., 2011, Siguret et al., 2000). To date, only enoxaparin has a recommended dose reduction for patients over 75 years being treated for acute ST-elevated myocardial infarction (0.75 mg/kg SC twice daily) (Sanofi-Aventis, 2011, White et al., 2007). Due to the paucity of data, both with prophylactic and therapeutic doses, no validated recommendations are available for dosing in the elderly (Gouin-Thibault et al., 2010).

At prophylactic doses, reduction in clearance (39% reduction) and bioaccumulation of the anti-FXa activity occur in patients with severe renal impairment receiving enoxaparin. Therefore, for patients with a $Cl_{Cr} \leq 30$ mL/min who require pharmacologic VTE prophylaxis, a dose of 30 mg is recommended (Mahé et al., 2007, Sanderink et al., 2002). There is limited data for the other LMWHs (Garcia et al., 2012).

When used in full therapeutic doses, reduction in clearance and bioaccumulation in patients with severe renal impairment may occur with enoxaparin (Chow, 2003), dalteparin (Schmid et al., 2009b), and nadroparin (Mismetti et al., 1998), while apparently not with tinzaparin (Siguret et al., 2000). The recommended treatment dose of enoxaparin for patients with a $Cl_{Cr} \leq 30$ mL/min who have ACS or VTE is 50% of the usual dose (i.e. 1 mg/kg once daily) (Sanofi-Aventis, 2011). No specific recommendations have been made for other LMWH preparations, and appropriate dosing of LMWH in patients with severe renal impairment remains uncertain (Garcia et al., 2012).

Bemiparin, the LMWH with the lowest mean MW and the highest anti-FXa:anti-FIIa activity ratio, has been on the market in several countries since 1998 (marketing authorizations in 58 countries and actively marketed in 54 countries as of 2014), and it has been administered to over 12.7 million patients as of 2014, many of whom are older than 75 years of age (Martinez-Gonzalez and Rodriguez, 2010). However, no specific study has assessed the influence of age and renal impairment on the potential bioaccumulation of bemiparin after prophylactic and therapeutic doses of bemiparin. We conducted a multicenter, open-label, 2-period, parallel study (Rico et al., 2014) to assess the pharmacodynamics of bemiparin after prophylactic and therapeutic doses in elderly and renal impairment subjects, and evaluated the potential need for dose adjustment in these populations. The results and conclusions of this study are presented in Chapter 6.

Chapter 2

Hypotheses

CHAPTER 2 - HYPOTHESES

The underlying hypotheses upon which the present doctoral dissertation is based are:

A. Clinical trial 1 - Comparative pharmacodynamic time-course of bemiparin and enoxaparin in healthy volunteers

Considering that bemiparin has the lowest mean molecular weight and the highest *in vitro* anti-FXa:anti-FIIa activity ratio among commercially available LMWHs, bemiparin will show a superior *in vivo* pharmacodynamic profile (higher anti-FXa activity, lower anti-FIIa activity, higher anti-FXa:anti-FIIa activity ratio, lower aPTT and TT, a lower release of TFPI, and a lower or equal TP-TmT), as compared to enoxaparin when administered to young healthy volunteers.

B. Clinical trial 2 - Safety and pharmacokinetics of a novel ultra low molecular weight heparin (RO-14) in healthy volunteers – a first time in human (FTIH) single ascending dose study

RO-14, a novel ULMWH, will be safe and well tolerated as assessed by adverse event reporting, vital signs, electrocardiograms, and laboratory tests, when administered subcutaneously as single ascending doses ranging from 1,750 IU to 19,950 IU to healthy male volunteers.

RO-14, a novel ULMWH, possesses a lower mean molecular weight, higher *in vitro* anti-FXa activity, and lower anti-FIIa activity; than any of the currently commercially available LMWHs. These chemical and *in vitro* characteristics will translate into a higher and more sustained *in vivo* anti-FXa activity and almost null anti-FIIa activity than other LMWHs currently in the market, when administered to male healthy volunteers.

Subcutaneous administration of RO-14 doses, ranging from 1,750 IU and 19,950 IU, will exhibit dose proportional and linear increases in anti-FXa activity when administered as single ascending doses to healthy male volunteers.

C. Clinical trial 3 - Pharmacodynamics assessment of bemiparin after multiple prophylactic and single therapeutic doses in adult and elderly healthy volunteers and in subjects with varying degrees of renal impairment

As LMWHs rely on the kidney as the primary route of elimination, the administration of multiple prophylactic (3,500 IU) and therapeutic doses (115 IU/kg) of bemiparin to elderly subjects or to patients with varying degrees of renal impairment will result in a higher exposure to anti-FXa activity than in adult healthy volunteers.

Given that there is a physiological age-related decrease in renal function, and that LMWHs reliance on this route for elimination is inversely correlated with their mean molecular weight, the administration of bemiparin will result in anti-FXa activity exposures directly correlated to age, and inversely correlated to creatinine clearance.

Potential differences in the pharmacodynamic profiles (anti-FXa and anti-FIIa activities) after the administration of bemiparin between adult healthy volunteers, elderly volunteers, and patients with varying degrees of renal insufficiency, in the pharmacodynamic profiles (anti-FXa anti-FIIa activities) after the administration of bemiparin may be significant enough to justify a dose adjustment recommendation.

Chapter 3

Objectives

CHAPTER 3 - OBJECTIVES

The objectives of this work were:

A. Clinical trial 1 - Comparative pharmacodynamic time-course of bemiparin and enoxaparin in healthy volunteers

To compare the pharmacodynamic time-course (anti-FXa activity, anti-FIIa activity, anti-FXa/FIIa activity ratio, aPTT, TT, total TFPI release and TP-TmT) of prophylactic doses of bemiparin (3,500 IU) and enoxaparin (4,000 IU) in healthy volunteers, and assess if chemical differences are translated into different activities *in vivo*.

B. Clinical trial 2 - Safety and pharmacokinetics of a novel ultra low molecular weight heparin (RO-14) in healthy volunteers – a first time in human (FTIH) single ascending dose study

To assess the safety and tolerability of single ascending doses of RO-14 (1,750-19,950 IU) in healthy male volunteers, as assessed by adverse event reporting, vital signs, electrocardiograms, and laboratory tests, with special emphasis on clotting tests (platelet count, aPTT, PT, TT, fibrinogen, and AT).

To evaluate the pharmacodynamic profile of 12 single ascending doses of RO-14 in healthy male volunteers, as assessed by the time-course anti-FXa activity and anti-FIIa activity.

To assess dose proportionality and linearity of anti-FXa activity profiles after the administration of 12 single ascending doses of RO-14 in healthy male volunteers.

C. Clinical trial 3 - Pharmacodynamics assessment of bemiparin after multiple prophylactic and single therapeutic doses in adult and elderly healthy volunteers and in subjects with varying degrees of renal impairment

To evaluate the pharmacodynamic profile (anti-FXa and anti-FIIa activity) of multiple prophylactic (3,500 IU) and single therapeutic doses (115 IU/kg) of bemiparin in elderly subjects and patients with varying degrees of renal failure, as compared to adult healthy volunteers.

To evaluate the correlation of age and creatinine clearance with anti-FXa activity after the administration of repeated prophylactic doses (3,500 IU) and single therapeutic doses (115 IU/kg) of bemiparin in healthy volunteers, elderly volunteers, and patients with varying degrees of renal impairment.

To evaluate the need for dose adjustment of prophylactic (3,500 IU) and therapeutic doses (115 IU/kg) of bemiparin in elderly subjects and patients with varying degrees of renal impairment, if differences in the pharmacodynamic profiles (anti-FXa and anti-FIIa activities) are found between these groups and adult healthy volunteers.

Chapters 4-6

Publications

CHAPTER 4 - COMPARATIVE PHARMACODYNAMIC TIME-COURSE OF BEMIPARIN AND ENOXAPARIN IN HEALTHY VOLUNTEERS

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Comparative pharmacodynamic time-course of bemiparin and enoxaparin in healthy volunteers

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Key words
bemiparin – enoxaparin – low-molecular-weight heparin – pharmacodynamics – time course

Abstract. Low molecular weight heparins (LMWHs) are antithrombotic drugs that differ on biochemical and pharmacological properties. Objective: This study was conducted to compare the pharmacodynamic time-course of two LMWHs, bemiparin and enoxaparin, at high prophylactic doses. Methods: This was an open, randomized, single-blind, cross-over study to compare the pharmacodynamic time-course, safety and tolerability of two LMWHs, bemiparin 3500 IU and enoxaparin 4000 IU at subcutaneous single doses in 12 healthy male volunteers. Anti-Xa activity (main biomarker of heparin activity), anti-IIa activity, total and free tissue factor pathway inhibitor (TFPI), activated partial thromboplastin time (APTT), thrombin time (TT) and thromboplastin-thrombomodulin mediated time (Tp-TmT) were investigated. Results: Bemiparin 3500 IU achieved more anti-Xa activity than enoxaparin 4000 IU, measured by the area under the curve (geometric mean AUC_0^t) (bemiparin 3.69 vs. enoxaparin 3.33 IU h/ml; $p < 0.001$). Maximum anti-Xa activity was reached at 3 hours and there were anti-Xa measurable levels up to 16 h after subcutaneous administration. Anti-Xa activity half-life was 5.44 hours for bemiparin and 4.71 hours for enoxaparin. Anti-IIa activity was above the limit of quantification (0.05 IU/ml) in only 2 volunteers after bemiparin and in 8 after enoxaparin. The "in-vivo" anti-Xa:IIa ratios were: bemiparin 37.9 (95% CI: 28.0 – 55.3, n = 2) and enoxaparin 16.3 (95% CI: 12.2 – 23.4, n = 8). Enoxaparin induced a higher release of total TFPI, but not on free TFPI, and a longer prolongation of APTT and TT (E_{max}) than bemiparin, with no differences between groups on Tp-TmT. Adverse events (one in each group) were mild and transient. Conclusion: Bemiparin 3500 IU showed more anti-Xa activity and higher anti-Xa: anti-IIa relationship than enoxaparin 4000 IU in healthy volunteers. Both treatments were well tolerated.

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Introduction

Low molecular weight heparins (LMWHs) are antithrombotic drugs of first choice for prophylaxis and treatment of venous thromboembolism (VTE) [Hirsh and Raschke 2004, Weitz 1997]. LMWHs are a heterogeneous mix of polysaccharide chains of different lengths and weights derived from unfractionated heparin (UFH) by chemical or enzymatic depolymerization. Depending on the manufacturer's procedure, the LMWH products obtained have different chemical composition and pharmacological properties. Consequently, each LMWH is a unique chemical entity and the results of clinical trials or pharmacokinetic studies cannot be extrapolated from one product to another [Christidou 2005, Barrett 2001, Fareed and Walenga 2007, Racine 2001].

LMWHs exert their main antithrombotic effect through the inhibition of Factor Xa (Stuart-Prower factor), an enzyme of the coagulation cascade [Hirsh and Raschke 2004, Weitz 1997]. This main anti-Xa antithrombotic effect may be modulated by other secondary effects, such as the release of tissue factor pathway inhibitor (TFPI) from endothelium [Broze 2003].

Factor Xa occupies a critical juncture in the coagulation cascade earlier than Factor IIa

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Table 1. Pharmacological characteristics of different LMWHs.

LMWH	Mean MW (Daltons)	Depolymerization process	Anti-Xa:IIa ratio**	Reference
Bemiparin	3600	Alkaline depolymerization	8	Falkon et al. 1995, 1997
Enoxaparin	4500	Benzylation and alkaline depolymerization	3.8	Racine 2001, Lovenox® package insert
Dalteparin	6000*	Nitrous acid depolymerization	2.7	Racine 2001, Fragmin® package insert
Tinzaparin	6500*	Enzymatic depolymerization with heparinase	2.0*	Racine 2001, Innohep® package insert

MW = molecular weight, LMWH = low molecular weight heparin, *European Pharmacopeia, 5th edition, Directorate for the Quality of Medicines of the Council of Europe. Strasbourg (France), 2004, **Related to the anti-Xa:IIa activities, in IU/mg of the dried substance "in vitro".

(thrombin). Inhibition earlier in the sequence of coagulation factor interactions has greater antithrombotic potential [Ansell 2007, MacFarlane 1964]. Clinical studies suggest that heparins with the highest anti-Xa:IIa activity are more effective and, at the same time, are safer, suggesting that the more anti-Xa activity a product has, the safer and more effective it is [Ansell 2007]. The lower the molecular weight (MW), the higher the anti-Xa:IIa activity ratio. Bemiparin is a new LMWH with the lowest MW (3600 Da) and the highest "in vitro" anti-Xa:IIa activity ratio (8:1) of commercially available LMWHs (Table 1) [Falkon et al. 1995, 1997, Racine 2001, Lovenox® package insert 2004, Fragmin® package insert 2004, Innohep® package insert 2004]. Enoxaparin, which is usually the reference LMWH used in comparative studies with new antithrombotics, has a higher MW (4500 Da) and a lower anti-Xa:IIa ratio (3.8:1) "in vitro" [Racine 2001, Lovenox® package insert 2004] than bemiparin.

The aim of the present study was to compare the pharmacodynamic time-course of bemiparin and enoxaparin in healthy volunteers, in order to assess if chemical differences are translated into different activities "in vivo".

Materials and Methods

Study design

This was a single-center, open-label, controlled, randomized, single-blind, cross-over study comparing two treatments with two study periods in healthy male volunteers. The protocol was approved by the Spanish Medicines Agency and the Ethics Committee of Hospital de la Santa Creu i Sant Pau. The study was conducted in compliance with the Declaration of Helsinki and Good Clinical Practice guidelines. All subjects gave written informed consent to participate in the study.

Subjects and treatments

Healthy, Caucasian male subjects, aged between 18 and 40 years of age, with a body mass index (BMI) between 19 – 26 kg/m^2, were screened for inclusion into the study. Subjects were excluded if they had known history of hypersensitivity to drugs, coagulation disorders (e.g., von Willebrand disease, hemophilias), conditions with an increased bleeding risk (e.g., peptic ulcer, hemorrhoids, acute gastroenteritis, or sensitivity to nasal bleeding) abnormal coagulation tests, positive serology for hepatitis B, C, or HIV virus, trauma or surgery in the 3 months prior to the study, use of any medication 15 days prior to the trial, or history of drug abuse or chronic disease.

Subjects were randomly assigned to receive either a single subcutaneous dose of bemiparin 3500 IU (Hibor®, Laboratorios Farmaceúticos Rovi, S.A.) or enoxaparin 4000 IU (Aventis Pharmaceuticals) contained in pre-filled syringes with 0.2 ml and 0.4 ml of solution for injection, respectively. The chosen dosages are those authorized for

thromboprophylaxis in patients at high risk of VTE. Each of the subjects included in the study received both medications separated by a washout period of at least 7 days, according to a cross-over design.

Subjects visited the trial unit (Institut de Recerca of Hospital de la Santa Creu i Sant Pau, Barcelona) the day before the study drug administration to undergo pre-treatment coagulation tests. Subjects stayed in the unit on each experimental day under fasting conditions. 24 hours after drug administration, subjects underwent physical examination, electrocardiogram (ECG) and laboratory tests (hematology, biochemistry and coagulation tests and urinalysis) before discharge.

Safety and tolerability

Subjective tolerability was evaluated by questioning subjects about any adverse events and by subjects spontaneously reporting them. Objective tolerability was assessed by monitoring heart rate, blood pressure, ECG and laboratory tests on the screening visit and also during the two experimental sessions. Platelet counts were measured before, during, and after the two experimental sessions.

Pharmacodynamic variables and pharmacokinetic-derived parameters

Anti-Xa activity (main biomarker of heparin activity), anti-IIa (antithrombin) activity, anti-Xa:IIa activity ratio, free and total TFPI release, and the interference on global clotting tests [activated partial thromboplastin time (APTT), thrombin time (TT), and thromboplastin-thrombomodulin-mediated time (TP-TmT)] were assessed at baseline and during the two experimental sessions. APTT, which examines the effects of drugs on coagulation stimulated by the intrinsic clotting pathway, is widely used to monitor the effects of UFH, but is not usually altered by LMWH to a great extent. TT measures the final step of the clotting pathway, the conversion of fibrinogen to fibrin, and is an indirect marker of anti-IIa activity. TP-TmT is a modified prothrombin time (PT) in the presence and in the absence of thrombomodulin, which may be used for screening the procoagulant capacity of plasma [Borrell et al. 2002].

The following pharmacokinetic-derived parameters were calculated for pharmacodynamic variables during study treatment: area under the pharmacodynamic variable-time curve (geometric mean AUC_0^t) during a 24h period from drug administration (if a pharmacodynamic variable had a pre-treatment value ≠ 0, the AUC was calculated from the differences between values at time points post-treatment and values at pre-treatment); maximum pharmacodynamic effect (E_{max}); time to reach maximum pharmacodynamic effect (t_{max}); and half-life ($t_{1/2}$).

Sample analysis

Blood samples to assess pharmacodynamic variables were collected in tubes containing sodium citrate (0.129 M) through an indwelling catheter in the antecubital vein at the time of drug administration and at regular intervals thereafter (0.5, 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, and 24 hours). Plasma samples were obtained by centrifugation and were frozen and stored at –20 °C until analysis at the laboratories at the Hospital de la Santa Creu i Sant Pau, Barcelona (Spain). Anti-Xa activity was measured with a commercial chromogenic assay, Coatest® LMW Heparin [Chromogenix, Instrumentation Laboratory (IL), Milan, Italy]. Anti-IIa activity was measured as described by Larsen et al. [1978], adapted to CPA (Chemistry Profile Analyser) (Coulter, USA). Total and free TFPI were measured using an enzyme-linked immunosorbent assay (ELISA) method (Diagnostica Stago Roche). TP-TmT was measured as described by Borrell et al [2002]. APTT and TT were measured in the analyser ACL 3000 (IL, Milan, Italy) using IL test APTT and IL test Thrombin Time (IL, Milan, Italy), respectively.

Statistical analysis

Parameters obtained after non-compartmental pharmacokinetic analysis of anti-Xa activity, geometric mean AUC_0^t and E_{max}, were compared between treatment groups by

Table 2. Baseline characteristics.

Variable	n = 12
	Mean ± SD
Demographic data	
Age, years	24.8 ± 3.7
Weight, kg	71.0 ± 7.4
Height, cm	179.5 ± 8.6
Quetelet index, kg/m^2	22.1 ± 2.0
Laboratory parameters	
Anti-Xa activity (IU/ml)	0.0 ± 0.0
Anti-IIa activity (IU/ml)	0.0 ± 0.0
Free TFPI (IU/ml)	12.2 ± 2.9
Total TFPI (IU/ml)	61.0 ± 15.2
APTT (ratio)	1.1 ± 0.1
TT (seconds)	21.2 ± 1.0
Tp-TmT (ratio)	1.1 ± 0.1
Creatinine (µmol/l)	98.1 ± 6.8

Table 3. Parameters derived from non-compartmental pharmacokinetic analysis of anti-Xa and anti-IIa activities after a single dose of LMWH

Parameter	Bemiparin 3,500 IU	Enoxaparin 4,000 IU	p value
Anti-Xa activity	n = 12	n = 12	
GM_AUC$_0^t$ (IU·h/ml)	3.69 ± 0.88	3.33 ± 0.80	< 0.001
E_{max} (IU/ml)	0.45 ± 0.07	0.45 ± 0.08	0.958
t_{max} (h)	3 (2 – 4)	3 (1 – 4)	0.755
$t_{1/2}$ (h)	5.44 ± 1.60	4.71 ± 1.77	0.232
Anti-IIa activity	n = 2	n = 8	
GM_AUC$_0^t$ (IU·h/ml)	0.09 ± 0.05	0.18 ± 0.10	0.057
E_{max} (IU/ml)	0.05 ± 0.00	0.08 ± 0.02	< 0.001
t_{max} (h)	2.5 (2 – 3)	3 (2 – 3.5)	0.560
Anti-Xa:IIa activity	n = 2	n = 8	
Ratio (95% CI)*	37.9 (28.0 – 55.3)	16.3 (12.2 – 23.4)	< 0.001

Values are means plus/minus standard deviation, except for t_{max}, which is median (1st and 3rd quartile). GM_ = geometric mean. *Based on AUC$_0^t$.

analysis of variance (ANOVA), controlling sequence, volunteers (clustered in the sequence), period and formulation. t_{max} was analyzed using Wilcoxon's non-parametric test and $t_{1/2}$ using paired-t-test. Main statistical analysis was performed using WinNonlin-Pro version 2.1 (Pharsight Corp, Cary, NC, USA). GraphPad Software was used to calculate 95% confidence intervals for anti-Xa: anti:IIa ratios.

Results

Study population

22 subjects were initially screened. 10 of them were not recruited for the following reasons: history of illegal drug intake or positive result on urine illegal drug screen (5 subjects), no agreement to perform the study (2 subjects), thalassemia (1 subject), blood pressure over normal values (one subject), laboratory values outside the accepted range (1 subject). Finally, 12 subjects were randomized and received medication. All of them completed the whole study. Demographics and baseline values for randomized subjects are shown in Table 2.

Safety and tolerability

Both treatments were well tolerated. Adverse events (one headache in each treatment group) were mild and transient. There were no clinically relevant abnormalities in hematology or biochemistry tests or urinalyses. There were no cases of heparin-induced thrombocytopenia. There were no statistically significant changes of the systolic blood pressure, pulse rate or ECG between treatment groups or with respect to baseline values.

Pharmacodynamics

The geometric mean (± SD) anti-Xa activity (AUC) was significantly higher in subjects on bemiparin 3500 IU than in those on enoxaparin 4000 IU (3.69 ± 0.88 vs. 3.33 ± 0.80; $p < 0.001$) (Table 3), indicating a more potent activity after bemiparin than after enoxaparin administration (11% higher in relative terms), despite the bemiparin dose administered was lower than that of enoxaparin. E_{max} and t_{max} were similar for both formulations. Anti-Xa activity was firstly detected at 30 minutes after the SC administration. The anti-Xa $t_{1/2}$ was 5.44 ± 1.60 after bemiparin administration and 4.71 ± 1.77 after enoxaparin administration.

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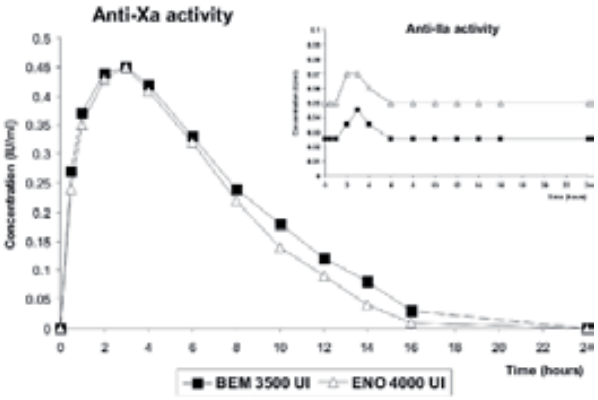

Figure 1. Time course of anti-Xa (left panel) and anti-IIa (right panel) activities after bemiparin 3500 IU or enoxiparin 4000 IU (n = 12 male healthy volunteers for both drugs in anti-Xa but n = 2 for bemiparin and n = 8 for enoxaparin in anti-IIa)

Table 4. Secondary parameters derived from non-compartmental pharmacokinetic analysis of pharmacodynamic variables after a single dose of LMWH (n = 12).

Parameter	Bemiparin 3500 IU	Enoxaparin 4000 IU	p value
Free TFPI			
GM_AUC_0^t (IU·h/ml)	309.37 ± 67.0	324.9 ± 48.6	0.160
E_{max} (IU/ml)	33.9 ± 9.5	38.4 ± 10.1	0.072
t_{max} (h)	1 (0.5 – 2)	1 (0.5 0 – 2)	0.630
Total TFPI			
GM_AUC_0^t (IU·h/ml)	1491.6 ± 245.7	1517.6 ± 233.6	0.028
E_{max} (IU/ml)	92.8 ± 14.9	95.7 ± 15.2	0.200
t_{max} (h)	0.8 (0.5 – 3)	2 (0.5 – 3)	0.443
APTT			
GM_AUC_0^t (ratio·h/ml)	27.5 ± 1.4	27.7 ± 2.0	0.458
E_{max} (ratio)*	1.3 ± 0.1	1.4 ± 0.1	0.039
t_{max} (h)	3 (1 – 4)	3 (2 – 4)	0.932
TT			
GM_AUC_0^t (seconds·h/ml)	494.9 ± 34.2	524.4 ± 34.1	0.014
E_{max} (seconds)	23.7 ± 1.7	27.1 ± 2.6	0.001
t_{max} (h)	3 (1 – 6)	3 (2 – 4)	0.671
Tp-TmT			
GM_AUC_0^t (ratio·h/ml)	27.3 ± 2.1	27.6 ± 1.5	0.2314
E_{max} (ratio)*	1.3 ± 0.1	1.3 ± 0.1	0.401
t_{max} (h)	2.5 (0.5 – 12)	1 (0.5 – 12)	0.347

Values are means plus/minus standard deviation, except for t_{max}, which is median (1st and 3rd quartile). GM_: geometric mean. *Ratio between coagulation time after heparin administration and control value.

Anti-IIa activity was absent before treatment, and its effect was above the limit of quantization (0.05 IU/ml) in 2 volunteers after the administration of bemiparin 3500 IU and in 8 volunteers after the administration of enoxaparin 4000 IU and. The geometric mean anti-IIa AUC_0^t presented a tendency to be lower in subjects on bemiparin than in those on enoxaparin (0.09 ± 0.05 vs. 0.18 ± 0.10; p = 0.058). The anti-Xa:anti-IIa ratio "in vivo" was 37.9 (95% CI: 28.0 – 55.3) for bemiparin and 16.3 (95% CI: 12.2 – 23.4) for enoxaparin (Table 3). Figure 1 shows the time-course curves of anti-Xa and anti-IIa activities after a single subcutaneous dose of bemiparin 3500 IU and enoxaparin 4000 IU.

With respect to secondary variables, no significant differences were observed between the kinetic profiles of free TFPI after the SC administration of both heparins (Table 4). Total TFPI (geometric mean AUC_0^t) after bemiparin administration was lower than that after enoxaparin administration (Table 4). APTT and TT (E_{max}) was significantly increased in subjects after enoxaparin treatment as compared to bemiparin treatment (Table 4), indicating that bemiparin interferes with APTT and TT to a lesser extent than enoxaparin. No differences were observed between treatment groups in Tp-TmT (Table 4).

Discussion

Our study shows that bemiparin and enoxaparin differs in their pharmacodynamic profile. Bemiparin 3500 IU achieved more anti-Xa activity and higher anti-Xa:IIa relationship than enoxaparin 4000 IU. The level of anti-Xa activity persisted longer with bemiparin than with enoxaparin. These differences are probably related to their different MW and chain composition, as a consequence of different manufacturing processes. The anti-Xa activity observed in our study is consistent with those previously reported in either healthy subjects or patients after the subcutaneous administration of bemiparin 3500 IU [Depasse et al. 2003, Kakkar et al. 2000, Planes et al. 2001] or enoxaparin 4000 IU [Collignon et al. 1995, Cornelli and Fareed 1999, Samama and Gerotziafas 2000] at single subcutaneous doses.

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The main antithrombotic effect of LMWH, mediated by its anti-Xa activity, may be modulated by secondary LMWH effects, such as release of TFPI, suppression of von Willebrand factor (vWF) release, interaction with heparin cofactor II, inhibition of leukocyte procoagulant actions, promotion of fibrinolysis, and modulation of vascular endothelium [Broze 2003, Racine 2001]. In our study, enoxaparin 4000 IU released a higher amount of total TFPI compared to bemiparin 3500 IU, although no statistically significant differences were observed when comparing the free TFPI levels. On the other hand, increased release of Von Willebrand factor appears to be a marker of platelet stimulation and adverse clinical outcomes, and a lower MW appears to be related to a higher suppression of vWF [Montalescot et al. 2000, Ray et al. 2005]. The possible correlation of MW on distinct effects of LMWHs and their clinical implications requires additional investigations.

APTT is a global test that screens the intrinsic pathway of the coagulation system, and prolonged APTT values are associated with bleeding risk [Hirsh 2004]. In our study, individuals on enoxaparin presented a higher APTT (E_{max}) than individuals on bemiparin. Enoxaparin induced a higher anti-IIa (antithrombin) activity than bemiparin. This higher anti-IIa activity was also confirmed with the TT, which is a more sensitive test. Tp-TmT increased very early after heparin administration and reached its maximum value at 1 hour. This t_{max} was the same as that observed for TFPI but was earlier than the peak of maximum activity of anti-Xa that was reached at 3 hours. This response suggests that Tp-TmT may be sensitive to the TFPI released from the endothelium by LMWH.

This study has some limitations. It was not double blind. However, double blinding is not commonly used in this type of studies and it is not relevant for study results, since main endpoint is a biochemical marker. We assessed pharmacodynamic parameters following a single dose of LMWH, instead of a multiple-dose study with measurement of parameters at steady state. Nevertheless, single-dose study is the commonly used design in this kind of studies because LMWHs have a linear pharmacokinetics and the results obtained after single dose administration are predictive of what will happen at steady state. Furthermore, we avoid unnecessary additional exposure of volunteers to study treatments.

In conclusion, bemiparin 3500 IU showed more anti-Xa activity and higher anti-Xa:IIa relationship than enoxaparin 4000 IU in healthy volunteers translating the chemical differences of both LMWH into different activities "in vivo". However, further investigation is needed to assess the clinical implications of these pharmacological differences.

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CHAPTER 5 - SAFETY AND PHARMACOKINETICS OF A NOVEL ULTRA LOW MOLECULAR WEIGHT HEPARIN (RO-14) IN HEALTHY VOLUNTEERS – A FIRST TIME IN HUMAN (FTIH) SINGLE ASCENDING DOSE STUDY

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Regular Article

Safety Assessment and Pharmacodynamics of a Novel Ultra Low Molecular Weight Heparin (RO-14) in Healthy Volunteers – A First-Time-In-Human Single Ascending Dose Study☆

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ABSTRACT

Introduction: RO-14 is a novel ultra low molecular heparin. The purpose of this study was to evaluate the safety and pharmacodynamic profile of RO-14 in healthy males.
Materials and methods: We conducted a two-stage, single-center, open-label, randomized study. Two cohorts of 6 volunteers were randomly assigned to 12 single, ascending subcutaneous doses (1750–19950 IU of anti-FXa activity) in an alternating crossover fashion. Safety was assessed by spontaneous/elicited adverse events, medical examination and laboratory tests. Anti-FXa activity and anti-FIIa activity were assessed throughout the 24 hours after dosing. Dose proportionality and linearity of the anti-FXa activity were evaluated.
Results: All doses were well tolerated and there were no bleeding events. At the lowest dose, anti-FXa activity A_{max} was 0.16 (±0.02) IU/mL and $AUC_{0\text{-}24}$ was 1.11 (±0.24) IU*h/mL, At the highest dose anti-FXa activity A_{max} was 1.67 (±0.15) IU/mL; $AUC_{0\text{-}24}$ was 21.48 (±4.46) IU*h/mL and t½ was 8.05 h. Mean T_{max} (all doses) was 2.86 (±0.39) h. RO-14 showed proportional and linear pharmacodynamics [normalized A_{max} among doses ($p=0.594$) and normalized $AUC_{0\text{-}24}$ ($p=0.092$), correlations between A_{max}-dose ($R^2=0.89$, $p<0.001$) and $AUC_{0\text{-}24}$-dose ($R^2=0.86$, $p<0.001$)]. Anti-FIIa activity was below the detection limit (0.1 IU/ml) at all dose levels. No clinically significant changes were observed in the platelet count, APTT, PT, TT, fibrinogen and antithrombin.
Conclusions: In this phase I study, RO-14 exhibited a good safety profile, anti-FXa activity for either prophylaxis or treatment of venous thromboembolism, linear pharmacodynamics, a longer elimination half-life than currently marketed low molecular weight heparin and no anti-FIIa activity.

Introduction

Venous thromboembolism (VTE), encompassing deep vein thrombosis (DVT) and pulmonary embolism (PE), is one of the major common endpoints in human disease and a frequent complication among hospital inpatients [1]. Since their development, 2 decades ago, low molecular weight heparins (LMWH) have addressed some of the disadvantages shown by unfractioned heparin (UFH). LMWHs produce a more predictable anticoagulant response than UFH, reflecting their better bioavailability, longer half-life, and dose-independent clearance [2]. These characteristics have eliminated the need for monitoring in most cases and reduced the risk of bleeding [2,3]. Due to their advantages, LMWHs have largely replaced UFH for many indications[3] and are nowadays standard care for the prevention and treatment of VTE [4,5].

Several LMWHs have been developed (e.g enoxaparin, dalteparin, tinzaparin, bemiparin, etc.). All have slightly different properties and

Abbreviations: APTT, Activated partial thromboplastin time; AE, Adverse event; AEMPS, Agencia Espanola de Medicamentos y Productos Sanitarios; AT, Antithrombin; $AUC_{0\text{-}24}$, Area under the anti-FXa activity-time curve in the 24 hours after drug administration; $AUC_{0\text{-}\infty}$, Area under the anti-FXa-time curve extrapolated to infinity; Cl/F, Clearance; DVT, Deep vein thrombosis; ECG, Electrocardiogram; t½, Elimination half-life; FOBT, Fecal occult blood test; FTIH, First-Time-In-Human; GMP, Good manufacturing practice; A_{max}, Maximum anti-FXa activity; MRSD, Maximum recommended starting dose; NOAEL, No Observable Adverse Effect Level; PT, Prothrombin time; PE, Pulmonary embolism; T_{max}, Time to reach maximum anti-FXa activity; TT, Thrombin time; VTE, Venous thromboembolism; Vd/F, Volume of distribution; ULMWH, Ultra Low Molecular Weight Heparin; UFH, Unfractioned heparin.

☆ The study was presented in part at the 22nd Congress of the International Society on Thrombosis and Haemostasis, Boston, USA, 15–16 July, 2009

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[1] In memoriam – Manel J. Barbanoj passed away in the company of his family and friends on December 12th, 2010. We have lost a great mentor, an outstanding researcher and a wonderful friend. May he rest in peace.

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licenses for different risk situations [6]. Each LMWH is a unique chemical entity and the results of clinical trials or pharmacodynamic studies cannot be extrapolated from one product to another [7–9].

Despite the recent development of new options in antithrombotic therapy (e.g. orally administered direct factor Xa inhibitors and direct thrombin inhibitors), there is still room for improvement [5]. One of the lines of research that has received attention is the development of LMWHs with an optimized profile. RO-14 is a new ultra low molecular weight heparin (ULMWH) currently under clinical development. The main theoretical advantage is conferred by the lower mean molecular weight, by the enhanced anti-FXa activity that has been suggested to be involved with an improved anti-thrombotic efficacy [10], the potential longer elimination half-life and by the larger anti-FXa:FIIa activity ratio. In regards to this last supposed advantage, it is noteworthy that there seems to exist a larger safety margin with anti-FXa inhibition with respect to bleeding in comparison to inhibition of thrombin [11–13].

As part of the drug development program, the aim of this first-time-in-humans (FTIH) study was to provide essential information about the safety, tolerability and preliminary pharmacodynamics of RO-14.

Materials and methods

Study design

This was a two-stage, two cohort, alternating crossover, single-center, open-label, controlled, randomized, single ascending dose study to evaluate the safety, tolerability and preliminary pharmacodynamics of RO-14 in healthy male volunteers. The protocol was approved by the Hospital de la Santa Creu i Sant Pau - Clinical Research Ethics Committee and the Spanish Drug Agency (AEMPS). The study was conducted in compliance with the Declaration of Helsinki and Good Clinical Practice guidelines. All subjects gave written informed consent to participate in the study and were paid for their collaboration. The study is registered at clinicaltrials.gov (ref. #NCT00629733).

Study drug

The study drug is the sodium salt of a novel ULMWH denominated RO-14 (Laboratorios Farmaceúticos Rovi, S.A., Spain). RO-14 is obtained by selective chemical despolyimerization of UFH in a non-aqueous medium, following a beta-elimination method [14].

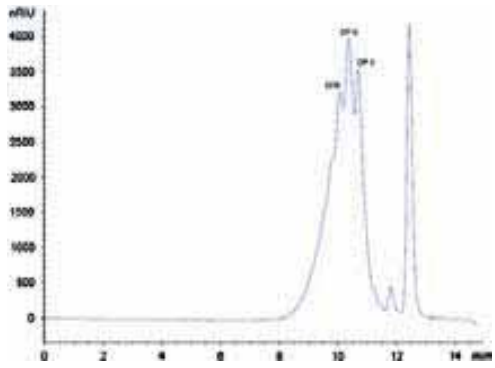

Fig. 1. GPC chromatogram of RO-14: RO-14 has a mean molecular weight of 2,200 Da is composed of 25–40% oligosaccharides with a mean MW lower than 2000 Da, 40–75% oligosaccharides with MW between 2000 and 6000 Da and less than 15% oligosaccharides with a MW higher than 6000 Da (DP: Degree of Polymerization).

RO-14 has an anti-factor Xa in vitro activity between 80 and 140 IU/mg and an anti-factor IIa in vitro activity lower than or equal to 7 IU/mg. RO-14 has an anti-factor Xa/anti-factor IIa ratio higher than 20.

RO-14 is composed of mixtures of fragments of heparin or oligosaccharides (Fig. 1). Its mean molecular mass by weight is between 1,800 and 3,000 Da. Its weight-average molecular weight ranges with a mean value of 2,200 Da. Its composition is the following:

- from 25 to 40% of oligosaccharides of molecular mass under 2,000 Da;
- from 40 to 75% of oligosaccharides of molecular mass between 2,000 and 6,000 Da; and
- less than 15% of oligosaccharides of molecular mass over 6,000 Da.

The investigational product was manufactured according to current good manufacturing practice (GMP) procedures and dispensed in individually-sealed boxes, labeled according to a randomization code. The randomization code was generated with SPSS Statistics 17.0 by a person not involved in the conduct of the study.

RO-14 was supplied in 0.5 mL pre-filled syringes containing sterile and endotoxin-free aqueous solution for subcutaneous injection. The following RO-14 strengths were used: 1750 IU anti-FXa/0.2 mL, 2450 IU anti-FXa/0.2 mL, 3500 IU anti-FXa/0.2 mL, 4550 IU anti-FXa/0.2 mL, 5600 IU anti-FXa/0.3 mL, 6650 IU anti-FXa/0.3 mL, 7700 IU anti-FXa/0.4 mL, 10150 IU anti-FXa/0.8 mL, 12600 IU anti-FXa/0.8 mL, 15050 IU anti-FXa/0.8 mL, 17500 IU anti-FXa/0.8 mL and 19950 IU anti-FXa/0.8 mL. Doses of 10150 IU and above were divided evenly in 2 syringes containing 0.4 mL each. An International Unit of anti-FXa activity is defined according to the standards established by the 1st International Standard for Low Molecular Weight Heparins (National Institute for Biological Standards and Control, United Kingdom) [15].

Maximum recommended starting dose

The maximum recommended starting dose (MRSD) in this FTIH study was determined following the Food and Drug Administration (FDA) guideline entitled "Estimating the Maximum Safe Starting Dose in Clinical Trials for Therapeutics in Adult Healthy Volunteers" [16].

Given that the No Observable Adverse Effect Level (NOAEL) in the most sensitive species (i.e. Beagle dog) derived from toxicology studies was 500 IU anti-FXa/kg SC [17], the MRSD was calculated to be 27 IU anti-FXa/kg. This was further rounded to 25 IU anti-FXa/kg for practical purposes.

Subjects and study conduct

Healthy male subjects, aged between 18 and 45 years of age and weighing 65-75 kg, were screened for inclusion in the study. Subjects had to be healthy as judged by medical history, physical examination, vital signs, electrocardiogram (ECG) and clinical laboratory results. They were excluded if they were smokers of more than 10 cigarettes a day, and if they had known history of hypersensitivity to drugs, coagulation disorders (e.g. von Willebrand disease, hemophilias), conditions with an increased bleeding risk (eg. peptic ulcer, hemorrhoids, acute gastroenteritis, or sensitivity to nasal bleeding), abnormal coagulation tests, urinalysis positive for hematuria, positive fecal occult blood test (FOBT), positive serology for hepatitis B, C, or HIV virus, trauma or surgery in the 6 months prior to the study, any medication 15 days prior to the trial, or history of drug abuse or chronic disease. Importantly, subjects were known not to have taken aspirin or aspirin-containing medications in the 10 days before the study.

The study was divided into two stages, A and B. Stage A assessed 6 ascending dose levels of RO-14 (1750, 2450, 3500, 4550, 5600, 6650 IU anti-FXa), each administered as a single subcutaneous injection in the abdominal region. After these 6 doses, it was deemed

appropriate to further characterize the activity of RO-14 with higher doses, and according to an approved protocol amendment, Stage B was performed to assess 6 additional strengths of RO-14: 7700, 10150, 12600, 15050, 17500 and 19950 IU anti-FXa.

In both stages, subjects were randomly assigned to two cohorts of 6 participants each. A minimum 14-day washout period elapsed before any given cohort was administered another dose. In as many cases as possible, volunteers from Stage A were recruited in Stage B of the trial (this occurred in 7 cases). No formal sample size determination was performed.

Clinical assessments

For every experimental session, participants attended the Drug Research Center (Centre d'Investigació de Medicaments) the morning before the study drug administration to undergo a urine drug screen. They were admitted to the study unit the same night and were administered the study on awakening the next morning (~8:00 am) in fasting state. All participants then remained at the study site for 24-hours post-drug administration, during which time blood samples were collected for pharmacodynamic analysis, and urine and fecal samples were collected for detection of blood presence. Twenty-four hours after drug administration, subjects underwent a physical examination, an ECG and laboratory tests, after which they were discharged. Seven days later they returned to the unit for a safety follow-up visit.

Safety and tolerability

Subjective tolerability was evaluated by indirect questioning and reporting about adverse events. Objective tolerability was evaluated by monitoring clinically evident adverse events, including bleeding, and by evaluating injection site tolerability. Causality Assessment of Adverse Events was performed according to the World Health Organization (WHO) – Uppsala Monitoring Centre system for standardized case causality assessment [18]. Objective tolerability was also evaluated by monitoring vital signs, ECG and laboratory tests (including urinalysis and FOBT) during all visits. Special emphasis was placed on changes at the end of each experimental period in the clotting tests comprising platelet count, activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT), fibrinogen and antithrombin (AT).

Safety and tolerability data were only described when clinically relevant values and outside reference range were detected.

Pharmacodynamic analysis

No validated and reproducible technique is yet available to determine LMWHs in plasma or other body fluids. For this reason, anti-FXa activity is normally used as a surrogate indicator of the time course of LMWHs [2]. Experimental techniques have demonstrated a very strong correlation between the concentration of LMWHs and anti-FXa activity, making anti-FXa activity the variable of choice for pharmacodynamic analysis [19].

Sample analysis

Blood samples were taken from the antecubital vein through an indwelling catheter and collected in tubes containing sodium citrate (0.129 M) at the time of drug administration and at regular intervals thereafter (0.5, 1, 2, 3, 4, 6, 8, 10, 12, 14, 18, and 24 hours post drug administration). Plasma samples were separated by centrifugation, frozen, and stored at −20 °C until analysis at the Hemostasis and Thrombosis Unit, Department of Hematology, Hospital de la Santa Creu i Sant Pau, Barcelona (Spain). Anti-FXa activity was measured with a commercial chromogenic assay, Coatest® LMW Heparin [Chromogenix, Instrumentation Laboratory (IL), Milan, Italy]. This assay is linear up to 2.0 IU/mL and the detection threshold is 0.1 IU/mL. Anti-FIIa activity was measured as described by Larsen et al. [20], adapted to CPA (Chemistry Profile Analyser) (Coulter, USA). The assay is linear up to 1.0 IU/mL and the detection threshold is 0.1 IU/mL.

Pharmacodynamic variables

Anti-FXa activity (main biomarker of heparin activity) and anti-FIIa activity were assessed at baseline and during all experimental sessions. The mean time course of anti-FXa activity at all dose levels was plotted in normal and semi-logarithmic scale.

Using WinNonlin-Pro version 2.1 (Pharsight Corp, Cary, NC), anti-FXa activity determinations in the 24-hour period after drug administration were analyzed using a non-compartmental approach. The following parameters were calculated for all volunteers and dose levels: time to reach maximum anti-FXa activity (T_{max}); maximum anti-FXa activity (A_{max}); elimination half-life (t ½); area under the anti-FXa activity-time curve in the 24 hours after drug administration (AUC_{0-24}); area under the anti-FXa-time curve extrapolated to infinity ($AUC_{0-\infty}$); volume of distribution (Vd/F) and clearance (Cl/F).

Statistical analysis

Statistical analysis was performed using SPSS Statistics 17.0. The parameters resultant of non-compartmental analysis of anti-FXa activity were analyzed for volunteers who completed all the study procedures of at least one dose level.

Descriptive statistics (mean and standard deviation) were calculated for all parameters.

Exploratory dose proportionality evaluation was carried out as follows:

a) Dose vs. pharmacodynamic parameter (A_{max}, $AUC_{0-\infty}$, AUC_{0-24}, T_{max}, t ½, Vd/F y Cl/F) plots were assessed.
b) We compared log-transformed dose-normalized pharmacodynamic parameters ($AUC_{0-\infty}$, AUC_{0-24} and A_{max} of anti-FXa activity) between groups using one-way analysis of variance (ANOVA) or the Kruskal-Wallis test if conditions for parametric analysis were not met (e.g. heterogeneous variances).
c) A linear regression analysis was conducted between dose and $AUC_{0-\infty}$, AUC_{0-24} and A_{max} of anti-FXa . Pearson's correlation test was first performed, and the correlation matrix was evaluated for direction, magnitude and significance. If the correlation was positive, linear regression analysis was performed following a least squares means approach. The coefficient of determination (R^2) was evaluated to determine the percentage of the dependent variable variance that could be explained by the independent variable (i.e. dose). The significance of the differences between the sum-of-squares values of the regression model and of the null hypothesis model was evaluated through the ANOVA method. The resulting coefficients were used for the construction of the linear regression equation according to the following expression: $Y=\beta_0+\beta_1X_1$ where β_0 is the intersect or constant, β_1 are the respective parameters of each independent variable.

In all cases, the statistical significance level required was equal or inferior to 5% ($[\alpha]=0.05$).

Results

Subject disposition and demographics

A total of 18 volunteers fulfilled all inclusion/exclusion criteria and were enrolled in the study. They received one or more doses of RO-14. Thirteen of the volunteers participated in Stage A and 12 volunteers participated in Stage B. Seven volunteers participated in both stages of the study. In Stage A one volunteer had to abandon the study

Table 1
Baseline characteristics.

Variable	Stage A		Stage B	
	Cohort 1 *n=6*	*Cohort 2* *n=7*	*Cohort 1* *n=6*	*Cohort 2* *n=6*
	Mean ± SD	*Mean ± SD*	*Mean ± SD*	*Mean ± SD*
Demographic data				
Age, years	30.83 ± 6.74	27.57 ± 2.70	31.17 ± 6.68	29.33 ± 2.34
Weight, kg	68.17 ± 2.54	70.90 ± 3.76	72.57 ± 4.44	72.80 ± 2.41
Height, cm	173.50 ± 6.50	175.14 ± 6.47	172.33 ± 8.71	176.50 ± 6.89
Body Mass Index, kg/m^2	22.42 ± 1.89	23.16 ± 1.34	24.48 ± 1.54	23.43 ± 1.53

prematurely owing to a serious adverse event (SAE). This volunteer and the volunteer that replaced him in the last dose level of Stage A both participated in Stage B.

Demographics for randomized subjects are shown in Table 1.

Clinical assessments

Safety and tolerability

RO-14 at all dose levels was subjectively and objectively well-tolerated. Six volunteers experienced a total of 14 adverse events (AE's), only one of which was serious. The SAE was a cycling accident during Stage A of the study. Volunteer 03 experienced an acromio-clavicular dislocation and had to be surgically intervened, hospitalized

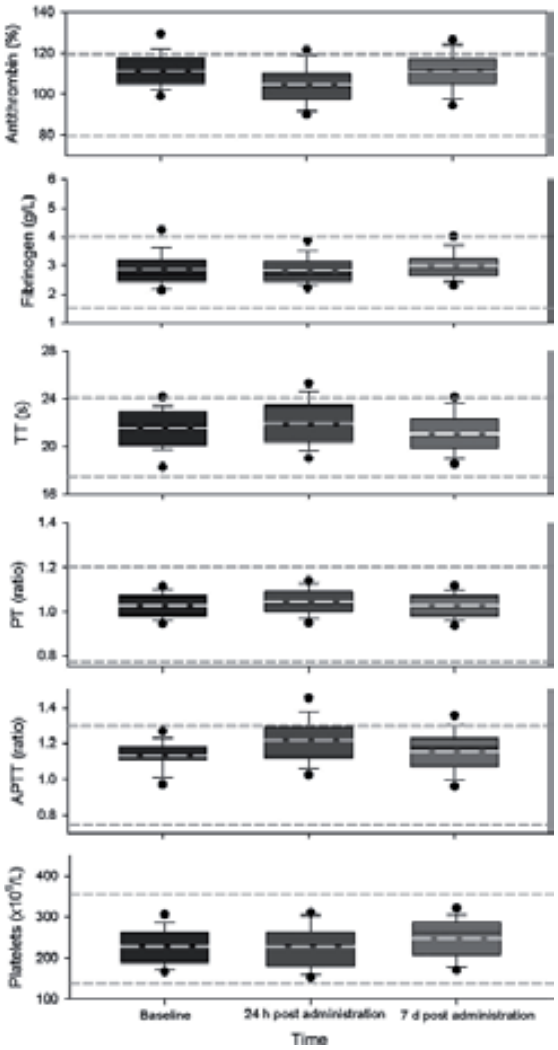

Fig. 2. Vertical box plots displaying the descriptive statistics of the platelet count, APTT, PT, TT, fibrinogen and antithrombin measured at baseline, 24 hours post drug administration and 7 days post drug administration at all tested doses. The mean is displayed in dashed white lines and the median is displayed as solid black lines within the boxes. Boxes display the standard deviation, whiskers display the 95% CI and the solid black dots show outliers. On gray dotted lines the upper and lower reference ranges of normality are indicated.

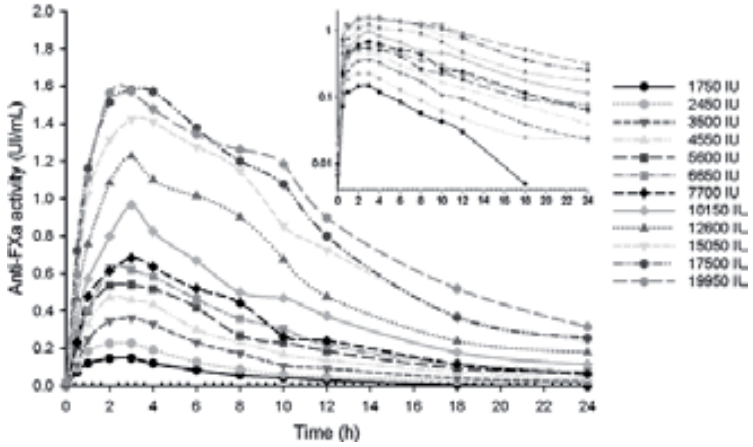

Fig. 3. Time course of anti-FXa activity after single subcutaneous doses of RO-14 ranging from 1750 IU to 19950 IU (n = 6 volunteers per dose level). Normal and semi-logarithmic scale.

and he was prescribed multiple doses of opioid and non-opioid analgesics. The SAE led to the premature withdrawal from the study of this volunteer, who was replaced by another subject (3R) who received the dose level of RO-14 that was foreseen for the volunteer who suffered the SAE. Volunteer 03 recovered satisfactorily without sequela. SAE was deemed not to be related to the study drug by the principal investigator. Noteworthy, during Stage B, both volunteers participated because at least 6 months had elapsed and the former volunteer was fully recovered. The 13 remaining AE's were not dose related, infrequent, mild in severity and transient.

There were no bleeding events in the study and no evidence of occult blood loss neither in urinalysis nor in the FOBT. There were no cases of heparin-induced thrombocytopenia. In relation to injection site tolerability, there were 2 reports of hematomas of less than 3 cm of diameter at the site of injection in one volunteer. Hematomas were considered probably associated with the administration study drug.

Vital signs, ECGs and laboratory test results (including platelet count, activated APTT, PT, TT, fibrinogen and AT) at baseline, 24 hours and 7 days post drug administration were within normal ranges or abnormalities were non-clinically significant. In Fig. 2, the mean, standard deviation and 95% CI are shown for the platelet count, activated APTT, PT, TT, fibrinogen and AT.

Pharmacodynamics

Anti-FXa activity. The time course of anti-FXa activity at all dose levels is shown in Fig. 3. The mean (SD) parameters of anti-FXa activity after all doses of RO-14 are summarized in Table 2. Maximal response to RO-14 was produced by the highest dose administered (i.e. 19950 IU), which yielded a mean A_{max} of 1.67 IU/mL at 3.17 hours post drug administration (T_{max}), and a mean $AUC_{0-\infty}$ of 25.38 IU*h/mL post drug administration. The elimination half-life after the highest dose was 8.05 h and clearance was 826.20 mL/h. Volume of distribution was 9259.25 mL. Noteworthy, anti-FXa activity 24 h post drug administration of RO-14 19950 IU was 0.31 IU/mL.

Exploratory assessment of dose proportionality

a) Visual inspection: The time course of anti-FXa activity after 12 dose levels of RO-14 showed a dose proportional increase in mean A_{max}, AUC_{0-24}, and $AUC_{0-\infty}$ (Fig. 3).
b) Between treatment group comparison of log-transformed dose normalized pharmacodynamic parameters.
As variances were heterogeneous, we used the Kruskal-Wallis test instead of ANOVAs to compare log-transformed dose normalized parameters. This test showed that there were no statistical differences in the pharmacodynamic parameters that define the speed (A_{max}, $p = 0.594$), and magnitude ($AUC_{0-\infty}$ $p = 0.074$ and AUC_{0-24}, $p = 0.092$) of exposure to anti-FXa activity.
Taken together, these results show a dose-proportional relationship within the studied range.
c) Analysis between dose and $AUC_{0-\infty}$, AUC_{0-24} and A_{max} of anti-FXa using linear regression.

Table 2
Mean ± SD pharmacodynamic parameters of anti-FXa activity after single subcutaneous doses of RO-14 ranging from 1750 to 19950 IU (n = 6 healthy young volunteers).

Dose (IU)	T_{max} (h)	A_{max} (IU/mL)	$t_{½}$ (h)	AUC_{0-24} (IU*h/mL)	$AUC_{0-\infty}$ (IU*h/mL)	Vd/F (mL)	Cl /F (mL/h)
1750	2.33 ± 0.52	0.16 ± 0.02	3.83 ± 0.77	1.11 ± 0.24	1.16 ± 0.25	8379.19 ± 1004.71	1552.65 ± 256.09
2450	2.33 ± 0.82	0.24 ± 0.05	4.97 ± 3.30	1.88 ± 0.98	2.16 ± 1.28	7710.13 ± 2084.53	1588.37 ± 985.02
3500	2.67 ± 0.52	0.37 ± 0.06	5.41 ± 3.65	3.09 ± 1.08	3.38 ± 1.35	7645.99 ± 2616.56	1207.99 ± 535.00
4550	2.67 ± 0.82	0.49 ± 0.09	6.10 ± 2.82	4.35 ± 1.68	4.79 ± 2.05	8670.96 ± 3343.74	1127.52 ± 571.04
5600	2.67 ± 0.52	0.55 ± 0.02	6.91 ± 0.96	5.54 ± 0.52	6.31 ± 0.60	8852.07 ± 949.21	894.60 ± 85.93
6650	2.50 ± 0.55	0.64 ± 0.16	6.45 ± 1.70	6.59 ± 1.85	7.43 ± 2.40	8568.36 ± 1814.50	987.14 ± 347.14
7700	3.17 ± 0.75	0.70 ± 0.13	6.44 ± 3.07	6.90 ± 1.92	7.64 ± 2.54	9439.60 ± 3663.52	1093.31 ± 307.61
10150	3.00 ± 0.00	0.97 ± 0.30	8.24 ± 5.04	9.64 ± 3.35	11.33 ± 4.54	10595.76 ± 4735.40	1040.26 ± 441.72
12600	3.33 ± 1.37	1.26 ± 0.24	8.12 ± 3.38	13.66 ± 3.14	15.86 ± 3.69	9661.32 ± 4717.68	830.12 ± 187.70
15050	3.50 ± 0.55	1.49 ± 0.29	7.37 ± 0.88	18.12 ± 4.23	20.69 ± 5.06	8046.93 ± 1817.64	764.47 ± 182.65
17500	3.00 ± 0.89	1.63 ± 0.31	6.99 ± 1.22	19.91 ± 4.98	22.55 ± 5.70	8266.74 ± 2653.59	820.18 ± 211.55
19950	3.17 ± 0.98	1.67 ± 0.15	8.05 ± 2.76	21.48 ± 4.46	25.38 ± 5.71	9259.25 ± 2601.17	826.20 ± 219.29

There was a strong correlation between the dose administered and $AUC_{0-\infty}$, AUC_{0-24} and A_{max}.

A linear regression model can predict $AUC_{0-\infty}$, AUC_{0-24} and A_{max} to a great extent. Figs. 4 and 5 illustrate the relationship between the dose administered and $AUC_{0-\infty}$, and A_{max} of anti-FXa activity along with the 95% prediction intervals and the model equation. Within the studied range of doses, the pharmacodynamic behavior of RO-14 appears to be linear.

Anti-FIIa activity. Anti-FIIa activity was below the detection threshold (0.1 IU/mL) before and after treatment at all dose levels and for all subjects. We were therefore unable to perform the non-compartmental analysis.

Discussion

This is the first study in humans to assess the safety, tolerability and preliminary pharmacodynamic profile of a novel ULMWH after single ascending doses. Our results show that RO-14 possesses a good safety and tolerability profile. In the present study, no major safety issues were identified or reported and no bleeding events occurred. RO-14 did not modify the vital signs, ECGs or laboratory tests at any dose. In particular, there were no apparent differences in the clotting tests performed before and after drug administration.

The exploratory assessment of dose proportionality showed that the dose response relationship of RO-14 within the studied dose-range was dose-proportional and linear. The time-concentration profiles were monophasic at all doses. Mean A_{max} after the highest dose was 1.67 IU/mL. In comparison to the same IUs of other LMWH, like bemiparin (which reached a mean maximum activity of 2.03 IU/mL after single doses of 12500 IU), RO-14 showed lower peak activity; however the t ½ of RO-14 (8.05) is considerably longer than other marketed LMWHs (Table 3) [21]. This characteristic would potentially confer a better sustained anti-FXa activity at prophylactic and therapeutic doses. Noteworthy, the mean elimination half-life of RO-14 within the tested dose range was 8.05 h in spite of great variability. Inter-dose variability could be explained by the fact that the analytical method used to determine anti-FXa is not reliable under 0.1 IU/mL, rendering the accurate pharmacodynamic characterization of RO-14 after low doses troublesome.

RO-14 demonstrated no detectable anti-FIIa activity even at doses of 19950 IU. If the lack of anti-FIIa activity or very high ratio of anti-FXa-FIIa activity were confirmed in future studies, RO-14 could set a milestone in the development of LMWHs and the therapeutic implications of this would definitively have to be studied.

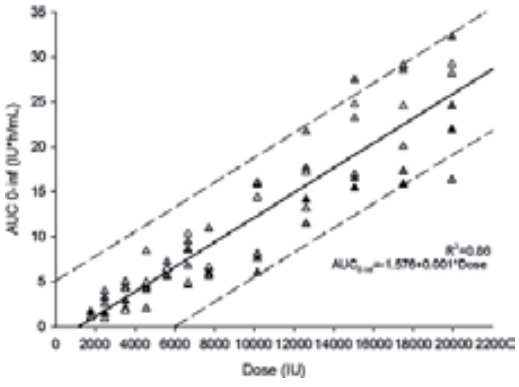

Fig. 4. Graphical representation of the relationship between 12 ascending doses of RO-14 and the AUC0-∞ of the anti-FXa activity. Least means squares linear regression line and 95% prediction intervals are shown as well as the determination coefficient of the relationship and the prediction equation.

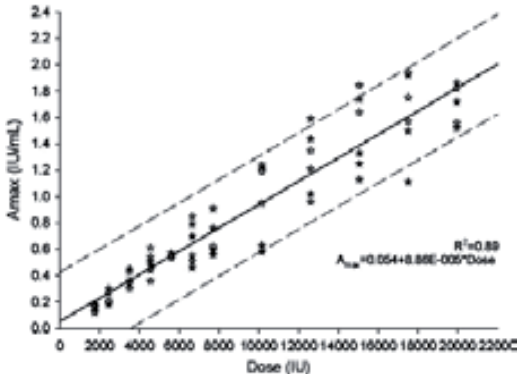

Fig. 5. Graphical representation of the relationship between 12 ascending doses of RO-14 and the A_{max} of the anti-FXa activity. Least means squares linear regression line and 95% prediction intervals are shown as well as the determination coefficient of the relationship and the prediction equation.

Besides RO-14, another ULMWH semuloparin (AVE5026, property of Sanofi-Aventis) is currently in phase III of clinical development [10,13,22]. Semuloparin is a hemisynthetic ULMWH that possesses a high anti-FXa activity (~160 IU/mg) with a residual anti-FIIa activity (~2 IU/mg) and a mean MW of 2400 Da. In the first phase I studies semuloparin showed a linear PK profile and dose proportionality after single and repeat dosing, within a dose range comprising ~800 – 16000 IU of anti-FXa activity. In spite of the great potency and long half-life (11 h), semuloparin also showed dose proportional increases in anti-FIIa activity and a slight increase in accumulation was observed in elderly volunteers. In a phase II clinical trial semuloparin has demonstrated a significant dose-related response in prevention of venous thromboembolism in patients undergoing orthopaedic surgery (TREK study) after being administered once daily doses of 20 or 40 mg for a period of 5 or 10 days. Semuloparin showed a superior efficacy and similar safety profile compared to that of enoxaparin 40 mg [10]. Although, very promising, its long half life might make it harder to handle in clinical situations such as surgery under neuraxial anesthesia.

A few limitations should be mentioned concerning this study. Firstly, this was not a double blind clinical trial. Double blinding, however, is not commonly used in this type of clinical trial (FTIH) and it is not relevant for our study results since main endpoints were objective safety and tolerability and the evaluation of a biochemical marker (anti-FXa activity). The alternating cross-over design could also be challenged. We chose this design because it facilitates volunteer recruitment, favors safety assessment, and diminishes interindividual variability. However, it also prevented us from getting

Table 3
Pharmacological characteristics of different LMWHs.

LMWH	Mean MW (Daltons)	Anti-FXa activity (IU/mg)	Anti-FIIa activity (IU/mg)	t ½ (h)
RO-14[17]	1800-3000	80-140	≤10	3.83–8.24
Semuloparin [22]	2000-3000	160	2	16-20
Bemiparin [24]	3600	80-120	5-20	5.2-5.4
Enoxaparin [25]	4500	105	27	4.0-4.7
Dalteparin [26]	6000	130	58	2.3-2.8
Tinzaparin [27]	65000	83	45	3

a better idea of the potential inter-subject variability of the parameters. Another limitation is the fact that we did not characterize the time-course of other variables such as APTT, TT, or tissue factor pathway inhibitor (TFPI) as has been done with other LMWHs [23]. These variables could be considered in future studies.

Considering the good safety and pharmacodynamic profile and the lack of anti-FIIa activity of RO-14 seen in this study, the possibilities of having an optimized LMWH in the armamentarium against thrombotic disorders are high and encouraging. Needless to say, there is still a long road ahead for this molecule and several studies will be required in order to prove its safety and efficacy as compared to existing drugs and those yet to be marketed.

Conflict of interest

J. Fontcuberta has worked as a consultant for Laboratorios Farmacéuticos Rovi S.A. for lecturing at educational meetings.

S. Rico and J. Fontcuberta have received honoraria from Laboratorios Farmacéuticos Rovi S.A. for lecturing at educational meetings.

M. Monreal and J. Martinez-Gonzalez are employees at Laboratorios Farmacéuticos Rovi S.A.

Role of the funding source

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The study sponsor did not have any involvement in the study design, in the collection, analysis or interpretation of data.

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CHAPTER 6 - PHARMACODYNAMICS ASSESSMENT OF BEMIPARIN AFTER MULTIPLE PROPHYLACTIC AND SINGLE THERAPEUTIC DOSES IN ADULT AND ELDERLY HEALTHY VOLUNTEERS AND IN SUBJECTS WITH VARYING DEGREES OF RENAL IMPAIRMENT

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Regular Article

Pharmacodynamics assessment of Bemiparin after multiple prophylactic and single therapeutic doses in adult and elderly healthy volunteers and in subjects with varying degrees of renal impairment☆

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ABSTRACT

Introduction: Aging and renal impairment may prolong the half-life and lead to accumulation of low molecular weight heparins. Correct dosing is critical to prevent bleeding or thrombosis.
Materials and Methods: Open, parallel study. Healthy adult [n = 13] and elderly (>65 yrs) [n = 12] volunteers; and subjects with mild (Cl_{Cr} ≥ 50 to ≤80 mL/min, n = 8), moderate (Cl_{Cr} ≥ 30 to <50 mL/min, n = 7), and severe (Cl_{Cr} < 30 mL/min, n = 8) renal impairment received four prophylactic doses (3,500 IU/24 h) and a single therapeutic dose (115 IU/kg) of bemiparin with an interim washout period. Anti-FXa activity and the potential need for dose adjustment were evaluated.
Results: There were statistically significant differences in the severe renal impairment group vs. adult volunteers in all anti-FXa related parameters, but no significant differences in any of the anti-FXa related parameters between the adult and the elderly. Anti-FXa simulations after 10 prophylactic doses predicted mean A_{max} = 0.59 IU/mL in subjects with severe renal impairment and 0.33–0.39 IU/mL in the rest. Simulations in the severe renal impairment group with dose adjustment (2,500 IU/24 h) predicted all individual A_{max} < 0.60 IU/mL (mean A_{max} = 0.42 IU/ml). Simulations after 10 therapeutic doses predicted mean A_{max} = 1.22 IU/mL in severe renal impairment group and 0.89–0.98 IU/mL in the rest. Simulations in the severe renal impairment group with 75% dose adjustment predicted individual A_{max} ≤ 1.60 IU/mL (mean A_{max} = 0.91 IU/mL).
Conclusions: No dose adjustments are required in elderly with preserved renal function. A dose adjustment of bemiparin is only advisable in patients with severe renal impairment when using prophylactic or therapeutic doses.

Introduction

Venous thromboembolism (VTE) is the third most common vascular disease after coronary heart disease and stroke, with approximately 300,000 new cases diagnosed annually in the United States [1]. Despite the development of other therapeutic options such as oral direct thrombin, or factor Xa inhibitors, low molecular weight heparins (LMWHs) continue to play a major role in the prevention and treatment of VTE [2–4]. LMWHs have a good safety profile, but unlike unfractioned heparin (UFH), they are predominantly cleared by the kidneys [5,6]. In patients with compromised renal function, such as the elderly or patients with renal impairment (RI), there could be a prolongation of the half-life and accumulation of LMWHs [6–9].

Aging is associated with a number of physiologic and pathophysiologic changes, such as differences in lean body mass and a reduction in

Abbreviations: ACS, Acute coronary syndrome; BMI, body mass index; Cl_{Cr}, creatinine clearance; DVT, deep vein thrombosis; ECG, electrocardiogram; FOBT, fecal occult blood test; GFR, glomerular filtration rate; LMWHs, low molecular weight heparins; OR, odds ratio; PE, pulmonary embolism; RR, relative risk; RI, renal impairment; UFH, unfractioned heparin; VTE, venous thromboembolism.

☆ Partial results of this study were presented at the 22nd Congress of the International Society on Thrombosis and Haemostasis, Boston, USA, 15–16 July, 2009 and in Fontcuberta Boj J. New frontiers with bemiparin: use in special populations. Drugs. 2010 Dec 14;70 Suppl 2:43–7.

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hepatic and renal function. These changes provoke pharmacokinetic alterations that affect drug absorption, volume of distribution, drug metabolism and renal clearance, and they may have an impact upon the safety, efficacy and dosing in elderly patients. Of all the physiologic changes, the most relevant is age-related reduction in renal clearance [9]. In patients with RI, the progressive decline in glomerular filtration and the associated uremic milieu leads to perturbations in hemostasis and thrombosis pathways, leading to the development of both a prothrombotic and pro-hemorrhagic state [10].

Elderly patients and those with RI, have higher risk of both, VTE [1, 11,12] and bleeding [13–15]. When treating these patients, physicians need to consider the patient's renal function, the thrombotic and bleeding risk, and the anticoagulant that is intended to be used [16]. Dosing decisions are of paramount importance, because underdosing of LMWHs in these patients could result in lack of efficacy and an increased risk of VTE and death [17].

Yet, dosing decisions are still made without robust scientific support. Randomized controlled trials evaluating LMWHs have generally excluded elderly patients and those with severe RI [18]. When available, dosing recommendations for the different LMWHs have been established empirically; and target ranges of anti-FXa levels vary according to LMWH and indication. Although pharmacodynamic studies in patients with RI have in general, demonstrated that clearance of the anti-FXa activity is highly correlated with creatinine clearance (Cl_{Cr}), such studies have not all followed the same methodology; therefore, results between the different LMWHs cannot be directly compared and recommendations should not be extrapolated from one LMWH to another [16, 18–23].

The present study assessed the pharmacodynamics of the LMWH, bemiparin, after prophylactic (3,500 IU) and therapeutic (115 IU/Kg) doses in elderly and RI subjects; and evaluated the potential need for dose adjustment in these populations.

Materials and methods

Study design

This multi-center, open-label, 2-period, parallel study was conducted at CIM-Sant Pau (Barcelona, Spain) and at the Clinical Hospital Centre (Zagreb, Croatia). The protocol was approved by the Clinical Research Ethics Committees at both institutions and by the corresponding health authorities. The study was conducted in compliance with the Declaration of Helsinki and Good Clinical Practice guidelines. All subjects gave written informed consent to participate in the study and received stipends for their collaboration. The study is registered at clinicaltrials.gov (ref.# NCT00413088).

Study drug

Bemiparin is a second-generation LMWH with the lowest mean molecular weight (3,600 Da), the longest elimination half-life (5.30 h), and the highest anti-FXa/anti-FIIa ratio (8/1) of activity among the current marketed LMWH [24].

Bemiparin was manufactured by Laboratorios Farmacéuticos Rovi S.A. (Madrid, Spain) and supplied in pre-filled syringes for subcutaneous (SC) injection. The following bemiparin solutions were used: 3,500 IU anti-FXa/0.2 mL (17,500 IU/mL) and 10,000 IU anti-FXa/0.4 mL (25,000 IU/mL).

Doses and dosing regimen

Bemiparin 3,500 IU (prophylactic dose) was administered on 4 consecutive days in period 1. Thereafter, once a minimum washout period of 7 days was completed, a single dose of bemiparin 115 IU anti-FXa/kg (therapeutic dose) was administered in period 2.

Subjects and clinical assessments

Five cohorts of volunteers participated in this trial: adult (18–65 years) and elderly (>65 years) healthy volunteers; and subjects with mild, moderate or severe RI (18–65 years). Subjects were screened for enrollment 4 weeks prior to the beginning of the trial.

Young and elderly volunteers who were screened for the study had a medical history, physical examination, vital signs, electrocardiogram (ECG) and laboratory results with no clinically significant findings. Their body mass index (BMI) had to be within 19–30 kg/m^2 and their glomerular filtration rate (GFR) had to be >80 mL/min/1.73 m^2 as assessed by Cl_{Cr}. Subjects with RI were grouped according to their Cl_{Cr} values: mild (≥50 and ≤80), moderate (≥30 and <50), and severe RI (<30 mL/min/1.73 m^2). Their renal function had to be stable for the last 3 months prior to inclusion in the study and their body weight had to be between 45 and 110 kg.

Healthy subjects were excluded if, had a known history of hypersensitivity to drugs, coagulation disorders, conditions with increased bleeding risk, abnormal coagulation tests, urinalysis positive for hematuria, positive fecal occult blood test (FOBT), positive serology for hepatitis B, C, or HIV virus, trauma or surgery in the 6 months prior to the study, use of any medication 4 weeks prior to the trial, contraindications for LMWHs; or history of drug abuse or chronic disease. Women were either post-menopausal or sterilized, or used a reliable method of contraception. Subjects with RI were excluded if they were on dialysis, had received treatment with ASA or nonsteroidal anti-inflammatory drugs within 10 days prior to their inclusion, had undergone changes in their pharmacological treatment within a month prior to their inclusion, or had a history of cardiovascular disease.

Cl_{Cr} was calculated according to the following formula: $Cl_{Cr} = U_{Cr}$ x 24-hour collected urine volume / P_{Cr} x 24 x 60, where U_{Cr} is urine creatinine concentration and P_{Cr} is the plasma creatinine concentration.

For both periods, participants attended the research site the night before bemiparin administration. Healthy subjects were administered bemiparin early in the morning in fasting state, unless they had a positive urine drug test, a platelet count below 100/mL and/or a serum potassium level >= 5.5 mEq/L (75/mL and/or >6.0 mEq/L, respectively, for subjects with RI).

Sample analysis

Blood samples to assess anti-FXa activity were collected in tubes containing sodium citrate (0.129 M) through an indwelling catheter in the antecubital vein. Blood draws took place before drug administration and at predefined intervals thereafter on days 1 and 4 of period 1 and on day 1 of period 2. Plasma samples were obtained by centrifugation and were frozen and stored at −80 °C until analysis. Anti-FXa activity was measured with a commercial chromogenic assay, Coatest® LMW Heparin (Chromogenix, Instrumentation Laboratory, Milan, Italy). The assay is linear up to 2.0 IU/mL and the detection threshold is 0.1 IU/mL. Intra-run precision coefficient of variation (CV) obtained with plasma supplemented with LMWH (0.88 IU/mL) was 0.9 % and inter-run precision CV was 1.5 %.

Pharmacodynamic analysis

Using Phoenix WinNonlin Professional Edition version 6.2.1 (Pharsight Corp, Cary, NC), determinations of anti-FXa activity in the 24-hour period after drug administrations were analyzed following a non-compartmental and a compartmental approach. With the non-compartmental approach the following parameters were calculated for all volunteers and study periods: maximum anti-FXa activity (A_{max}); time to reach maximum anti-FXa activity (T_{max}); elimination half-life ($t_{½}$); area under the anti-FXa activity-time curve during a 24 h period from drug administration (AUC_{0-24}); area under the anti-FXa-time curve extrapolated to infinity ($AUC_{0-\infty}$); apparent volume of

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distribution after a single administration (Vd/F), and at steady-state (Vd_{ss}/F); and apparent clearance (Cl/F).

A_{max} and T_{max} were determined from the time-activity curves. The apparent terminal elimination rate constant (λ_z) was estimated by semilogarithmic regression of time-activity data. Only data points that described the terminal decline were used in the regression and a minimum of three data points was utilized. $t_{1/2}$ was calculated as $\ln(2)/\lambda_z$. AUC_{0-24} was calculated using the linear trapezoidal rule from the time of drug administration to 24 hours post drug administration. The estimated last determination A_z, was used in extrapolating AUC to infinite time as $AUC_{0-\infty} = AUC_{0-24} + A_z / \lambda_z$. Vd/F and Vd_{ss}/F were calculated as $Vd/F = dose/ (AUC_{0-\infty} * \lambda_z)$ and $Vd_{ss}/F = Dose * AUMC / AUC^2_{0-\infty}$, where AUMC is the area under the first moment of the plasma concentration vs. time curve. Cl/F was calculated as $dose/ AUC_{0-\infty}$.

For the compartmental approach we considered models with one, two, or three disposition compartments and first-order absorption and elimination. Models were parameterized in terms of Vd/F, and intercompartment distribution rate constants (K). The need for a lag time in the absorption process was also tested. Best-fit model selection was based on the visual inspection of the observed vs. predicted values plots, on the Akaike information criterion and on the plausibility and coefficients of variation of the estimates.

To evaluate the need for dose adjustment after multiple therapeutic doses, simulations were performed using the mean parameters derived from the selected compartmental model and were visually compared. The optimal range for A_{max} was defined as between 0.35 and 0.60 IU/mL after prophylactic doses and between 1 and 2 IU/mL after therapeutic doses [25]. We simulated 10 administrations of bemiparin 3,500 IU QD and 115 IU/kg QD. Lower doses were explored based on the results of the initial simulations. Doses were chosen following the current approved doses for prophylaxis and treatment of VTE. Duration of treatment was chosen because 7 to 10 days are recommended for prophylaxis of VTE and 7 ± 2 days for its treatment.

Statistical analysis

Graphs were created using SigmaPlot Version 12.0 (Systat Software, San Jose, CA). Analysis of the parameters of anti-FXa activity was performed in all volunteers who completed all the study procedures for at least one experimental period. Statistical analysis was performed using SPSS for Windows, Rel. 17.0 (SPSS, Inc., Chicago). In all cases, the statistical significance level required was equal or inferior to 5% ($[\alpha] = 0.05$).

Descriptive statistics were calculated for A_{max}, $t_{½}$, AUC_{0-24}, $AUC_{0-\infty}$, Vd/F, Vd_{ss}/F (only Day 4), and Cl/F as well as for the parameters derived from the compartmental analysis (Vd/F and K's) of anti-FXa activity. For T_{max} values we calculated median and ranges.

To determine whether the treatment group had any effect on the parameters of pharmacodynamic activity in the three treatment days, log transformed and dose normalized (when required) parameters were analyzed via a two-way ANOVA model, with day (Day $1_{Period\ 1}$, Day $4_{Period\ 1}$, and Day $1_{Period\ 2}$) as within subjects factor and treatment group (adult, elderly, and subjects with mild, moderate and severe RI) as a between subjects factor. Pairwise comparisons of the parameters were performed between treatment days and post hoc Tukey tests were carried out between treatment groups. The non-parametric Kruskal-Wallis test was used to compare the anti-FXa T_{max} activity values between treatment groups in the 3 treatment days.

To study the potential association between renal function and anti-FXa activity after the administration of bemiparin, it was performed a linear regression analysis between Cl_{Cr} and A_{max}, Cl/F, AUC_{0-24} and $t_{1/2}$ of anti-FXa activity, using pooled data from all groups. Pearson's correlation test was first performed, and the correlation matrix was evaluated for direction, magnitude, and significance. If the correlation was statistically significant, a linear regression analysis was performed following a least squares means approach. The coefficient of determination (R^2) was evaluated to determine the percentage of the dependent variable variance that could be explained by the independent variable (i.e., Cl_{Cr}). The significance of the differences between the sum-of-squares values of the regression model and of the null hypothesis model was evaluated through the ANOVA method. The resulting coefficients were used for the construction of the linear regression equation according to the following expression: $Y = \beta_0 + \beta_1 X_1$ where β_0 is the intersect or constant, β_1 are the respective parameters of each independent variable. A multiple regression analysis incorporating age, weight, and gender as independent variables and A_{max} of anti-FXa activity after therapeutic doses (Day $1_{Period\ 2}$) as a dependent variable, was also carried out.

Results

Subject disposition and demographics

A total of 49 volunteers (14 adult, 12 elderly, and 8, 7 and 8 with mild, moderate and severe RI, respectively) were enrolled in the study. Of these, 48 received at least one bemiparin administration (one adult volunteer abandoned the study prior to bemiparin administration); a total of 46 completed the 2 periods (one adult volunteer and one subject with moderate RI suffered AE and did not complete the 2 periods). Baseline characteristics are shown in Table 1.

Pharmacodynamic results

Non-compartmental analysis

The mean time course of anti-FXa activity of bemiparin after the administration of prophylactic and therapeutic doses to all the treatment groups is represented in Fig. 1. The descriptive statistics of the non-compartmental analysis-derived parameters of anti-FXa activity are summarized in Tables 2 and 3.

The absorption rate of bemiparin was similar across the five treatment groups. T_{max} occurred around 3–4 h after the first prophylactic dose and around 2–4 h after the 4th prophylactic dose, while it took place between 4–5 h after the administration of the single therapeutic dose. There were no statistically significant differences in the median T_{max} values in any of the treatment groups at any of the experimental days.

Mean A_{max} values after four prophylactic doses were 25-57% lower in the two groups of healthy volunteers and in the subjects with mild and moderate RI, than in the subjects with severe RI. After the single therapeutic dose, A_{max} values were approximately 30% higher in the group of subjects with severe RI than in the rest of the treatment groups. A greater degree of variability in the subjects with RI was evidenced. There were statistically significant differences in the log-transformed and dose-normalized A_{max} values between the 3 treatment days ($P < 0.001$) and between the 5 treatment groups ($P = 0.002$). Pairwise comparisons showed that there were differences between Day $4_{Period\ 1}$ and the rest of the days ($P < 0.001$) and between the group of subjects with severe RI and the group of healthy volunteers ($P < 0.001$). There was also a significant day by group interaction ($P = 0.001$), largely due to the A_{max} observed at Day $4_{Period\ 1}$ in the group of subjects with severe RI.

Mean $t_{½}$ values after prophylactic doses were 2–4 h longer in the groups of subjects with RI than in the healthy volunteers groups. After the administration of the single therapeutic dose, t½ was between 4.08 h (in group of healthy volunteers) and 9.12 h (in the group of severe RI). There were no statistically significant differences in the $t_{½}$ values between the 3 treatment days ($P = 0.292$), but there were statistically significant differences between the 5 treatment groups ($P < 0.001$). Pairwise comparisons showed that there were differences between the groups with RI and the group of healthy volunteers ($P < 0.001$). There was not a significant day by group interaction ($P = 0.902$).

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Table 1
Baseline characteristics of the subjects participating in the study.

Parameter		Adult (n = 13)	Elderly (n = 12)	Mild RI (n = 8)	Moderate RI (n = 7)	Severe RI (n = 8)
Gender [n]	*Males*	8	8	5	4	2
	Females	5	4	3	3	6
Age [years]		33.54 (6.54)	67.17 (2.82)	53.38 (6.07)	57.29 (7.34)	53.13 (9.33)
Weight [kg]		69.15 (8.98)	72.38 (7.33)	79.24 (13.33)	77.11 (14.71)	76.25 (17.62)
Height [cm]		169.38 (7.89)	169.25 (7.31)	168.38 (9.49)	165.43 (5.62)	161.88 (6.64)
BMI [kg/m²]		24.05 (1.58)	25.14 (0.81)	27.85 (3.04)	27.94 (4.46)	28.96 (5.62)
Cl_{Cr} *[ml/min/1.73 m²]*		119.77 (27.05)	113.10 (14.81)	75.43 (13.14)	41.38 (8.59)	22.82 (4.46)
APTT [Ratio]		1.07 (0.13)	1.05 (0.10)	1.01 (0.05)	1.04 (0.09)	1.03 (0.11)
Prothrombin time [Ratio]		1.02 (0.05)	0.97 (0.04)	1.01 (0.08)	1.09 (0.18)	1.18 (0.12)
Thrombin time [s]		21.97 (1.41)	21.88 (1.94)	20.93 (2.58)	19.87 (2.56)	18.53 (2.64)
Fibrinogen [g/L]		2.92 (0.30)	3.41 (0.63)	3.57 (0.62)	5.11 (1.02)	4.93 (1.14)
Antithrombin [%]		108.62 (10.11)	108.58 (11.69)	102.59 (9.96)	106.06 (17.40)	106.98 (18.58)

All values (except for *Gender*) expressed as 'Mean (standard deviation)'. RI: renal impairment. BMI: body mass index. Cl_{Cr}: creatinine clearance. APTT: activated partial thromboplastin time.

Mean values of AUC_{0-24} and $AUC_{0-\infty}$ on the 3 treatment days were higher in the groups of RI patients and increased with the degree of RI, with the exception of Day $1_{Period\ 1}$, when values between the mild and moderate RI groups were very similar. There were statistically significant differences in the log-transformed and dose-normalized AUC_{0-24} and $AUC_{0-\infty}$ values between the 3 treatment days (AUC_{0-24}: $P < 0.001$ and $AUC_{0-\infty}$: $P < 0.001$) and the 5 treatment groups (AUC_{0-24}: $P < 0.001$ and $AUC_{0-\infty}$: $P < 0.001$). Pairwise comparisons showed that there were differences between Day $4_{Period\ 1}$ and the rest of the days ($P < 0.001$) and between the group with severe RI and the rest of the groups ($P < 0.001$). There was also a significant day by group interaction (AUC_{0-24}:$P = 0.001$ and $AUC_{0-\infty}$: $P < 0.001$), largely due to the values observed at Day $4_{Period\ 1}$ in the group with severe RI.

There were statistically significant differences in the Vd/F values between the 3 treatment days ($P = 0.002$). Pairwise comparisons showed that there were differences between Day $4_{Period\ 1}$ and the rest of the days ($P < 0.001$). There was also a significant day by group interaction ($P = 0.007$) that was driven by the fact that the groups of patients with RI showed a decreased Vd/F after the administration of prophylactic doses and a higher Vd/F after the administration of single therapeutic doses.

Cl/F mean values after the administration of the first and fourth prophylactic doses and after the single therapeutic dose were lower in the group with severe RI as compared to the rest of the groups. There were statistically significant differences in the Cl/F values between the 3 treatment days ($P < 0.001$) and between the 5 treatment groups ($P < 0.001$). Pairwise comparisons showed that Cl/F values were higher on Day $1_{Period\ 1}$ than in the rest of the days ($P < 0.001$) and that there were differences between the groups with mild and severe RI and the group of healthy volunteers ($P < 0.001$). There was also a significant day by group interaction ($P = 0.037$), largely due to the Cl/F values decreasing at Day $4_{Period\ 1}$ in the groups with mild and severe RI.

Anti-FXa activity - assessment of the effect of renal function

There was an inverse relationship between Cl_{Cr} and A_{max}, AUC_{0-24} and $t_{½}$ after the administration of multiple prophylactic doses (Fig. 2). Cl_{Cr} accounted for 36.2% (Day $1_{Period\ 1}$) and 36.8% (Day $4_{Period\ 1}$) of the variation in A_{max}, and for 49% (Day $1_{Period\ 1}$) and 51% (Day $4_{Period\ 1}$) of the variation in AUC_{0-24}. After the administration of single therapeutic doses, this relationship was practically null for A_{max} (3%) and weaker for AUC_{0-24} (18%), but it remained important for the $t_{½}$ (60%). The gradient and the pattern of the data showed a strong positive relationship between Cl_{Cr} and Cl/F after the administration of prophylactic doses. Cl_{Cr} accounted for 60% and 51% of the variation in Cl/F. After therapeutic doses, it only accounted for 19% of the variation in Cl/F. A multiple linear regression analysis model incorporating Cl_{Cr}, age, weight and gender was not successful in predicting A_{max} ($R^2 = 0.210$, $P = 0.076$).

Compartmental analysis and simulations

A one-compartment model with first-order absorption and elimination from the central compartment and no lag time was selected, as this model was best describing plasma sample measurement results (Table 4).

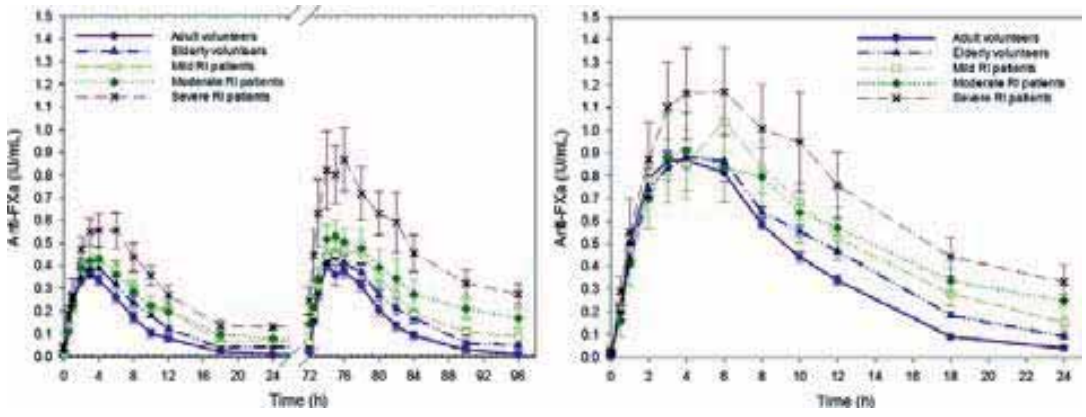

Fig. 1. Mean (±SE) time course of anti-FXa activity on Days 1 (1–24 h) and 4 (72–96 h) after daily subcutaneous administration of prophylactic doses of bemiparin (3,500 IU) and on Day 1 (1–24 h) in healthy adult and elderly volunteers and in subjects with mild, moderate and severe renal impairment.

Table 2
Descriptive statistics of the non-compartmental analysis–derived parameters of anti-FXa activity elicited by bemiparin after a single (Period 1-Day 1) and four once daily (Period 1- Day 4) prophylactic doses (3,500 IU) in healthy adult and elderly volunteers and in subjects with renal impairment.

Parameter	Adult (n = 13)	Elderly (n = 12)	Mild RI (n = 8)	Moderate RI (n = 7)	Severe RI (n = 8)
Period 1 - Day 1					
T_{max} (h)	3.00 (2.00-4.00)	4.00 (2.00-4.00)	3.00 (2.00-4.00)	4.00 (2.00-4.00)	3.00 (3.00-6.00)
A_{max} (IU/mL)	0.35 (0.06)	0.40 (0.10)	0.45 (0.11)	0.45 (0.13)	0.60 (0.16)
$t_{½}$ (h)	4.20 (1.48)	4.60 (0.63)	6.44 (1.91)	8.02 (4.74)	8.23 (0.74)
AUC_{0-24} (IU/mL·h)	2.42 (0.76)	3.12 (0.85)	4.10 (1.19)	3.96 (2.60)	7.26 (2.32)
$AUC_{0-\infty}$ (IU/mL·h)	3.17 (0.87)	4.04 (0.89)	5.70 (1.25)	5.60 (2.68)	8.87 (2.62)
Vd/F (mL)	6928.0 (2392.5)	6028.3 (1579.9)	5775.7 (1349.8)	7841.1 (5137.0)	5042.4 (1618.2)
Cl/F (mL/h)	1190.8 (381.4)	900.6 (176.9)	646.6 (182.4)	716.8 (264.3)	429.1 (150.6)
Period 1 - Day 4					
T_{max} (h)	2.00 (2.00-4.00)	3.00 (2.00-4.00)	3.00 (2.00-6.00)	3.00 (2.00-4.00)	4.00 (2.00-4.00)
A_{max} (IU/mL)	0.41 (0.08)	0.44 (0.11)	0.49 (0.13)	0.53 (0.18)	0.94 (0.48)
$t_{½}$ (h)	4.51 (1.38)	5.59 (1.84)	5.80 (1.97)	8.94 (4.28)	9.71 (3.69)
AUC_{0-24} (IU/mL·h)	2.97 (1.08)	3.83 (1.73)	5.37 (1.91)	6.55 (4.12)	12.10 (5.84)
$AUC_{0-\infty}$ (IU/mL·h)	3.71 (1.23)	5.03 (1.68)	6.54 (2.41)	9.13 (6.33)	16.29 (8.75)
Vd_{ss}/F (mL)	6581.0 (2925.7)	5883.8 (2018.8)	4642.2 (1302.6)	5851.9 (1916.7)	3425.4 (1533.4)
Cl/F (mL/h)	1025.2 (282.7)	757.3 (215.3)	613.3 (262.4)	624.4 (509.9)	282.2 (156.2)

All values are expressed as 'Mean (standard deviation)', except values for T_{max} which are shown as 'Median (minimum, maximum)'. RI: renal impairment. Tmax: time to reach the maximum anti-FXa activity. Amax: the maximum anti-FXa activity. t ½: elimination half-life. AUC_{0-24}: area under the anti-FXa activity-time curve during a 24-hours period. $AUC_{0-\infty}$: area under the anti-FXa activity-time curve during extrapolated to infinity. Vd/F: apparent volume of distribution after a single administration. Cl/F: apparent clearance. Vd_{ss}/F: apparent volume of distribution at steady state.

On Day $1_{Period\ 1}$ there were no statistically significant differences between the treatment groups in the absorption rate constant, K_{01} ($P = 0.590$), but there were statistically significant differences in the elimination rate constant, K_{10} ($P = 0.004$). The group with severe RI showed the highest values, followed by the groups of moderate and mild RI, elderly and healthy volunteers. Results on Day $1_{Period\ 2}$ were very similar. There were no statistically significant differences between the treatment groups in the K_{01} ($P = 0.801$), but there were statistically significant differences in the K_{10} ($P < 0.001$). On both cases, pairwise comparisons revealed that the significance was driven by differences between the subjects with severe RI and the adult healthy volunteers. There were no statistically significant differences between the groups with RI.

Using the mean estimates of the compartmental analysis by treatment group, we performed pharmacodynamic simulations with 10 hypothetical administrations of bemiparin at prophylactic (3,500 IU QD) and therapeutic doses (115 IU/kg QD). Simulation time was 240 h (Fig. 3). In all simulations it was observed that steady state is reached after the first 2 administrations of bemiparin. In the simulations with a 3,500 IU QD dose, the predicted A_{max} at steady state was between 0.33 and 0.39 IU/mL in the groups of healthy volunteers and subjects with mild and moderate RI, whereas it was close to 0.60 IU/mL in the group with severe RI. In the simulations with a 115 IU/kg QD dose (for an average person or 70 kg = 8,050 IU QD) the predicted A_{max} at steady state was between 0.80 and 0.93 IU/mL in the groups of adult and elderly healthy volunteers and subjects with mild and moderate RI, whereas it was 1.11 IU/mL in the group with severe RI. Although the simulations results in subjects with severe RI fulfilled the optimal range for A_{max} proposed by Kearon et al. [25] two additional simulations were carried out with a hypothetical prophylactic dose of 2,500 IU QD and a therapeutic dose of 86.25 IU/kg (for an average person or 70 kg = 6,037.5 IU QD; 25% lower than the regular therapeutic dose). This simulation showed that the A_{max} at steady state at the lower prophylactic dose would be between ~0.42 IU/mL and ~0.84 IU/mL at the lower proposed therapeutic dose.

Discussion and conclusions

This study evaluated the pharmacodynamics of bemiparin after multiple prophylactic doses and a single therapeutic dose in adult and elderly volunteers and in subjects with different degrees of RI. Previous pharmacodynamic studies with other LMWHs have either assessed prophylactic or therapeutic doses, have not included an in-study control group or an elderly volunteers group; and/or have been observational studies with less rigorous control [16].

Results in the group of healthy volunteers were in agreement with the pharmacodynamic profile described for bemiparin [26,27]. There were no significant differences in any of the analyses comparing the adult and elderly volunteer groups, indicating no need for dose adjustment in elderly patients with preserved renal function. After the administration of multiple prophylactic doses and a single therapeutic dose of bemiparin, the mean absorption rate and exposure to anti-FXa activity was significantly higher in patients with severe RI as compared to adult healthy volunteers. In spite of these differences, observed A_{max} values in subjects with severe RI were close to the optimal range

Table 3
Descriptive statistics of the non-compartmental analysis–derived parameters of anti-FXa activity elicited by bemiparin a single therapeutic dose (115 IU/kg) in healthy adult and elderly volunteers and in subjects with renal impairment.

Parameter	Adult (n = 13)	Elderly (n = 12)	Mild RI (n = 8)	Moderate RI (n = 7)	Severe RI (n = 8)
T_{max} (h)	4.00 (2.00-6.00)	4.00 (3.00-6.00)	5.00 (2.00-6.00)	4.00 (3.00-6.00)	5.00 (3.00-10.00)
A_{max} (IU/mL)	0.90 (0.20)	0.90 (0.10)	1.00 (0.30)	0.90 (0.30)	1.30 (0.50)
$t_{½}$ (h)	4.08 (0.54)	5.42 (0.69)	6.18 (1.34)	8.76 (0.67)	9.12 (1.17)
AUC_{0-24} (IU/mL·h)	8.24 (1.37)	10.45 (1.34)	12.48 (5.00)	12.58 (4.88)	17.17 (7.69)
$AUC_{0-\infty}$ (IU/mL·h)	9.48 (0.92)	11.47 (1.24)	14.27 (5.71)	15.54 (5.25)	21.76 (10.72)
Vd/F (mL)	4910.8 (725.8)	5598.1 (1214.1)	6445.2 (3543.1)	8339.6 (3976.8)	6355.8 (2677.7)
Cl/F (mL/h)	838.4 (96.9)	718.0 (139.7)	797.6 (602.7)	651.1 (283.8)	507.5 (266.4)

All values are expressed as Mean (SD), except values for T_{max} which are shown as median (minimum, maximum). RI: renal impairment. Tmax: time to reach the maximum anti-FXa activity. Amax: the maximum anti-FXa activity. t ½: elimination half-life. AUC_{0-24}: area under the anti-FXa activity-time curve during a 24-hours period. $AUC_{0-\infty}$: area under the anti-FXa activity-time curve during extrapolated to infinity. Vd/F: apparent volume of distribution after a single administration. Cl/F: apparent clearance.

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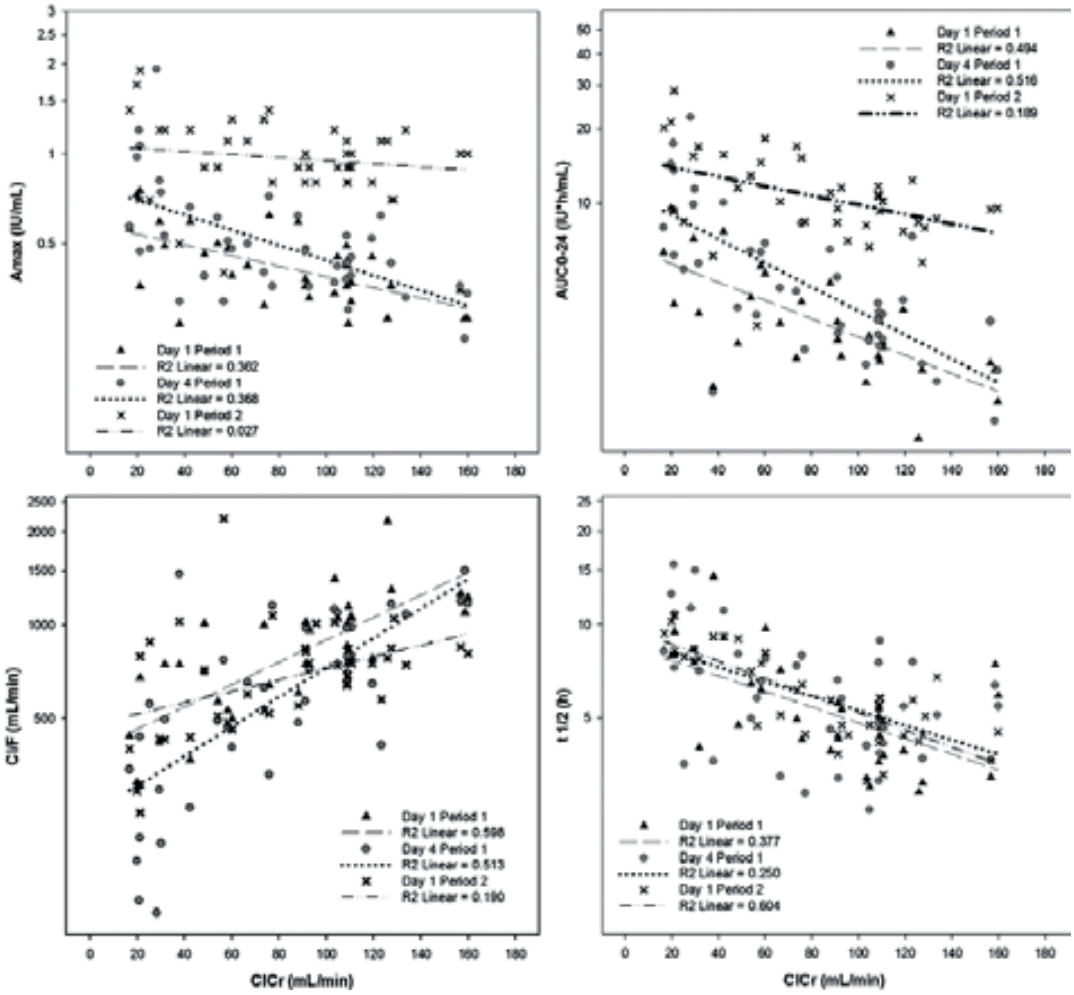

Fig. 2. Scatterplots with regression lines of the relationship between Cl_{Cr} and A_{max}, AUC_{0-24}, $t_{½}$ and Cl/F of anti-FXa activity on Days 1-$4_{Period\ 1}$ and Day $1_{Period\ 2}$.

Table 4
Descriptive statistics of the parameters derived from compartmental analysis of anti-FXa activity elicited by bemiparin after a single prophylactic (Period 1-Day 1) and a single therapeutic dose (Period 2-Day 1).

Period, Day & Dose	Parameter	Adult		Elderly		Mild RI		Moderate RI		Severe RI	
		Mean	CV (%)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)
Period 1 - Day 1	*N*	11		9		7		4		5	
(3500 IU)	*Vd/F (mL)*	4832.51	33.85	5443.15	34.79	4762.74	25.58	6026.74	50.88	4311.11	38.47
	K_{01} (h^{-1})	0.43	30.68	0.50	29.79	0.43	35.76	0.53	31.00	0.53	41.64
	K_{10} (h^{-1}) $^{\Delta}$	0.28	33.56	0.20	35.69	0.18	44.31	0.15	48.14	0.11	19.24
Period 2 - Day 1	*N*	11		11		8		4		6	
(115 IU/kg)	*Vd/F (mL)*	4158.23	25.38	4631.13	15.00	4932.50	29.39	7722.64	51.43	4840.85	47.71
	K_{01} (h^{-1})	0.38	50.35	0.34	32.48	0.32	29.25	0.42	13.62	0.39	23.70
	K_{10} (h^{-1}) $^{\Delta}$	0.21	23.93	0.16	19.60	0.16	26.17	0.09	9.81	0.11	54.92

RI: renal impairment. Vd/F: apparent volume of distribution after a single administration. K_{01} = Absorption rate constant; K_{10} = Elimination rate constants. $^{\Delta}$Statistically significant differences between treatment groups. Pairwise comparisons revealed that the significance was due to differences between healthy volunteers and subjects with severe RI.

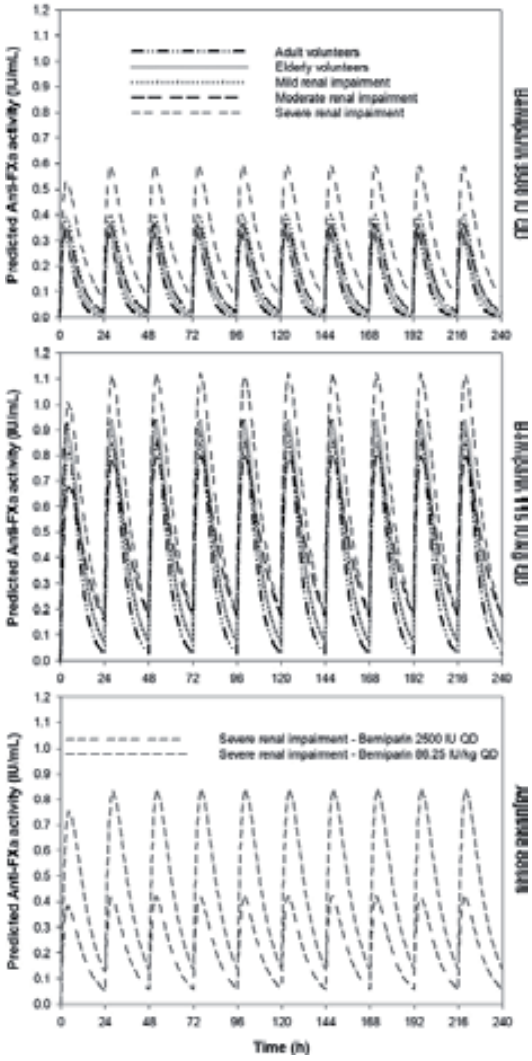

Fig. 3. Simulations of anti-FXa activity after 10 hypothetical administrations of bemiparin 3,500 IU QD (prophylactic dose), 115 IU/kg QD (therapeutic dose) using mean parameters derived from compartmental analysis in healthy adult and elderly volunteers and in subjects with mild, moderate and severe renal impairment. The bottom plot represents a simulation with adjusted doses of 2,500 IU QD (prophylactic dose) and 86.25 IU/kg QD (therapeutic dose) in the group of subjects with severe renal impairment.

proposed by Kearon et al. [25]. Clearance was decreased in all the groups of patients with RI and there were significant differences between subjects with severe RI and healthy volunteers.

The simulation we carried out using the parameters derived from the group of patients with severe RI resulted in a predicted A_{max} at steady state between 0.33 and 0.60 IU/mL with the 3,500 IU dose, and between 0.80 and 0.93 IU/mL with the dose of 115/IU/kg. Lowering the prophylactic dose to 2,500 IU yielded a mean predicted A_{max} of 0.42 IU/mL, a value within the same range observed without dose adjustment. However, lowering the therapeutic dose by 25% resulted in a noticeable lower predicted A_{max} of 0.84 IU/mL, a value within the range observed in the other groups. Based on these results, a dose adjustment of prophylactic and therapeutic doses in patients with severe RI would result in anti-FXa activity profiles similar to the ones observed in other patient populations.

By and large, there was a greater degree of interindividual variability in the time course of anti-FXa activity in volunteers with RI. This phenomenon has already been reported by other authors, [16,28,29] and might not entirely be explained by the differences in renal function, but by differences in the type of renal injury or other underlying clinical conditions [30].

There is limited published information available concerning comparative PK/PD analysis in healthy adult and elderly subjects with other LMWHs. To date, only enoxaparin has a recommended dose reduction for patients over 75 years being treated for acute ST-elevated myocardial infarction (0.75 mg/kg SC BID) [31].

It is generally accepted that there is potential for lower clearance and accumulation of anti-FXa activity in patients with severe RI with most LMWHs after multiple therapeutic doses. Accumulation after multiple prophylactic doses has been studied less but it is still observed [18]. Our results are in line with those reported by other groups who assessed enoxaparin in patients with RI [29,32–35]. Cadroy et al. [32] reported that clearance of a single therapeutic dose of enoxaparin (0.5 mg/kg SC) was 2 times lower and elimination half-life was 1.7 times longer in patients with severe RI. Sanderink et al. [33] performed a clinical trial with a similar design to ours, but only tested prophylactic doses of enoxaparin (40 mg SC QD for 4 days) and did not include a group of elderly volunteers. They found that the elimination half-life increased with the degree of RI and anti-FXa exposure was significantly different between healthy volunteers and patients with severe RI. In addition, median A_{max} values in healthy volunteers, and in mild, moderate and severe RI patients were very similar to the results of our simulations.

There is less information on the rest of the LMWHs. Prophylactic doses of dalteparin show decreased anti-FXa activity clearance and prolonged half-life in patients with RI as compared with healthy volunteers [36]. No clinically relevant bioaccumulation has been reported when dalteparin is used for prophylaxis of VTE, but significant bioaccumulation in the group of patients with severe RI when used at therapeutic doses [37,38].

Studies with tinzaparin have shown no reduction in clearance or accumulation of anti-FXa activity [29,39,40]. The apparent difference in tinzaparin clearance in patients with severe RI may reflect metabolism by hepatic mechanisms, possibly due to the higher molecular weight of tinzaparin compared with other LMWHs [18].

Other simulation approaches have been used in the past to explore dose adjustments with LMWHs. Green et al. [41] developed a population pharmacokinetic model using data of 38 patients (age range 44–87) with acute coronary syndrome (ACS) and a mean GFR of 32 (range 16–117) mL/min; receiving enoxaparin (0.5 and 1.0 mg/kg SC BID). Based on stochastic simulations they recommended that patients with Cl_{Cr} between 30 to 39 mL/min should receive 0.5 mg/kg BID, whereas patients with a Cl_{Cr} of 10 to 19 mL/min should receive 0.3 mg/kg BID. A model developed by Hulot et al. [42] based on data of 532 patients with ACS and varying degrees of renal function receiving full dose enoxaparin, concluded that enoxaparin clearance was decreased by 31% in patients with Cl_{Cr} between 30 to 49 mL/min and by 44% in patients with $Cl_{Cr} \leq 30$ to 49 mL/min. Simulations suggested that a loading dose of 1 mg/kg followed by a regimen of 0.8 mg/kg BID in patients with moderate RI and 0.66 mg/kg BID in patients with severe RI would result in therapeutic anti-FXa levels. A similar approach but with lower doses (0.75 mg/kg BID and 0.5 mg/kg in patients with moderate and severe RI, respectively) was suggested by Kruse and Lee [34].

Our results are also in agreement with those that have been obtained in therapeutic clinical trials. In a subanalysis of a trial that compared bemiparin 115 IU/kg with UFH for the treatment of deep vein thrombosis (DVT). bemiparin was not associated with an increased incidence of

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major bleeding in elderly patients or those with RI [43]. On the other hand, in the ANCIANOS study, very elderly subjects showed a trend towards a lower incidence of total bleeding than younger patients and a similar incidence of major bleeding when they received prophylactic doses of bemiparin [44].

Accurate dosing is of paramount importance, because the elderly and patients with RI are at an increased risk of bleeding, [45–49] but their risk for VTE is also known to be higher than younger patients and those with preserved renal function [12,17,50–53]. The risk of major bleeding and anti-FXa levels in patients receiving LMWH who had severe RI ($Cl_{Cr} \leq 30$ mL/min), was compared with those in patients with a $Cl_{Cr} > 30$ mL/min in a meta-analysis [54]. In 12 studies involving 4,971 patients given LMWH, the odds ratio (OR) for major bleeding was 2.25 (95% CI, 1.19-4.27; P = 0.013) in patients with severe RI compared with that of those with a $Cl_{Cr} > 30$ mL/min. Therapeutic doses of enoxaparin were associated with a further increase in major bleeding in patients with severe RI (8.3% vs 2.4%; OR, 3.88; 95% CI, 1.78-8.45), but this was not observed when enoxaparin was empirically dose reduced (0.9% vs 1.9%; OR, 0.58; 95% CI, 0.09-3.78).

A higher bleeding risk is only part of the equation. Recently, different studies have stressed that advanced aged and RI are not only independent risk factors for bleeding, but also for VTE. The results of the Longitudinal Investigation of Thromboembolism Etiology (LITE) study indicated that after adjusting for other variables of cardiovascular risk, patients with grade 3/4 RI (eGFR = 15-59 ml/min per 1.73 m^2) have a relative risk (RR) for VTE of 1.71 (95% CI = 1.18 - 2.49) as compared with subjects with preserved renal function [55]. The higher VTE risk might be more life-threatening than the risk of bleeding. Findings from the "*Registro Informatizado de la Enfermedad Tromboembolica*" (RIETE) registry suggest that the risk of fatal pulmonary embolism (PE, 3.7% incidence) is of greater concern than the risk of fatal bleeding (0.8% incidence) in patients over 80 years, [56] and that the risk of death for PE and DVT clearly exceeds the risk of fatal bleedings in patients with RI [17]. All these data support the use of full-dose anticoagulant therapy, even in patients with severe RI [12].

Furthermore, recent RIETE data indicate there is an increased rate of 15-day mortality fatal PE for UFH compared with LMWH, while showing a similar rate of fatal bleeding [57]. This is in line with the CERTIFY study in which certoparin (3000 IU QD) was as efficacious as UFH (5000 IU TID) in patients with severe RI but had a reduced risk of bleeding [51]. The IRIS study, comparing UFH treatment to tinzaparin, yielded different results. It was stopped prematurely due to a difference in mortality favoring the UFH group (11.5 vs. 6.3%; P = 0.035). Interestingly, rates of clinically relevant bleedings were similar in the tinzaparin (11.9%) and UFH (11.9%) groups, as were rates of confirmed recurrent VTE (2.6 vs. 1.1%; P = 0.34) [58].

Some authors and current guidelines in the prophylaxis and treatment of VTE still recommend using other anticoagulants, monitoring anti-FXa activity or adjusting the dose of LMWH's [18,59–63]. However none of these options seems particularly convenient and the supporting evidence for these recommendations is of low quality (Grade 2C, weak recommendation) [4]. Arguably, there is more robust evidence supporting the use of full doses in patients with RI.

Using other anticoagulants could prevent patients from receiving a convenient cost-effective drug and the use of UFH has not been associated with better outcomes in recent studies [57,58]. Monitoring anti-FXa activity could be challenging for practical reasons and may provide flawed information for patient management due to the inherent characteristics of the test that measures neither the essential molecule nor the effect [64,65]. The net anticoagulant effect is not only the result of the anti-FXa activity. It is co-determined by plasma characteristics such as the concentration of antithrombin, the level of heparin-binding proteins and the thrombin-forming power of the hemostatic system [66]. Although anti-FXa activity is the closest we have gotten to a practical risk predictor of bleeding when using LMHW therapy, there is a rather poor correlation between the anti-FXa activity and safety, [67] but Montalescot et al. found a correlation with efficacy [68]. Although some studies reported that high anti-FXa levels were associated with an increased bleeding risk, several other studies failed to show a relationship between anti-FXa levels and bleeding [18]. Furthermore, prophylactic and therapeutic anti-FXa target ranges vary from one LMWH to another and have not been validated [18]. If monitoring is considered in patients receiving therapeutic dose LMWHs, appropriate target ranges for peak anti-FXa levels should be used and so far, no anti-FXa based guidelines have been issued [67].

Gouin-Thibault et al. [67] published a review highlighting the caveats behind dose-adjustment in the elderly and in patients with RI. They noted that the efficacy and safety of LMWHs used at reduced initial dosages have not been evaluated in the treatment of VTE. In the widely known MEDENOX study, [69] that assessed the safety and efficacy of enoxaparin for the prevention of VTE in acutely ill medical patients, it was shown that a full 40 mg QD dose of enoxaparin was particularly effective in the group of patients over 80 years of age (87% risk reduction vs. 63% in the general study population). Given that the lower dose tested in this study (20 mg QD) did not differ from placebo, any recommendation for dose reduction would presumably have a negative impact on efficacy; as was the case when a dose-adjustment proposal of enoxaparin was evaluated in the treatment of ACS and found that too low doses of enoxaparin could be as ineffective as placebo [70].

LMWH dosing in patients with severe RI is in fact a complicated issue. Clinicians have to be careful with empirical dose adjustments as underdosing might result in thrombosis and an overall increase in mortality. The best course is to balance the risk of VTE carefully against the risk of bleeding for each patient.

One potential limitation of our study is the relatively small sample size enrolled in the trial. Nevertheless, this sample size is in accordance with recommendations issued by the European Medicines Agency and the U.S. Food and Drug Administration for the conduct of trials assessing the pharmacokinetics of drugs in patients with impaired renal function [71,72]. Our sample size was also greater than the ones included in most trials with other LMWHs [16].

Another limitation that may be mentioned is the lack of multiple dose administration at therapeutic doses. However, the well-established linearity and dose proportionality of anti-FXa activity with bemiparin in the range of prophylactic and therapeutic doses, [27,73–75] allows for reliable results when performing simulations using the right model.

Finally, the mean age of 67 years in the elderly group is also a limitation. In the interest of recruiting elderly volunteers who were free of comorbidity and concomitant medications, we may have recruited a younger patient population than one that is currently representative of this age group.

Conclusions

Age does not constitute a factor that affects the pharmacodynamic profile of bemiparin at prophylactic or therapeutic doses and no dose adjustment is required in elderly subjects with preserved renal function. The administration of multiple prophylactic and therapeutic doses of bemiparin in patients with mild and moderate RI does not result in a clinical significant accumulation of anti-FXa activity and hence dose adjustment is not required in that case either. In patients with severe RI, there is a potential risk of bioaccumulation of anti-FXa activity, however no-bleeding relationship has been thoroughly described and no anti-FXa bleeding thresholds have been set. Nonetheless, as a precautionary measure a dose adjustment is recommended for both, prophylaxis (2,500 IU) and treatment doses (86.25 IU/kg) for VTE.

Conflict of interest statement

J. Fontcuberta has worked as a consultant for Laboratorios Farmacéuticos Rovi S.A. for lecturing at educational meetings.

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S. Rico and J. Fontcuberta have received honoraria from Laboratorios Farmacéuticos Rovi S.A. for lecturing at educational meetings.

J. Martinez-Gonzalez, I. Ayani and I. Gutierro are employees at Laboratorios Farmacéuticos Rovi S.A.

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Chapter 7

Discussion and conclusions

CHAPTER 7 - DISCUSSION AND CONCLUSIONS

Venous thromboembolism, which includes DVT and PE, is the third most common cardiovascular disorder having an estimated annual incidence of 0.1% and affecting 2% to 5% of the population during their lifetimes. Approximately 20% of patients with PE will die before diagnosis or on the first day. For those surviving more than 1 day, up to 11% may die in the first 3 months even with adequate therapy, although many of these patients succumb to comorbidities associated with VTE (e.g. cancer) rather than from PE itself (Wells and Anderson, 2013).

The mainstay of VTE treatment is anticoagulation. Multiple therapeutic modes and options exist for VTE treatment with small but nonetheless important differential effects to consider. Anticoagulants will probably always increase bleeding risk, necessitating tailored treatment strategies that must incorporate etiology, risk, benefit, cost, and patient preference. Although great progress has been made, further study to understand individual patient risks is needed to make ideal treatment decisions (Wells et al., 2014). Prior to the development of anticoagulant therapy, untreated VTE was often fatal (30% of cases), but anticoagulant therapy effectively treats symptoms and decreases recurrent VTE and death; however, its use increases the risk of major hemorrhage, which may be fatal in up to 25% of cases (Nieto et al., 2010, Wells et al., 2014).

VTE treatment can be divided into 3 phases, acute (first 5-10 days), long-term (first 3 months), and extended (beyond 3 months). The objectives of the acute treatment are prevention of DVT extension or prevention of PE occurrence or recurrence and relief of acute symptoms while averting hemodynamic collapse or death. When diagnostic testing for VTE is delayed, empirical administration of therapeutic dosages of LMWH is indicated (Blondon et al., 2012, Wells et al., 2014).

Initial VTE treatment requires therapeutic dosages of UFH (IV or SC), LMWH, rivaroxaban, dabigatran, apixaban, or fondaparinux. Although any of these drugs can be used, a recent meta-analysis of 23 studies (n = 9587), comparing fixed-dose LMWH with UFH administered as either an adjusted dose IV or an adjusted or fixed dose SC, found that LMWH was associated with significantly fewer deaths (OR 0.77; 95% CI 0.63-0.93), less major hemorrhage (OR 0.58; 95% CI 0.40-0.83), and lower rates of thrombotic complications (OR 0.70; 95% CI 0.57-0.85) (Erkens and Prins, 2010). LMWH is effective and easily administered, making it the preferred anticoagulant irrespective of VTE treatment in the outpatient or inpatient setting (Wells et al., 2014).

Once systemic anticoagulation is initiated and after initiation of parenteral therapy, vitamin K antagonists (VKAs) can be started for long-term or extended treatment. Alternatively, a new class of oral anticoagulant drugs is now available; these drugs are direct inhibitors of either FXa (rivaroxaban, apixaban, edoxaban, betrixaban) or thrombin (dabigatran). These drugs share in common low MW, reasonably short half-lives of 8 to 16 h, direct inhibition of activated clotting factors, oral administration, and no need to monitor the anticoagulant effect. Ease of administration is attractive, but more clinical data is needed to support their efficacy and safety, and adequate assessment of their advantages needs to be performed as these medications are costly, and may not all be appropriate for patients with renal insufficiency (Wells et al., 2014).

A. LMWHs – Outstanding questions

LMWHs have been at the forefront of the prophylaxis and treatment of thrombosis for the past couple of decades, yet much remains to be learned about these life-saving compounds. For instance, what is the influence of a lower MW distribution on the *in vitro* and *in vivo* characteristics of these compounds? What is the role of residual anti-FIIa activity? How do these pharmacological characteristics influence clinical outcomes (efficacy and safety) in the prophylaxis and treatment of VTE? How might the physicochemical characteristics and pharmacological profiles of LMWHs affect their numerous pleiotropic effects? Are LMWHs safe to use in elderly patients and in patients with chronic kidney disease or should we avoid their use in these populations? How do LMWHs compare to the NOACs? What will be their future place in the therapeutic armamentarium?

1. Bemiparin - Pharmacodynamic impact of a lower mean molecular weight

In an attempt to learn more about the influence of a lower mean MW distribution and higher *in vitro* anti-FXa:anti-FIIa activity ratio on the *in vivo* performance of LMWHs, we performed a long overdue pharmacodynamic assessment of bemiparin, the commercially available LMWH with the lowest mean MW (mean MW 3,600 Da), vs. enoxaparin (mean MW 4,500 Da), the first and most commercially successful LMWH to date (Antonijoan et al., 2009).

In our study, the administration of bemiparin 3500 IU resulted in higher exposure to anti-FXa activity, lower exposure to anti-FIIa activity, and thus a higher *in vivo* anti-FXa/FIIa activity ratio (more than double), vs. enoxaparin 4,000 IU. Bemiparin administration also led to a more sustained anti-FXa activity than enoxaparin (Antonijoan et al., 2009). Hypothetically, a more sustained anti-FXa activity should re-

sult in an improved 24-h antithrombotic coverage, and higher anti-FXa over anti-FIIa activity selectivity, should be indicative of an improved safety profile. However, to date there is no clinical evidence to support these assertions.

Mean MW of LMWHs is typically correlated with loss of anti-FXa activity *in vitro*. This can be explained by a partial destruction of the AT-binding sequences by their cleavage or denaturation by side reactions. Given that longer heparin chains are required to inhibit FIIa, the decline of anti-FIIa is proportionally larger than that of anti-FXa. Higher selectivity of anti-FXa vs. anti-FIIa activity *in vitro* has been reported to be associated with lower bleeding *in vivo*. For example, when bleeding was qualitatively examined in a model of venous thrombosis in rats that were administered semuloparin or enoxaparin, equipotent doses of semuloparin did not show increased bleeding as opposed to enoxaparin when compared vs. controls (Viskov et al., 2009). Furthermore, in several other *in vivo* experiments, bleeding effects seem to follow a MW-dependent pattern (Gray-Shah, 2012). That said, the theoretically better safety profile of a LMWH with a higher anti-FXa:anti-FIIa activity ratio has yet to be proven in the clinic. Such an assessment will most probably prove too difficult to undertake as a clinical trial focused on bleeding would require an enormous sample size to be adequately powered.

Anti-FXa activity was not only more sustained with bemiparin than with enoxaparin, but the anti-FXa:anti-FIIa activity ratio was consistently higher. It is well known that different chain length/sequence permutations lead to time dependent variation in anti-FXa:anti-FIIa circulating activity. Anti-FIIa activity is absorbed more slowly and eliminated more quickly than anti-FXa activity (Sanderink et al., 2002). This has been clearly recognized for heparins where the higher MW species are cleared from the circulation more rapidly than the lower MW species. This differential clearance results in accumulation *in vivo* of the lower MW species, which have a lower ratio of anti-FIIa to anti-FXa activity (Hirsh, 2001).

Making *in vitro-in vivo* correlations with LMWHs has proven challenging. Even though two LMWHs may have the same anti-FXa:anti-FIIa ratio as measured before injection, differences in polysaccharide length/sequence permutations would likely lead to varying rates of absorption and elimination and thus, different circulating anti-FXa:anti-FIIa ratios. A real world example can be found in the comparison of enoxaparin and nadroparin. The two drugs have a similar MW distribution and anti-FXa:anti-FIIa ratio. Nevertheless, nadroparin has a significantly longer T_{max} and a significantly higher Cl for anti-FXa activity. Furthermore, despite demonstrating an equivalent anti-FXa:anti-FIIa activity before injection, the two drugs diverge after injection to demonstrate different *in vivo* ratios (Stiekema et al., 1993).

Given that the pharmacological correlations are not direct, one has to be even more cautious when drawing clinical inferences from pharmacological profiles. The improved 24-h antithrombotic coverage conferred by the more sustained anti-FXa activity should be relevant for VTE management. However, it is hard to conclusively state that this improved 24-h antithrombotic coverage translates into lower VTE recurrence rates. A recent update of a meta-analysis assessing randomized clinical trials in which LMWH given once daily is compared with LMWH given twice daily (resulting in a more sustained activity) for the initial treatment of VTE (Bhutia and Wong, 2013), suggests that once daily treatment with LMWH is as effective and safe as twice daily treatment with LMWH. Nevertheless, in a previous report of this meta-analysis the 95% confidence interval implied that there is a possibility that the risk of recurrent VTE might be higher when people are treated once daily (van Dongen et al., 2005). Head to head comparisons of LMWHs in clinical trials assessing efficacy or safety with different dosing schemes would provide information as to the clinical impact of a more sustained anti-FXa activity profile, but they are not likely to be performed. Therefore, on the basis of our study, it is proposed that a once daily regimen should preferably be considered only with a LMWH with a longer elimination half-life of anti-FXa activity.

In our study, enoxaparin 4,000 IU released a higher amount of total and free TFPI compared to bemiparin 3,500 IU, although no statistically significant differences were observed when comparing free TFPI levels. Interestingly, with bemiparin T_{max} of total TFPI release occurred more than 1 h prior to that of enoxaparin. The clinical translation of these differences is yet to be established. Enoxaparin elicited higher aPTT (E_{max}) ratios than bemiparin. This could explain the higher total TFPI levels and could have important implications, as it has been reported that prolonged aPTT values are associated with bleeding risk (Hirsh and Raschke, 2004). Another indicator of potentially higher bleeding risk is the higher anti-FIIa activity of enoxaparin, which was confirmed with TT measurements. A parameter that would have potentially provided additional safety information is vWF release. Increased release of vWF appears to be a marker of platelet stimulation and adverse clinical outcomes, and a lower mean MW appears to be related to a higher suppression of vWF (Montalescot et al., 2000a, Ray et al., 2005). We did not measure for this effect, but future investigations should take this parameter into consideration.

Tp-TmT, a potential indicator of thrombosis risk sensitive to raised levels of factor X (Borrell et al., 2002), increased very early after enoxaparin administration and reached its maximum value at 1 h, whereas T_{max} of Tp-TmT for bemiparin was 3 h. This suggests that Tp-TmT may be sensitive to the total TFPI release from the endothelium by LMWHs.

Lower MW fractions of LMWHs typically stimulate a lower release of TFPI (Mousa, 2013), but this correlation does not seem to always hold true. Although tinzaparin showed higher total (~30%) and free TFPI peak levels than bemiparin (Depasse et al., 2003), bemiparin induced release of TFPI to a greater extent than dalteparin and UFH (Falkon et al., 1995a, Ciccone et al., 2014). This is important because TFPI is ideally situated to modulate the proangiogenic biological actions of TF/VIIa (Holroyd et al., 2012). TFPI may also regulate angiogenesis independently of TF, through sequences within its polybasic carboxyl terminus (TFPI C), by directly blocking VEGF-receptor-2 activation and attenuating the migratory capacity of endothelial cells (Holroyd et al., 2012). Heparin has been postulated to have an anti-metastatic effect due to a decrease in neoangiogenesis (Collen et al., 2000, Mousa, 2013). However, the influence of the physicochemical and *in vitro*/*in vivo* profiles of LMWHs in modulating neoangiogenesis is yet to be elucidated. Antiangiogenic actions seem to be mediated not only exclusively by TFPI release but also by binding to vascular endothelial growth factors, cytokines, and adhesion molecules. So far, analysis of trials of heparin treatment in cancer patients indicates an improved rate of survival and meta-analyses performed specifically to assess the effects of UFH and LMWH treatment on survival in cancer patients have indicated positive effects in embolic outcomes with no differences in major or minor bleeding (Kuderer et al., 2007, Akl et al., 2011b), although no clear differences have been observed between UFH and LMWHs. Therefore, the interplay between anti-thrombotic efficacy, risk of bleeding, and proangiogenic biological actions has to be further explored in this patient population.

2. RO-14 – An ULMWH

In our second study (Rico et al., 2011), we assessed the pharmacodynamic profile of an ULMWH with an even lower mean MW than bemiparin. This was the first study in humans to assess the safety, tolerability, and preliminary pharmacodynamic profile of RO-14 (mean MW 2,200 Da), after twelve single ascending doses. The time-concentration profiles of anti-FXa activity obtained after the administration of RO-14 were monophasic at all doses. Dose-response relationship of RO-14 within the studied dose-range was dose-proportional and linear. Mean A_{max} after the highest dose (19,950 IU) was 1.67 IU/mL. In comparison, in spite of a lower *in vitro* anti-FXa activity, bemiparin reached a mean A_{max} of 2.03 IU/mL after single doses of 12,500 IU (Antonijoan et al., 2009). The lower *in vivo* potency of RO-14 as compared to bemiparin is hard to explain and additional studies are warranted. Interestingly, RO-14 showed more sustained anti-FXa activity (t ½ = 8.05 h) vs. other LMWHs such as bemiparin (t ½ = 5.2 h) (Antonijoan et al., 2009), or enoxaparin (t ½ = 4.5 h), and this characteristic could have important clinical implications both for prophylaxis and treatment of VTE.

RO-14 demonstrated no detectable anti-FIIa activity even at doses as high as 19,950 IU. As explained above, there seems to exist a larger safety margin for FXa inhibition with respect to bleeding, than for FIIa inhibition (Ahmad et al., 2003, Leadley, 2001, Viskov et al., 2009), so this could also be an important characteristic in the clinic. However, since residual anti-FIIa activity seems to be important for the non-anticoagulant effects of LMWHs, more information is needed to assess the potential trade-offs of an ULMWH resembling the synthetic pentasaccharide, fondaparinux.

Besides RO-14, another ULMWH, semuloparin (AVE5026, property of Sanofi-Aventis), has been developed (Dubruc et al., 2009, Hoppensteadt et al., 2013, Lassen et al., 2009, Viskov et al., 2009). Semuloparin is a hemisynthetic ULMWH that possesses a high anti-FXa activity (~160 IU/mg) with a residual anti-FIIa activity (~2 IU/mg) and a mean MW of 2400 Da. Depolymerization of heparin using a phosphazene base preserves the AT binding sequences from destruction, leading to an enrichment of AT-binding oligosaccharides in the hexasaccharide to at least the hexadecasaccharide fraction of semuloparin. Semuloparin differs from fondaparinux in that, due to the presence of a small amount of higher MW oligosaccharides, it retains a small level of anti-FIIa activity and a low but measurable anticoagulant effect in plasma. This small level of anti-FIIa activity allows semuloparin to preserve heparin's other binding sites to retain effects that are not mediated by AT, such as stimulation of TFPI release from the vascular endothelium (Gray-Shah, 2012, Viskov et al., 2009). Semuloparin had an equally effective antithrombotic effect and a substantially lower bleeding profile than other heparins (i.e. UFH, enoxaparin and bemiparin), as measured in the various animal models (Gray-Shah, 2012).

In the first phase I studies, semuloparin showed a linear PK profile and dose proportionality after single and repeat dosing, within a dose range comprising ~800 – 16,000 IU of anti-FXa activity. In spite of the great anti-FXa potency and long half-life (11 h), semuloparin also showed dose proportional increases in anti-FIIa activity and a slight increase in accumulation was observed in elderly volunteers (Lassen et al., 2009). In a phase II clinical trial, semuloparin demonstrated significant dose-related response in prevention of VTE in patients undergoing orthopedic surgery (TREK study[6]) (Lassen et al., 2009) after being administered once daily doses of 20 or 40 mg, for a period of 5 or 10 days. Semuloparin showed a superior efficacy and similar safety profile compared to enoxaparin 40 mg. Three phase III studies compared semuloparin and enoxaparin after major orthopedic surgery: elective TKR (SAVE-KNEE[7]), elective THR

[6] Prevention of Venous Thromboembolism in Patients Undergoing Elective Total Knee Replacement Surgery study
[7] Three Phase III studies compared semuloparin and enoxaparin after major orthopedic surgery: elective knee replacement (SAVE-KNEE), elective hip replacement (SAVE-HIP1) and hip fracture surgery (SAVE-HIP2)

(SAVE-HIP1), and hip fracture surgery (SAVE-HIP2) (Lassen et al., 2012). All studies were multinational, randomized, and double-blind. Semuloparin and enoxaparin were administered for 7-10 days after surgery. In total, 1,150, 2,326, and 1,003 patients were randomized in SAVE-KNEE, SAVE-HIP1, and SAVE-HIP2, respectively. In all studies, the incidences of DVT, non-fatal PE, or all-cause death were numerically lower in the semuloparin group vs. the enoxaparin group, but the difference was statistically significant only in SAVE-HIP1. In SAVE-HIP1, clinically relevant bleeding and major bleeding were significantly lower in the semuloparin vs. the enoxaparin group. In SAVE-KNEE and SAVE-HIP2, clinically relevant bleeding tended to be higher in the semuloparin group, but rates of major bleeding were similar in the two groups. In summary, semuloparin was superior to enoxaparin for VTE prevention after THR, but failed to demonstrate superiority after TKR surgery and hip fracture surgery (Lassen et al., 2012).

In a multicenter, double-blind, placebo-controlled trial (SAVE-ONCO) (Agnelli et al., 2012), once-daily SC semuloparin at 20 mg administered to 1,608 patients with metastatic or locally advanced solid tumors, who were beginning to receive a course of chemotherapy, significantly prevented VTE (HR 0.36; 95% CI 0.21-0.60) without increasing major bleeding (HR 1.05; 95% CI 0.55-1.99), suggesting that semuloparin thromboprophylaxis can be beneficial in cancer patients receiving chemotherapy (Gras, 2012).

Sanofi-Aventis eventually sought approval for semuloparin before the European Medicines Agency (EMA) and the FDA, in September 2011, for VTE in patients receiving chemotherapy for locally advanced or metastatic solid tumors. However, in 2012 The Oncologic Drugs Advisory Committee of the FDA voted overwhelmingly to reject the use of semuloparin for this indication, based on absence of a survival benefit (FDA, 2012, Mulcahy, 2012). After this, Sanofi-Aventis decided to withdraw all applications globally following comments by regulatory agencies (EMA, 2012).

In spite of its failure to get approval, the study of semuloparin resulted in a better understanding of the determinants of the pharmacological actions of LMWHs. AT seems to be the main cofactor required for the mediation of the anticoagulant and antithrombotic effects of LMWHs, whereas an interaction with HCII plays a relatively minor role. Owing to the high number of AT-binding sequences preserved during the synthesis process, semuloparin had an equally as effective *in vivo* antithrombotic effect and a substantially lower bleeding profile in comparison to other LMWHs (Gray-Shah, 2012).

One area that has received a lot of attention in the past few years is the study of the pleiotropic effects of LMWHs. Although it is generally agreed that LMWHs are unique compounds, the clinical relevance of

this uniqueness in the context of VTE is not fully understood and is less understood when discussing the myriad of pleiotropic effects.

One of the pleiotropic effects of LMWHs is angiogenesis modulation. A better understanding of the determinants of this effect is necessary, as this could have important implications for cancer-related thrombosis, and to modulate the neovascularization process that is important for the progression of atherosclerotic plaques (Da Pozzo et al., 2012, Ciccone et al., 2014).

In vitro evidence provided by Da Pozzo et al.,(2012) seems to indicate that bemiparin modulates angiogenesis better than enoxaparin or fondaparinux. This group treated human umbilical vein endothelial cells or human endothelial progenitor cells with bemiparin, fondaparinux, or UFH, at concentrations reflecting the doses used in clinical practice. Bemiparin gave a significant decrease of *in vitro* angiogenesis as shown by the reduction of endothelial cell tubule network, while both fondaparinux and UFH did not show any significant effect. In assays of Matrigel sponge invasion in mice, UFH was able to stimulate angiogenesis and, conversely, bemiparin inhibited angiogenesis. Furthermore, both bemiparin and fondaparinux caused a significant reduction in an *in vitro* vasculogenesis-like model, as demonstrated by the decrease of tubule network after co-seeding of endothelial progenitor cells and human umbilical vein endothelial cells. In addition, UFH but not bemiparin was able to increase AKT phosphorylation.

During the last decade, some papers have studied the effects of different LMWH and UFH on *in vitro* angiogenesis models (Rak and Weitz, 2003). One study in particular examined effects of several heparin fractions, with a wide range of MW (6,000 Da and 3,000 Da LMWH, and UFH) (Khorana et al., 2003). Interestingly, results showed that only intermediate heparin fractions are able to reduce angiogenesis, with little or no activity of the larger and smaller heparin fractions. Furthermore, concerning cell proliferation, different heparins and oligosaccharides exerted a significant inhibition, while no effect was observed with heparin tetrasaccharide, octasaccharide, or fondaparinux. On the contrary, Collen and colleagues(2000) didn't observe any effect on Human Umbilical Vein Endothelial Cells (HUVEC) angiogenesis by either UFH or LMWH (reviparin) (Da Pozzo et al., 2012).

An explanation for the difference exerted on HUVEC angiogenesis by the drugs, in accordance to Khorana and colleagues (Khorana et al., 2003), may reflect the differences in the chemical composition of individual heparins, above all regarding the polysaccharide chain lengths. It is possible that the effects on endothelial cells observed in this study might result from the different proportions of short and long

polysaccharide chains contained in the drug formulation. Polysaccharides heparan sulfate can indeed modulate the activities of angiogenic growth factors, such as VEGF, by altering the interaction of growth factor with its receptor. Structural variations in anticoagulant sulphated chains may result in differential effects on VEGF signaling. This might be consistent with the findings of Da Pozzo et al.(2012) and might explain the different modulation of endothelial cell organization, depending on the anticoagulant MW. Regarding LMWHs, heparin fragments with less than 18 saccharide residues have been reported to inhibit the binding of VEGF to its receptors on endothelial cells. In contrast with UFH, it has been shown that LMWH , can hinder the binding of growth factors to their high-affinity receptors as a result of its smaller size (Castelli et al., 2004). Small molecular heparin fractions have also been shown to inhibit VEGF-mediated angiogenesis *in vivo*, in contrast with UFH. The effects of heparins on oncogenesis appear to be also due to their interference with the activity of VEGF or FGF-2 (Smorenburg and Van Noorden, 2001). One study reported that LMWH might inhibit angiogenesis by competing with cellular heparan sulphates for the binding of these growth factors, blocking the positive effect on angiogenesis exerted by heparan sulphate (Giraux et al., 1998, Da Pozzo et al., 2012).

The antiangiogenic effects of LMWHs have also been assessed in the chick embryo chorioallantoic membrane model, a model that has been long used for the studies on tumor angiogenesis and metastasis, as well as the studies of the macromolecules with angiogenic or antiangiogenic activity. Bemiparin, enoxaparin, nadroparin, and tinzaparin sodium demonstrated antiangiogenic effects in this model, but nadroparin and tinzaparin showed a higher potency, highlighting potential differences due to their higher mean MW (Dogan et al., 2011).

There are several mechanisms through which LMWHs modulate angiogenesis, and in spite of some evidence indicating that a higher mean MW could result in a more potent antiangiogenic effect, ULMWHs are not devoid of this property. For example, Vignoli et al. (2011) demonstrated that RO-14 and bemiparin inhibit *in vitro* the angiogenic response of microvascular endothelium stimulated by tumor-cell-conditioned media (TCM) from human leukemia, lung cancer, and breast cancer cells. Bemiparin and RO-14 dose dependently inhibited the increase of capillary-like tube formation (Matrigel-based assay) and endothelial migration (wound-healing assay) induced by TCM. Both drugs also inhibited angiogenic response elicited by purified VEGF and FGF-2 (Vignoli et al., 2011, Ciccone et al., 2014).

LMWHs may also differ in their antiangiogenic and other pleiotropic activities potentials due to several factors which may include the mean fragment size, the manufacturing process, the assay used to assess

the effect, and the type of angiogenesis reaction studied (Dogan et al., 2011). Given the multiple individual biological behaviors of these agents, it has become evident that pharmacopoeial parameters such as anti-FIIa and anti-FXa potency and MW have become insufficient to characterize such agents (Jeske et al., 2011).

3. Efficacy and safety of LMWHs in elderly patients and patients with chronic kidney disease

In our last study, we generated evidence-based guidance on dosing for elderly patients and CKD patients receiving bemiparin. Dose adjustment of LMWHs in patients with decreased renal function has been a polemic and poorly studied issue in the past. In spite of more than 20 years of clinical research of LMWH, many of the individual recommendations on the use of LMWHs in these patient populations remain empirical or based on partial data.

We evaluated the pharmacodynamics of bemiparin after four prophylactic doses (3,500 IU) and a single therapeutic dose (115 IU/kg) in young and elderly volunteers, and in subjects with different degrees of RI. Previous pharmacodynamic studies with other LMWHs had either assessed prophylactic or therapeutic doses, had not included an in-study control group or an elderly volunteers group, and/or had been observational studies with less rigorous control and with less strong evidence (Schmid et al., 2009c).

Results in the group of healthy volunteers were in agreement with the pharmacodynamic profile described for bemiparin (Falkon et al., 1995b, Antonijoan et al., 2009). There were no significant differences in any of the analyses comparing the young and elderly volunteer groups, indicating no need for dose adjustment in elderly patients with preserved renal function. After the administration of multiple prophylactic doses and a single therapeutic dose of bemiparin, mean absorption rate and exposure to anti-FXa activity was significantly higher in patients with severe RI as compared to young healthy volunteers. In spite of these differences, observed A_{max} values in subjects with severe RI were close to the optimal range proposed by Kearon et al.(2008) Clearance was decreased in all the groups of patients with RI and there were significant differences between subjects with severe RI and healthy volunteers.

By and large, there was a greater degree of inter-individual variability in the time course of anti-FXa activity in patients with RI. This phenomenon has already been reported by other authors (Schmid et al., 2009c, Grand'Maison et al., 2005, Mahé et al., 2007), and might not entirely be explained by the differ-

ences in renal function, but rather by differences in the type of renal injury or other underlying clinical conditions (Nagge et al., 2002).

Compartmental analysis and simulations allowed us to evaluate the potential need for dose adjustment especially after prophylactic and therapeutic doses. The simulations allowed us to observe the net effect that a lower elimination rate constant has in the anti-FXa activity profile of bemiparin in patients with severe RI. After 10 simulated repeated prophylactic (3,500 UI/24 h) and therapeutic (115 IU/kg QD) doses of bemiparin, the group of patients with severe RI would have mean anti-FXa A_{max} values around 20-25% higher than the rest of the groups (between 0.33 and 0.60 IU/mL with the 3,500 IU dose, and between 0.80 and 0.93 IU/mL with the dose of 115 IU/kg). Lowering the prophylactic dose to 2,500 IU/24 h yielded a mean predicted A_{max} of 0.42 IU/mL, a value within the same range observed without dose adjustment. However, lowering the therapeutic dose by 25% to 86.25 IU/kg QD, resulted in a noticeably lower predicted A_{max} of 0.84 IU/mL, a value within the range observed in the rest of the groups. Based on these results, a dose adjustment of prophylactic and therapeutic doses in patients with severe RI would result in anti-FXa activity profiles similar to the ones observed in other patient populations.

Age-related alterations in renal function, protein binding, and increased bleeding risk must be considered prior to administering anticoagulants to the increasing elderly population (Dinwoodey and Ansell, 2008). The safety of LMWHs in the elderly population has been explored from different perspectives in several studies (Alikhan et al., 2003, Berges et al., 2007, Kucher et al., 2005, Mahé et al., 2007, Martínez-González et al., 2005, Pautas et al., 2002, Pautas et al., 2001, Rodriguez-Manas et al., 2010, Simoneau et al., 1992). There is limited published information available on the comparative pharmacokinetic/pharmacodynamic (PK/PD) analysis in healthy young and elderly subjects with other LMWHs. One of the earliest published comparisons in this regard was performed by Simoneau et al.(1992) According to this group, the time course of anti-FXa activity after single administrations of dalteparin at prophylactic (2,500 IU) and therapeutic (10,000 IU) doses in young and elderly subjects did not differ. Likewise, according to the FDA's review of enoxaparin, repeated administration of daily prophylactic doses of 40 mg of enoxaparin over 10 days did not result in statistically significantly different PD parameters in young and elderly subjects (FDA, 1993). The issue of dose adjustment in the elderly has been studied in some therapeutic clinical trials (Alikhan et al., 2003, Depasse et al., 2003, Kucher et al., 2005, Siguret et al., 2000, Montalescot et al., 2000b). Prospective data evaluating LMWH use in elderly patients have been mostly limited to inpatient treatment (Clark, 2008) and most of the studies in the elderly have been in patients with some degree of RI, and are discussed below (Siguret et al., 2000, Bauersachs,

2012, Mismetti et al., 1998, Mahe et al., 2007, Pautas et al., 2002). To date, only enoxaparin has a recommended dose reduction for patients over 75 years being treated for acute ST-elevated myocardial infarction (0.75 mg/kg SC BID) (Sanofi-Aventis, 2011).

LMWH's are polar, hydrophilic drugs that are approximately 80% renally eliminated. In patients with RI, accumulation could potentially occur with standard doses. This increases the risk of bleeding (Barras, 2013). It is generally accepted that there is potential for lower clearance and accumulation of anti-FXa activity in patients with severe RI with most LMWHs after multiple therapeutic doses. Accumulation after multiple prophylactic doses has been studied less but it is still observed (Garcia et al., 2012). Our results are in line with those reported by other groups who assessed enoxaparin in patients with RI (Cadroy et al., 1991, Sanderink et al., 2002, Mahé et al., 2007, Kruse and Lee, 2004, Bazinet et al., 2005). Cadroy et al.(1991) reported that clearance of a single therapeutic dose of enoxaparin (0.5 mg/kg SC) was 2 times lower and elimination half-life was 1.7 times longer in patients with severe RI. Sanderink et al.(2002) performed a clinical trial with a similar design to ours, but only tested prophylactic doses of enoxaparin (40 mg SC QD for 4 days) and did not include a group of elderly volunteers. They found that elimination half-life increased with the degree of RI and anti-FXa exposure was significantly different between healthy volunteers and patients with severe RI. In addition, median A_{max} values in healthy volunteers, and in mild, moderate, and severe RI patients were very similar to the results of our simulations.

There is less information on the rest of the LMWHs. Dalteparin has been assessed in a number of studies in patients with RI (Stobe et al., 2006, Schmid et al., 2009b, Douketis et al., 2008, Shprecher et al., 2005). Stobe et al.,(2006) assessed the anti-FXa activity profile after the administration of a single IV bolus of dalteparin (50 IU anti-FXa/kg; roughly higher than prophylactic doses when corrected for relative bioavailability) in patients with normal function, moderate and severe RI, and hemodialysis-dependent CKD. They found that anti-FXa clearance is significantly decreased in both groups with RI and that there is a 50% prolongation in the elimination half-life in the groups with RI when compared with healthy volunteers. Shprecher et al.,(2005) compared the peak steady-state anti-FXa levels of therapeutic doses of dalteparin (100 IU/kg SC BID) in patients with adequate (n = 11, Cl_{Cr} > 80 mL/min) and compromised renal function (n = 11, Cl_{Cr} < 40 mL/min) and found no meaningful differences between groups after three days of administration. Nevertheless, there was no 24-h characterization of the anti-FXa profile, and patients with moderate and severe RI were pooled in one category. Douketis et al.,(2008) enrolled 156 critically ill patients with severe RI to a single-arm, open-label trial where they received prophylactic

doses of dalteparin (5,000 IU SC QD) and reported no bioaccumulation (defined as a trough anti-FXa level > 0.40 IU/mL). However, peak anti-FXa levels have been shown to be lower in intensive care unit patients because vasopressors may decrease resorption and therefore bioavailability of SC administered LMWH (Schmid et al., 2009a, Dorffler-Melly et al., 2002). Schmid et al., performed two prospective cohort studies where patients with various degrees of RI received prophylactic (n = 42; 5,000 IU SC QD) (Schmid et al., 2009a), or therapeutic doses (n = 32; 100 IU/kg SC BID) of dalteparin (Schmid et al., 2009b) and peak anti-FXa activity levels were measured 2-3 times/week for up to three weeks. They concluded that there was no clinically relevant bioaccumulation (< 30%) of dalteparin used for prophylaxis of VTE. However, when used at therapeutic doses, they found that there is significant bioaccumulation in the group of patients with severe RI (Ratio $A_{max\ Day6/Day\ 1} = 2.32$ and recommended a dose adjustment based on the individual patients' condition. According to the product information for dalteparin, monitoring only needs to occur after the patient has received three to four doses (Barras, 2013).

Studies with tinzaparin have shown no reduction in clearance or accumulation of anti-FXa activity (Siguret et al., 2000, Pautas et al., 2002, Mahé et al., 2007) Siguret et al.,(2000) carried out a prospective study with therapeutic doses of tinzaparin (175 IU/kg SC QD) administered over 10 days to elderly patients with age-related RI (n = 30, Cl_{Cr} = 40.6±15.3 mL/min (range 20-72)). There was no bioaccumulation of the anti-FXa and anti-FIIa activities, determined at 5 h post-administration, over the 10 day treatment period. In a similar study, very elderly patients (mean age 85.2, n = 200, mean Cl_{Cr} = 51.2 ± 22.9 mL/min) received therapeutic doses of tinzaparin (175 IU/kg SC QD) for up to 30 days (Pautas et al., 2002). A similar dispersion of anti-FXa activity levels was observed in the four Cl_{Cr} subgroups studied (Cl_{Cr} = >65, 50-64, 35-49 and 20-34 mL/min). A 20% dose adjustment was necessary in 20 patients who were distributed throughout the four Cl_{Cr} subgroups, and a further 20% adjustment was necessary in 3 patients (no group specified). No correlation was found between anti-FXa activity and Cl_{Cr} or age. Mahé et al.,(2007) performed a randomized trial with elderly patients (n = 55; Cl_{Cr} range = 20-50 mL/min), who received prophylactic doses of enoxaparin (4,000 IU SC QD) or tinzaparin (4,500 IU SC QD) for 8 consecutive days and found significant accumulation of anti-FXa activity for enoxaparin ($A_{max\ Day8/Day\ 1} = 1.22$, P < 0.0001) but not for tinzaparin ($A_{max\ Day8/Day\ 1} = 1.05$, P = 0.29). The apparent difference in tinzaparin clearance in patients with severe RI may reflect metabolism by hepatic mechanisms, possibly due to the higher MW of tinzaparin compared with other LMWHs (Garcia et al., 2012). To date, no complete

pharmacodynamic characterization in patients with varying degrees of RI has been performed with tinzaparin.

Goudable et al.,(1991) investigated the pharmacodynamics of nadroparin after a single bolus intravenous injection (41.3 IU/kg) in patients with moderate and severe RI, including patients receiving hemodialysis (total n = 19). There were significant differences in the exposure to anti-FXa activity and a longer elimination half-life in the patients with RI when compared with the healthy subjects. Mismetti et al.(1998) found that after multiple administrations of therapeutic doses of nadroparin (180 IU/kg QD for 6-10 days), accumulation of the anti-FXa activity was observed in healthy elderly volunteers and in elderly patients but not in the healthy young subjects (accumulation factor = 1.3). Clearance of the anti-FXa and of the anti-FIIa activities were 1.4 and 2 times higher respectively than those calculated in the healthy elderly.

Lineal regression analyses in our study, demonstrated a moderately strong correlation between Cl_{Cr} and the majority of the non-compartmentally-derived parameters of anti-FXa activity calculated after the administration of prophylactic doses. A lower Cl_{Cr} predicts a higher A_{max} and a lower Cl of anti-FXa activity. This correlation was less evident after therapeutic doses. A multiple regression analysis model incorporating Cl_{Cr}, age, weight, and gender showed that only gender had some effect in predicting A_{max} of anti-FXa activity. Female patients with severe RI apparently would have higher A_{max} of anti-FXa activity than male patients. Body weight could explain part of this variation, but the sample size is too small to draw any conclusions (Al Dieri et al., 2006). Results of regression analyses assessing the correlation between Cl_{Cr} and anti-FXa activity have yielded conflicting results. With some exceptions (Siguret et al., 2000, Pautas et al., 2002, Goudable et al., 1991, Cadroy et al., 1991), studies have demonstrated that clearance of the anti-FXa effect of LMWH is highly correlated with Cl_{Cr} (Green et al., 2005, Becker et al., 2002, Stobe et al., 2006, Mismetti et al., 1998, Garcia et al., 2012, Chow, 2003). In the study performed by Mismetti et al., nadroparin clearance, but not tinzaparin clearance, was shown to be correlated with Cl_{Cr} ($R^2 = 0.49$, $P = 0.002$) (Mismetti et al., 1998). This was also observed in a study performed by Becker et al., in which a strong linear relationship was reported between Cl_{Cr} and enoxaparin clearance ($R^2 = 0.85$, $P = 0.001$) (Becker et al., 2002). A linear inverse correlation was also shown between Cl_{Cr} and anti-FXa levels ($R^2 = 0.58$, $P = 0.0005$) after multiple therapeutic doses of enoxaparin (Garcia et al., 2012, Chow, 2003). Small sample sizes and a high degree of variability diminish the power of this analysis approach and may help explain the different results obtained by other groups.

Other simulation approaches have been used in the past to explore dose adjustments with LMWHs. Green et al.(2005) developed a population pharmacokinetic model using data of 38 patients (age range 44-87) with ACS and a mean GFR of 32 (range = 16-117) mL/min, receiving enoxaparin (0.5 and 1.0 mg/kg SC BID). Based on stochastic simulations they recommended that patients with Cl_{Cr} between 30 to 39 mL/min should receive 0.5 mg/kg BID, whereas patients with a Cl_{Cr} of 10 to 19 mL/min should receive 0.3 mg/kg BID. A model developed by Hulot et al.(2004) based on data of 532 patients with ACS and varying degrees of renal function receiving full dose enoxaparin, concluded that enoxaparin clearance was decreased by 31% in patients with Cl_{Cr} between 30 to 49 mL/min and by 44% in patients with $Cl_{Cr} \leq$ 30 to 49 mL/min. Simulations suggested that a loading dose of 1 mg/kg followed by a regimen of 0.8 mg/kg BID in patients with moderate RI and 0.66 mg/kg BID in patients with severe RI would result in therapeutic anti-FXa levels. A similar approach, but with lower doses (0.75 mg/kg BID and 0.5 mg/kg in patients with moderate and severe RI, respectively), was suggested by Kruse and Lee (2004).

Our results are also in agreement with those that have been obtained in therapeutic clinical trials. Martínez-González et al.(2005) performed a subanalysis of a trial that compared bemiparin 115 IU/kg with UFH for the treatment of DVT. This subanalysis included patients of any age with RI and elderly patients without RI. Bemiparin was not associated with an increased incidence of major bleeding in either the RI group or in the elderly group. Moreover, in the ANCIANOS study[8] (Rodriguez-Manas et al., 2010), very elderly subjects showed a trend towards a lower incidence of total bleeding than younger patients and a similar incidence of major bleeding when they received prophylactic doses of bemiparin.

Until recently, the increased bleeding risk, as a result of decreased clearance and increase in half-life of LMWHs in patients with severe RI, has attracted most of the attention (Saltiel, 2010). Thorevska et al.(2004) performed a retrospective cohort study comparing the rates of bleeding complications in patients with RI who received anticoagulation therapy with therapeutic doses of UFH or enoxaparin. Major bleeding rates were 26.3 per 1,000 person-days for UFH and 20.7 per 1,000 person-days for enoxaparin, but patients with severe RI had a 154% excess incidence of minor bleeding compared to those receiving UFH (incidence ratio, 2.54; 95% CI 1.01-6.36). Spinler et al. (2003) published the results of a retrospective analysis of the ESSENCE[9] and TIMI 11B[10] trials which compared enoxaparin to UFH in patients with

[8] Thromboprophylaxis with the Low-Molecular-Weight Heparin Bemiparin Sodium in Elderly Medical Patients in Usual Clinical Practice

[9] Efficacy and Safety of Subcutaneous Enoxaparin in Non-Q-Wave Coronary Events. The ESSENCE trial evaluated the efficacy of enoxaparin vs. UFH, plus aspirin, in patients with rest angina or non-Q-wave infarction.

ACS. Patients with a Cl_{Cr} ≤ 30 mL/min receiving therapeutic doses of enoxaparin had an increased risk for major hemorrhage (RR = 6.1; 95% CI 2.47-14.88). Another prospective study (Cestac et al., 2003) in patients with either VTE or ACS treated with therapeutic doses of enoxaparin or tinzaparin, showed that the decrease of Cl_{Cr} (10 mL/min) was associated with an increased haemorrhagic risk (RR = 1.34, 95% CI 1.12-1.65). Moreover, a Cl_{Cr} ≤ 20 mL/min was associated with a RR of 2.8 (95% CI 1.0-7.8) for bleeding complications. In a retrospective observational study (n = 106) (Gerlach et al., 2000) medical charts of patients receiving multiple doses of enoxaparin were reviewed to compare the frequency of bleeding complications from enoxaparin in patients with normal renal function versus patients with RI. Total bleeding complications occurred in 22% of patients with normal renal function and 51% with RI (P < 0.01). Major bleeds were also significantly different, 2% and 30%, respectively (P < 0.001).

One of the most cited studies on the issue of increased risk of bleeding is the meta-analysis performed by Lim and associates (2006). They compared the risk of major bleeding and anti-FXa levels in patients receiving LMWH who had severe RI (Cl_{Cr} ≤ 30 mL/min) with those patients with a Cl_{Cr} > 30 mL/min. Data were insufficient to assess the relationship between anti-FXa activity and renal function for prophylactic doses of enoxaparin and therapeutic doses of tinzaparin or dalteparin. In 12 studies involving 4,971 patients given LMWH, the OR for major bleeding was 2.25 (95% CI 1.19-4.27) in patients with severe RI, compared to those with a Cl_{Cr} > 30 mL/min. Therapeutic doses of enoxaparin were associated with a further increase in major bleeding in patients with severe RI (8.3% vs 2.4%; OR, 3.88; 95% CI 1.78-8.45), but this was not observed when enoxaparin was empirically dose reduced (0.9% vs 1.9%; OR, 0.58; 95% CI 0.09-3.78).

Unfortunately, less emphasis has been given to another equally and potentially higher risk for these populations, namely VTE. Accurate dosing is of paramount importance, because the elderly and patients with RI are at an increased risk of bleeding (Clark, 2008, Dinwoodey and Ansell, 2008, Campbell et al., 1996, Mahe et al., 2002, Lacut et al., 2008), but their risk for VTE is also known to be higher than younger patients and those with preserved renal function (Monreal et al., 2006, Daneschvar et al., 2008, Bauersachs et al., 2011, Bauersachs, 2012, Falga et al., 2007, Samama, 2011). Literature indicates that advanced age and RI are not only independent risk factors for bleeding, but also for VTE. The RIETE[11]

[10] Thrombolysis in Myocardial Infarction trial. The TIMI 11B trial tested the benefits of a strategy of an extended course of uninterrupted antithrombotic therapy with enoxaparin compared with standard treatment with unfractionated heparin for prevention of death and cardiac ischemic events in patients with unstable angina/non–Q-wave myocardial infarction.

[1111] *"Registro Informatizado de la Enfermedad Tromboembolica"*

international registry of thromboembolic disease has provided interesting information in this regard. Findings from the analysis of patients over 80 years suggest the risk of fatal PE (3.7% incidence) is of greater concern than the risk of fatal bleeding (0.8% incidence) (Lopez-Jimenez et al., 2006). Additional RIETE data reported that the incidence of fatal PE in patients with moderate and severe RI (2.6%, and 6.6%, respectively) was higher than that of fatal bleeding (0.3%, and 1.2% of the patients, respectively). These data support the use of full-dose anticoagulant therapy, even in patients with severe RI (Monreal et al., 2006). The results of the LITE[12] study indicated that after adjusting for other variables of cardiovascular risk, patients with grade 3/4 RI (GFR = 15-59 ml/min per 1.73 m^2) have a RR for VTE of 1.71 (95% CI 1.18-2.49) as compared with subjects with preserved renal function (Wattanakit et al., 2008). Moreover, the higher VTE risk might be more life-threatening than the risk of bleeding. Findings from the RIETE registry suggest that the risk of fatal pulmonary embolism (PE, 3.7% incidence) is of greater concern than the risk of fatal bleeding (0.8% incidence) in patients over 80 years (Lopez-Jimenez et al., 2006), and that the risk of death for PE and DVT clearly exceeds the risk of fatal bleedings in patients with RI (Falga et al., 2007). All these data support the use of full-dose anticoagulant therapy, even in patients with severe RI (Monreal et al., 2006).

Furthermore, recent RIETE data also supports the use of LMWH over UFH, even in patients with severe RI. Propensity score-matching was used to compare patients treated with UFH (n = 2167) with those treated with LMWHs (n = 34,665) in 3 groups stratified by Cl_{Cr} levels at baseline: > 60 mL/min (n = 1598 matched pairs), 30-60 mL/min (n = 277 matched pairs), or < 30 mL/min (n = 210 matched pairs). In this study the group of patients with Cl_{Cr} levels < 30 mL/min showed an increased 15-day mortality for UFH compared with LMWH (15% vs. 8.1%; P = 0.02), an increased rate of fatal PE (5.7% vs. 2.4%; P = 0.02), and a similar rate of fatal bleeding (0.5% vs 0.0%) (Trujillo-Santos et al., 2013). This is in line with the CERTIFY[13] study in which certoparin (3,000 IU QD) was as efficacious as UFH (5,000 IU TID) in patients with severe RI but had a reduced risk of bleeding (Bauersachs et al., 2011). The IRIS study[14], comparing UFH treatment to tinzaparin, yielded different results. It was stopped prematurely due to a difference in mortality favoring the UFH group (11.5 vs. 6.3%; P = 0.035). Interestingly, rates of clinically relevant bleedings were similar in the tinzaparin (11.9%) and UFH (11.9%) groups, as were rates of confirmed recurrent VTE (2.6 vs. 1.1%; P = 0.34). Because the mortality difference could not be explained by

[12] Longitudinal Investigation of Thromboembolism Etiology study
[13] A randomized, double-blind study of certoparin vs unfractionated heparin to prevent venous thromboembolic events in acutely ill, non-surgical patients
[14] Innohep® in Renal Insufficency Study

bleedings or recurrent VTE, a post-hoc analysis helped identify six baseline characteristics significantly correlated with mortality, of which five were over-represented in the tinzaparin group reflecting an imbalance of mortality risk factors at baseline (Leizorovicz et al., 2011).

Some authors and current guidelines in the prophylaxis and treatment of VTE still recommend using other anticoagulants, monitoring anti-FXa activity or adjusting the dose of LMWH's (Robert-Ebadi et al., 2009, Kearon et al., 2012, Garcia et al., 2012, Harenberg, 2004, Hirsh et al., 2008a, Hirsh et al., 2008b). However, none of these options seem particularly convenient and the supporting evidence for these recommendations is of low quality (Grade 2C, weak recommendation) (Guyatt et al., 2012). Arguably, there is more robust evidence supporting the use of full doses in patients with RI.

Using other anticoagulants could prevent patients from receiving a convenient cost-effective drug and the use of UFH has not been associated with better outcomes in recent studies (Leizorovicz et al., 2011, Trujillo-Santos et al., 2013) Monitoring anti-FXa activity is still advocated by some authors (Barras, 2013), but could be challenging for practical reasons and may provide flawed information for patient management due to the inherent characteristics of the test which measures neither the essential molecule nor the effect (Hemker et al., 2005, Bounameaux and de Moerloose, 2004). The net anticoagulant effect is not only the result of the anti-FXa activity. It is co-determined by plasma characteristics such as the concentration of AT, the level of heparin-binding proteins, and the thrombin-forming power of the hemostatic system (Al Dieri et al., 2006). Although anti-FXa activity is the closest we have gotten to a practical risk predictor of bleeding when using LMHW therapy, how the anti-FXa activity relates to a clinical effect remains poorly understood. Clinical trials of LMWHs used prophylactically or therapeutically have shown at best a poor correlation between the anti-FXa activity levels and clinical bleeding (Walenga, 1993, Gouin-Thibault et al., 2010). Nevertheless, important correlations with efficacy have been reported (Montalescot et al., 2000a). Although some studies reported that high anti-FXa levels were associated with an increased bleeding risk, several other studies failed to show a relationship between anti-FXa levels and bleeding. Furthermore, prophylactic and therapeutic anti-FXa target ranges vary from one LMWH to another and have not been validated (Garcia et al., 2012). If monitoring is considered in patients receiving therapeutic dose LMWHs, appropriate target ranges for peak anti-FXa levels should be used, and so far no anti-FXa based guidelines have been issued (Gouin-Thibault et al., 2010).

Empirical dose reductions can also be troublesome. Gouin-Thibault et al.(2010) published a review highlighting the caveats behind dose-adjustment in the elderly and in patients with RI. They noted that the efficacy and safety of LMWHs used at reduced initial dosages have not been evaluated in the treatment of VTE. In the widely known MEDENOX trial[15], that assessed the safety and efficacy of enoxaparin for the prevention of VTE in acutely ill medical patients (Alikhan et al., 2003), it was shown that a full 40 mg QD dose of enoxaparin was particularly effective in the group of patients over 80 years of age (87% risk reduction vs. 63% in the general study population). Given that the lower dose tested in this study (20 mg QD) did not differ from placebo, any recommendation for dose reduction would presumably have a negative impact on efficacy; as was the case when a dose-adjustment proposal of enoxaparin was evaluated in the treatment of ACS where it was found that too low doses of enoxaparin could be as ineffective as placebo (Montalescot et al., 2004). Analysis of patients over 75 years in the PREVENT trial[16], demonstrated superiority of dalteparin (5,000 IU QD) to placebo in reducing the incidence of a composite endpoint comprising: symptomatic VTE, fatal PE, sudden death, or asymptomatic proximal DVT (4.2% vs. 8.0%, RR, 0.52; 95% CI 0.31-0.87), without increasing the risk of major hemorrhage (1.1% vs. 0.7%, P = 0.12) (Leizorovicz et al., 2004, Gouin-Thibault et al., 2010, Kucher et al., 2005). In spite of this evidence, some authors still propose lowering LMWH dose in CKD to reduce hemorrhagic risk, even if reducing LMWH dose could impair drug effectiveness (Fabbian et al., 2011).

LMWH dosing in patients with severe RI is in fact a complicated issue. Clinicians have to be careful with empirical dose adjustments as underdosing might result in thrombosis and an overall increase in mortality. The best course is to carefully balance the risk of thrombosis against the risk of bleeding for each patient.

B. LMWHs and/or NOACs

In addition to LMWHs and ULMWHs other therapeutic agents have recently been developed for the prophylaxis and treatment of thrombosis (Blann and Khoo, 2009). For instance, a chemoenzymatic approach, relying on a series of HS biosynthetic enzymes mimicking the biosynthesis of heparin and HS, has been recently described. Using this method, two structurally homogenous ULMWH (MW 1,778.5 and 1,816.5 Da) with *in vivo* anti-FXa activity similar to that of fondaparinux have recently been reported (Xu et al., 2011). Furthermore, NOACs that directly inhibit thrombin, such as dabigatran etexilate

[15] Medical Patients with Enoxaparin Trial
[16] Prospective Evaluation of Dalteparin Efficacy for Prevention of VTE in Immobilized Patients Trial

(marketed as Pradaxa® by Boehringer Ingelheim) (Boehringer Ingelheim Pharmaceuticals, 2013); or FXa, such as rivaroxaban (marketed as Xarelto® by Bayer Pharma/Janssen Pharmaceuticals) (Janssen-Pharmaceuticals, 2014), apixaban (marketed as Eliquis® by Pfizer and Bristol-Myers Squibb) (Bristol-Myers-Squibb, 2014), edoxaban (marketed as Lixiana® in Japan by Daiichi-Sankyo) (Daiichi-Sankyo, 2014), betrixaban (in development by Portola Pharmaceuticals) (Portola-Pharmaceuticals, 2014) and AZD0837 (in development by AstraZeneca) (Johansson et al., 2011) are emerging options for VTE thromboprophylaxis and treatment. Although it has yet to be determined whether factor Xa or thrombin is a better target for anti-thrombotic therapy, clinical data show that both drug families are effective (Bounameaux, 2009, Agnelli et al., 2013b, Agnelli et al., 2013a). Table 8 shows the most relevant pharmacokinetic characteristics of these compounds.

Table 8. Pharmacokinetic characteristics of NOACs

Drug	Dabigatran etexilate*	Rivaroxaban*	Apixaban*	Edoxaban[+]	Betrixaban[Δ]
Mechanism of action	DTI	Direct FXa inhibitor	Direct FXa inhibitor	Direct FXa inhibitor	Direct FXa inhibitor
Oral bioavailability	6.5%	80-100%	50%	62%	34
T_{max} (h)	0.5-2	1-4	1-4	1-2	1
t ½ (h)	12-14	5-13	8-15	10-14	20
Renal excretion (%)	85	66	27	50	5 (Mainly excreted in bile)
Potential metabolic drug interaction	Potent P-gp inhibitors and P-gp inducers	Strong dual CYP3A4 and P-gp inhibitors/inducers	Strong dual CYP3A4 and P-gp inhibitors/inducers	Potential interactions with strong inducers of both CYP3A4 and P-gp	Potential interactions with P-glycoprotein inducers Not substrate for CYP450 system
Antidote	In development	No	In development	No	In development

*Data extracted from (Bauer, 2013, Gonsalves et al., 2013)
[+] Data extracted from (Mendell et al., 2013)
[Δ] Data extracted from (Palladino et al., 2013)

NOACs have been mainly evaluated VTE treatment, primary and secondary prevention in medical and surgical patients and in patients with atrial fibrillation to reduce risk of stroke and embolism. The most frequent comparators in clinical trials have been warfarin and enoxaparin. No head to head comparisons have been performed to date. It is still debated whether these new drugs are more efficacious, safer, have a better therapeutic window, or are more cost-effective than existing drugs, such as warfarin or

LMWHs (Hirsh et al., 2007, Mackman, 2008, Weitz and Linkins, 2007). Several studies have compared the efficacy and safety of NOACs vs LMWHs in VTE prophylaxis for major orthopedic surgery. A systematic review with meta-analysis of 37 RCTs of patients undergoing THR, TKR, or hip fracture surgery who received prophylaxis with a LMWH or another anticoagulant, was recently performed (Sobieraj et al., 2012). It was concluded that compared with patients who received UFH, patients who received LMWHs had fewer PE (OR = 0.48; 95% CI 0.24-0.95; number needed to treat (NNT) = 8), total DVT (RR = 0.80; 95% CI 0.65-0.99; NNT = 12–100), major bleeding (OR = 0.57; 95% CI 0.37-0.88; NNT = 41), and HIT events (OR = 0.12; 95% CI 0.03-0.43; NNT = 34–202). Compared with patients who received VKAs, patients who received LMWHs had fewer total DVT (RR = 0.66; 95% CI 0.55-0.79; NNT = 6–13) and distal DVT (RR = 0.56; 95% CI 0.43–0.73; NNT = 6–10) events but reported increased major bleeding (OR = 1.92; 95% CI 1.27-2.91; number needed to harm (NNH) = 57–220), minor bleeding (RR = 1.23; 95% CI 1.06-1.43; NNT = 18–218), and surgical site bleeding events (OR = 2.63; 95% CI 1.31-5.28; NNH = 23–64). Major efficacy end points such as symptomatic VTE, PE, and nonfatal PE showed similar benefits of therapy with LMWHs and VKAs. Compared with patients receiving FXa inhibitors, patients who received LMWHs had more major VTE (OR = 2.64; 95% CI 1.82-3.84; NNH = 22–314), PE (OR = 2.50; 95% CI 1.08-5.78; NNH = 223), total DVT (RR = 2.05; 95% CI 1.68-2.50; NNH = 8–119), proximal DVT (OR = 2.62; 95% CI 1.95-3.51; NNH = 44–122), and distal DVT (RR = 2.14; 95% CI 1.84-2.50; NNH = 10–126) events but fewer major bleeding events (OR = 0.65; 95% CI 0.49-0.86; NNT = 74–999). Compared with patients receiving DTIs, patients who received LMWHs had more major VTE (OR = 2.64; 95% CI 1.82-3.84; NNH = 22–314), total DVT (RR = 1.31; 95% CI 1.09-1.57; NNH = 18–41), and proximal DVT (RR = 1.31; 95% CI 1.09-1.57; NNH = 18–41) events without significantly negatively affecting bleeding. However, patients who received LMWHs had fewer distal DVT events versus those who received DTIs(RR = 0.80; 95% CI 0.68-0.93; NNT = 17–100). According to this meta-analysis, the balance of benefits to harms analysis seems favorable for FXa inhibitors or DTIs compared with LMWHs. The authors of this work also concluded with predominantly low-to-moderate strength of evidence, the known benefits in total DVT and distal DVT with LMWHs versus VKAs may not be sufficient to counteract the increased risk of bleeding (Sobieraj et al., 2012).

Another meta-analysis evaluated the benefits and harms of oral direct FXa inhibitors (including rivaroxaban, apixaban, edoxaban, YM150, TAK442, betrixaban and LY517717) vs. LMWHs in patients undergoing THR or TKR (22 RCTs) (Neumann et al., 2012). There were no statistically significant differences between FXa inhibitors and LMWHs in mortality at the end of treatment (OR = 1.27; 95% CI 0.63-2.55) or at the

end of follow-up (OR = 0.95; 95% CI 0.55-1.63). There was no statistically significant difference between treatments in nonfatal PE. There was a statistically significant benefit of reduced symptomatic DVT in patients treated with FXa inhibitors (OR = 0.46; 95% CI 0.30-0.70) which represented a reduction of three DVT (95% CI 1-5) events per 1,000 patients treated for one to five weeks. When the baseline risk from a large cohort study was used, FXa inhibitors were estimated to give a benefit of four (95% CI 3-6) fewer events per 1,000 treated patients. Analyses of major bleeding events and bleeding leading to reoperation were not statistically significant but suggested the possibility of harm; analysis of absolute differences showed an increase of two (95% CI 0.98-1.65) major bleeding events per 1,000 patients treated for one to five weeks. Subgroup and regression analyses indicated that excess bleeding events resulted from high (OR = 2.50, 95% CI 1.38-4.53) rather than low or intermediate doses of FXa inhibitors (no statistically significant difference).The authors concluded that compared with LMWHs, lower doses of oral FXa inhibitors can achieve a small absolute risk reduction in symptomatic DVT without increasing bleeding.

Gomez-Outes et al. (2012) conducted another meta-analysis of RCTs evaluating NOACs (rivaroxaban, dabigatran, or apixaban) vs. enoxaparin for VTE prophylaxis after THR or TKR. Sixteen trials were included. Rivaroxaban was associated with a significant reduction in risk of symptomatic VTE compared with enoxaparin (RR = 0.48; 95% CI 0.31-0.75). Compared with enoxaparin, neither dabigatran (RR = 0.71, 95% CI 0.23-2.12) nor apixaban (RR = 0.82; 95% CI 0.41-1.64) reduced the risk of symptomatic VTE. Compared with enoxaparin, the relative risk of clinically relevant bleeding was higher with rivaroxaban (RR = 1.25; 95% CI 1.05-1.49), similar with dabigatran (RR = 1.12; 95% CI 0.94-1.35), and lower with apixaban (RR = 0.82, 95% CI 0.69-0.98). The treatments did not differ on the net clinical endpoint (symptomatic VTE, major bleeding, and death) in direct or indirect comparisons. The authors concluded that a higher efficacy of NOACs was generally associated with a higher bleeding tendency. The new anticoagulants did not differ significantly for efficacy and safety.

Adam et al. (2013) analyzed the results six good-quality systematic reviews (including the ones mentioned above) comparing NOACs with LMWHs for thromboprophylaxis after THR or TKR. According to this analysis risk for symptomatic DVT, but not risk for death or nonfatal PE, was reduced in patients receiving FXa inhibitors compared with LMWHs (4 fewer events per 1,000 patients; 95% CI 3-6). Conversely, the risk for major bleeding increased (2 more events per 1,000 patients). Outcomes of dabigatran did not significantly differ from those of LMWHs. Indirect evaluation of NOACs by common comparison with LMWH showed non-significantly reduced risks for VTE with rivaroxaban compared with

dabigatran (RR = 0.68; 95% CI 0.21-2.23) and apixaban (RR = 0.59; 95% CI 0.26-1.33) but increased major bleeding. The authors concluded that clinical benefits of NOACs over LMWHs are marginal and offset by increased risk for major bleeding (Adam et al., 2013).

A higher potential for bleeding has indeed been an ongoing concern. Like all anticoagulants, dabigatran, rivaroxaban, apixaban and edoxaban can provoke bleeding. For instance, numerous reports of severe bleeding associated with dabigatran have been recorded since this drug was first marketed. Some situations are associated with a particularly high bleeding risk, including: even mild RI, advanced age, extremes in body weight, and drug-drug interactions, particularly with antiplatelet agents (including aspirin), non-steroidal anti-inflammatory drugs, and many drugs used in cardiovascular indications. In patients treated with dabigatran, rivaroxaban, or apixaban, changes in the INR (international normalized ratio) and aPTT do not correlate with the dose. The lack of a routine coagulation test suitable for monitoring these patients is a recognized downside, as is the fact that there is no antidote available for dabigatran, rivaroxaban, or apixaban. This last point may be relative, as it has not represented an important problem with LWMHs. Although protamine sulphate can be used to neutralize the effect of LMWHs, it is not 100% effective. For instance, protamine sulphate only neutralizes around 30% of the effect of bemiparin (Falkon et al., 1998b). That said, reversal of the anticoagulant effect may be urgently needed in some situations. While this is currently not a possibility with NOACs (Bounameaux, 2009, 2013a), active work is underway to develop a suitable reversal agent for dabigatran (Schiele et al., 2013), rivaroxaban (Perzborn et al., 2014), and betrixaban (Portola-Pharmaceuticals, 2014).

Other safety concerns have been raised for some of the newly developed drugs. In 2004 ximelagatran (Exanta®, Astra Zeneca) an orally active DTI, was not approved by the FDA, because liver-enzyme elevations were reported. Three patients died due to liver failure deemed attributable to the drug. An increase in cardiac events has also been described even after short-term exposure (Keisu and Andersson, 2010, FDA, 2004, Jeffrey, 2004). Since ximelagatran's failure, drug-induced liver injury (DILI) has been a concern. A meta-analysis where NOACs were compared against any control group, concentrated on this question. The primary outcome assessed was DILI (transaminases elevations >3x upper limit of normal (ULN) with total bilirubin > 2x ULN). Twenty-nine randomized clinical trials evaluating 152,116 patients (mean follow-up of 16 months) were included. The authors reported NOACs are not associated with an increased risk of DILI (RR = 0.90, 95% CI 0.72-1.13). Similar results were obtained for individual NOAC's (rivaroxaban, apixaban, dabigatran, edoxaban) and considering the different control groups (VKAs, LMWHs, and placebo). The risk of transaminases elevations (> 3x ULN) was lower among NOAC-treated

patients, in particular in comparison with LMWH-treated patients (RR = 0.71; 95% CI 0.59-0.85) (Caldeira et al., 2014).

The place of new compounds in special populations (e.g. patients with reduced renal or hepatic function, pregnant women, and children) will have to be carefully assessed (Bounameaux, 2009). Dose adjustment in patients with liver or kidney failure has not yet been resolved by the new compounds. Although dabigatran, for example, is generally safe in patients with hepatic impairment, differences in pharmacokinetics, attributable to variation in renal function, have been observed (Stangier and Clemens, 2009). Dabigatran requires dose adjustment in patients with moderate RI and is contraindicated in patients with severe RI (Harder, 2012). And in the case of rivaroxaban, for instance, it has been seen that plasma clearance correlates with Cl_{Cr}, thereby increasing exposure and pharmacodynamic effects (Ordovas Baines et al., 2009). Rivaroxaban can be administered as a fixed dose for the prevention of VTE in patients with moderate RI and should be used with caution in patients with severe RI. Apixaban excretion is also partly dependent on renal function, although the impact of RI has not been determined. Additional data on the safety of chronic dosing of NOACs in RI are awaited (Harder, 2012). Importantly, direct comparisons between NOACs are lacking, as are comparisons between NOACs and LMWHs other than enoxaparin.

Because of the above, some authors think that elucidation of the true efficacy and safety of NOACs requires more time (Abrams, 2013). When compared with warfarin, the long-term outcome of NOACs in patients who are elderly and who have suboptimal renal function is not completely understood. Furthermore, on average, the patients in clinical studies of NOACs have been younger than those found in typical clinical practices. Patients in clinical studies were typically not receiving drugs that might impact their renal function or otherwise affect the pharmacodynamics of the NOACs. Drug-induced changes in kidney function can lead to changes in the drug levels, which in turn may lead to changes in anticoagulation status that will go unrealized because monitoring is not part of the standard management of patients taking NOACs (Abrams, 2013).

LMWHs could have some advantages over NOACs. Firstly, it is very important to remember that when targeting factors in the coagulation cascade, the sequential activation of factors by proteolytic cleavage results in an amplification of each step. In this regard, the heparin family targets an upstream component of the cascade and its mechanism of action has been deemed more physiological than other suggested targets (Mackman, 2008). Moreover, it is unlikely that newer drugs will ever match the

polypharmacology of heparin. This is not only because the actions of LMWHs are limited to the inhibition of coagulation enzymes, but also because these drugs exhibit profound actions on endothelial sites and blood cells (Fareed et al., 2008), that give rise to a multitude of pleiotropic effects covered in the introduction.

Although DTIs and anti-FXa inhibitors have been slated to replace older anticoagulants (including LMWHs), this is not likely to happen soon. LMWHs will most likely continue to be important agents in the prophylaxis and treatment of VTE and, since great progress has been made in the understanding of the polypharmacology of LWMHs, clinical trials for new indications are currently underway (Page, 2013).

CONCLUSIONS

When administered to healthy volunteers, prophylactic doses of bemiparin (3,500 IU) showed a higher anti-FXa activity and higher anti-FXa/FIIa activity relationship than enoxaparin (4,000 IU). Bemiparin administration produced lower anti-FIIa activity, aPTT ratios, TT, and TFPI than enoxaparin. There were no differences between the two LMWHs when comparing free TFPI levels or Tp-TmT.

Subcutaneous administrations of twelve single ascending doses RO-14 (1,750-19,950 IU) to healthy male volunteers were well tolerated. No major safety issues were identified or reported, and no bleeding events occurred. RO-14 did not modify the vital signs, electrocardiograms (ECGs) or laboratory tests at any dose. In particular, there were no apparent differences in the clotting tests performed before and after drug administration.

When administered to healthy male volunteers, RO-14 showed a lower dose-adjusted peak anti-FXa activity than the one reported for other LMWH, although it was sustained for a longer time. There was no detectable anti-FIIa activity at any dose.

Upon administration of 12 single ascending doses of RO-14 to healthy male volunteers, anti-FXa activity increased in a dose-proportional and linear fashion.

There were no significant differences between adult healthy volunteers and elderly volunteers, in the pharmacodynamic profiles of anti-FXa and anti-FIIa activity after the administration of multiple prophylactic (3,500 IU) and a single therapeutic dose (115 IU/kg) of bemiparin.

Age *per se*, does not constitute a factor that affects the pharmacodynamic profile of bemiparin at prophylactic or therapeutic doses, whereas creatinine clearance does have an inverse correlation with anti-FXa exposure.

After the administration of multiple prophylactic doses and a single therapeutic dose of bemiparin, the mean absorption rate and exposure to anti-FXa activity was significantly higher in patients with severe renal insufficiency as compared to adult healthy volunteers.

Clearance was decreased in all the groups of patients with renal insufficiency and there were significant differences between subjects with severe renal insufficiency and healthy volunteers.

No dose adjustment is required in elderly subjects with preserved renal function or in patients with mild and moderate renal insufficiency. In patients with severe renal insufficiency, there is a potential risk of bioaccumulation of anti-FXa activity, therefore a dose adjustment is recommended for both, prophylactic (now 2,500 IU) and therapeutic doses (now 86.25 IU/kg) for venous thromboembolism.

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