In memory of Marc Vestraete (1925-2018)

FROM UNFRACTIONATED HEPARIN TO PENTASACCHARIDE: PARADIGM OF RIGOROUS SCIENCE GROWING IN THE UNDERSTANDING OF THE *IN VIVO* THROMBIN GENERATION

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Abstract

Following a chance discovery made by a medical student who was searching for a clot-promoting activity in tissue extracts, it took 15-20 years to attain the therapeutic use of standard unfractionated heparin (UFH), due to problems with the purification and extraction of the active material. Soon it was found that:

1) thrombin inactivation by UFH was associated with the formation of molecular complexes between antithrombin and the activated forms of factor X (FXa) and thrombin, 2) low-molecular-weight fractions of UFH lose their antithrombin activity while still interacting with FXa, 3) a pentasaccharide sequence of UHF increases FXa (but not thrombin) inactivation by antithrombin. Low-molecular-weight heparins (LMWHs) with little effect on thrombin and strongly active versus FXa were then developed. In patients, LMWHs (and the pentasaccharide sequence) came up as a useful class of drugs to prevent and treat thrombosis, their greatest advantage over UFH being the convenience of the once/twice daily subcutaneous injections at a fixed dose without any laboratory monitoring. In addition to providing major information on *in vivo* modulation of thrombin generation, the heparin saga served as a paradigm to support an alternative coagulation scheme that includes platelets and tissue factor as integral parts of the model. Forthcoming work with this scheme – also supported by studies in hemophilia and rare bleeding disorders – is expected to provide major hints for understanding why some patients benefit more than others from the small amount of thrombin they form and directions to tailor prevention and treatment of thromboembolic disorders.

Introduction

It has been stated that, in terms of improved of patients care, a limited number of compounds deserve to be singled out as true milestones.[1] Having facilitated the introduction of the artificial kidney for haemodialysis[2] and having contributed to the development of cardiopulmonary bypass,[3] heparin – one of the oldest drugs still in widespread use – belongs to this category. Heparin has been a successful anticoagulant for over 80 years and is on the WHO list of essential drugs. Despite the availability of other anticoagulants, it remains a major therapeutic choice especially in surgical interventions. The 1990s were a critical time for the study of heparins: after the evidence (1980s) that low-molecular-weight-heparins (LMWHs) were as effective as unfractionated heparin (UFH) for preventing venous thromboembolism (VTE), the 1990s were devoted to methodologically high-quality trials aimed at evaluating to what extent LMWH could be safely administered in outpatient settings (e.g. for long-term prophylaxis in high-risk individuals). Indeed, the most salient feature of LMWH underscored by Marc Vestraete in *Haemostasis* Journal in 1990, was that a lower bleeding incidence of LMWHs for equivalent antithrombotic efficacy was yet to be established in man.[4] The present review — started after his death (August 16th, 2018) — is an update several decades later of his review on heparin and thrombosis. The discovery and development of UFH and LMWH have been covered by excellent reviews (see for example [1, 5, 6]).

The question whether LMWH would be associated with a lower incidence of hemorrhagic side-effects than UFH was raised at an early stage of the development of this drug.[7] Maybe due to the different balance of sugar moieties – a major determinant of the binding to platelets,[8] or to the higher number of more negatively charged areas for the binding to positively charged regions on the cell surface, larger heparin species (e.g. UHF) interact more with platelets than LMWHs.[9-12] This suggested that, rather than to the reduced antithrombin (AT) effect,[13] the lower tendency to bleeding complications in animals receiving LMWH might be related to a reduced binding of this molecule to platelets.[14-16]

Heparin discovery.

Like many other biological substances, this highly sulfated glycosaminoglycan was discovered incidentally. Early observations on the anticoagulation of circulating blood were made by Maurice Doyon (1912) who was investigating the effect of peptone on coagulability of blood in dog livers.[17] In those years, William H. Howell, Professor of Physiology at Johns Hopkins University, was trying to isolate, from organ extracts, the well-known accelerator of coagulation tissue thromboplastin. Following his initial interest in this field whereby he analyzed the differences in coagulability between arterial and venous blood,[18] Howell developed the theory of a balance between a clotting inhibitor (*antithrombin*) and a procoagulant substance (*thromboplastin*), and claimed that, by neutralizing antithrombin and in turn activating prothrombin in the presence of Ca^{2+}, cephalin released from platelets and leukocytes was able to trigger clotting.[19] After funding and accomplishing his undergraduate studies at the University of California at Berkeley, Jay McLean approached Howell, asking for a project that he could complete quickly, as he only had sufficient funds for one year of school at Johns Hopkins University. He was instructed to make extracts from brain, heart, and liver and was assigned by Howell to examine the chemical purity of cephalin preparations, and to demonstrate that it was cephalin – and not a contaminant in the preparation – that accounted for the procoagulant activity. McLean finished this work early and went on to isolate other phosphatides from the samples. His original intent was to show that the removal of cephalin would reduce the procoagulant activity of the samples. To his surprise, he found that two of his samples had anticoagulant properties. He informed Howell of his discovery, but it is unclear how seriously either took it at the time. McLean described his discovery in 1916 and referred to the compounds carrying the anticoagulant activity as 'the phosphatides from heart and liver', but only briefly mentioned the prospect of an anticoagulant.[20] He also noticed that the clot-promoting thromboplastic property of liver extracts deteriorated when stored. Indeed, the oldest batches even prolonged the clotting time of test plasma. McLean then moved to Penn University for further research on cephalins, under the supervision of Richard Mills Pearce. In October 1917, he went back to Baltimore to Howell's laboratory. However, he abandoned his search for phosphatides with anticoagulant capability he had isolated the previous year, as he felt procoagulants would be more valuable for the ongoing Great World War efforts. McLean wrote down those details shortly before his fatal illness in 1957 (Appendix), and the account was published after his death.[21] According to his report, McLean

attempted several times to return to experimental work on heparin. However, he was engaged in clinical practice and was only honored after his death as the discoverer of heparin.

In 1918, Howell and another medical student, Emmett Holt Jr., carried on McLean's work and isolated a fat-soluble anticoagulant apparently distinct from that isolated by McLean. For its isolation, ether was employed to extract dried powdered liver, and the solution obtained was precipitated by acetone, re-dissolved in ether and re-precipitated by absolute alcohol at 50°. The process was repeated 12-20 times and yielded a mixture with a powerful *in vivo and in vitro* anticoagulant potential which differed from antithrombin in that it did not neutralise the action of thrombin.[22] The product was <5% pure and could only be used *in vitro* as it was toxic in experimental animals. McLean declined Howell's offer to be included as co-author of this paper, since he felt that his contribution in the work had not been sufficient to justify that.[23] In the description of such fat-soluble anticoagulant, Howell and Holt introduced for the first time the term heparin (after the tissue from which it was first isolated).[24]

Four years later (1922), during the Annual Meeting of the American Physiological Society, Howell presented an aqueous extraction protocol for isolating and purifying a material that he named heparin (the same name of the lipid extract isolated in 1918)[25] and, at the 12th International Physiological Congress in 1926, he presented refinements to this protocol, identified the water-soluble carbohydrate as glucuronic acid and published a detailed report on the chemical and physiologic reactions of water-soluble heparin.[26] This turned out to be a compound distinct from both the ones isolated by himself and Holt in 1918 and by McLean in 1916, and began to be produced commercially by Hynson, Westcott, and Dunning, a pharmaceutical company in Baltimore. Since it caused headaches, fevers and nausea,[27] Howell was concerned that such toxic effects might preclude its widespread use and that heparin production would cease.[28] Despite his fears – and although the pharmaceutical company did not advance its isolation procedures beyond Howell's original protocol, heparin continued to be commercially available. In 1931, Howell retired from his post at Johns Hopkins and did no further research on heparin.

Heparin purification.

The development of non-toxic heparin was carried out in Canada in 1933-36. Approximately at the time of William H. Howell's retirement, Canadian physiologist Charles H. Best, assistant director at the Connaught Laboratories in Toronto and Nobel Prize in 1923 for his discovery of insulin along with Frederick Banting, had been involved with the preparation of insulin and of beef liver extracts for administration to patients. Based on his experience with tissues, he began to show interest in the production of a more refined heparin. With the help of the organic chemist Arthur Charles and of David Scott, he carried out chemical work on heparin at the Connaught Laboratories. They first finalized a protocol for isolating a crude heparin preparation from bovine liver and documented that the amount of heparin yielded was maximal if the tissue was autolyzed.[29-32] They also reported that heparin contains large amounts of hexosamine, in a ratio of one mole of hexosamine per mole of uronic acid.[33] Finally, they showed that heparin could be found in many organs throughout the body – liver, muscle and lung tissues containing the largest quantities – and that only circulating blood did not contain significant quantities of heparin.

In 1929, the Swedish scientist Erik Jorpes visited the Connaught Laboratories in Toronto to observe the production process of insulin. He was also introduced to the heparin research and, when he returned to Stockholm, he began his own attempt to isolate and characterize heparin and avoid its side effects. At the Chemistry Department of the Karolinska Institutet in Stokholm, using the Charles and Scott extraction procedure, Eric Jorpes and his co-workers Holmgren and Wilander found that the Glisson's capsule (the connective tissue surrounding the liver) was very rich in mast cells and contained ten times more heparin than the liver parenchyma itself. Using meta-chromatic staining, they also found that the site of storage of heparin was the granules of the so-called mast cells discovered by Paul Ehrlich in 1877.[34] Early investigations by Jorpes and his co-workers on the chemical nature of heparin showed it to be uronic acid and hexosamine in a ratio of 1:1 with a weight content of 40% of sulfur in the ash. Later they found the hexosamine to be glucosamine. Only in the late 1960s, with the improvement of chemical techniques and the application of nuclear magnetic resonance technology, it was shown that the global composition of heparin (i.e. the sulfur-containing polysaccharide reported by Howell and the highly sulfated complex of glucuronic acid and glucosamine described by Charles and Best) was a sequence of alternating sulfated L-iduronic acid and D-glucosamine units.[35]

Heparin development: early studies on thrombosis.

Several years after the discovery of heparin, Gordon Murray – the surgeon who developed a practical artificial kidney in the 1940s[36]– started his work on experimental thrombosis at the Toronto General Hospital, and reported that heparin was effective in the prevention of vein occlusion in dogs undergoing mechanical or chemical injuries.[37] Almost in parallel, using a more purified heparin preparation, Hedenius and Wilander performed the first intravenous heparinization on themselves outside the hospital.[38] Meanwhile, the Swedish pharmaceutical company Vitrum AB (later bought by Kabi, which later joined Pharmacia) got interested in heparin and produced on a large scale a more purified heparin preparation. In April 1937, enough heparin to begin studies in patients was available without the fear of secondary effects. The first clinician to use heparin in patients was the Swedish surgeon Clarence Crafoord who had studied pulmonary embolism in postoperative patients and treated some of them by embolectomy.[39] He treated 325 post-surgery patients in his department at Lund University, and his colleague Per Wetterdal did similarly in 309 patients during the postpartum period at the Department of Obstetrics of the same University. Based on previous clinical experience, a frequency of 3-4% of serious or fatal pulmonary embolism was expected in these patients. Bleeding and other complications were also expected; no such problems occurred in the patients receiving heparin prophylaxis. These investigations allowed the Vitrum Company to register the first commercial preparation of heparin in Sweden for intravenous anticoagulation (1939). In 1937, clinical trials with heparin were also started by Murray et al[40] at the Toronto General Hospital. Using Connaught's heparin solutions, these investigators reported, in at risk patients, results as good as the Swedish team and the drug was introduced for clinical use in Canada. By 1941, Jay McLean succeeded in using heparin in subacute bacterial endocarditis[41] and, five years later, the use of heparin plus sulfapyridine in the treatment of endocarditis and gangrene was documented.[42] Once the prophylactic value of heparin was established, large-scale clinical studies with heparin in the US and in Switzerland documented its effectiveness in the treatment of patients with venous or arterial thrombosis (early 1940s). By 1942, in addition to heparin from both Connaught and Vitrum Laboratories, two additional sources of heparin were available for clinical use (Liquaemin-Roche Organon, Solution Laboratories and Heparin-Lederle).

Except for Schmitz and Fischer (the Biological Institute of the Carlsberg Foundation, Copenhagen, Denmark) who purified heparin from dog liver as brucine salt in 1933 (this preparation did not lead to any large-scale production),[43] beef lung was the preferred source for

all commercially available heparins. However, the cost for the preparation and in turn the average daily cost of therapy (20-25 US dollars), was exceedingly high for most potential users.[44] In May 1948, while Connaught Laboratories were continuing research to develop a protocol for mass-production of heparin, Edith M. Taylor and Peter J. Moloney at the Toronto University (Ontario, Canada) applied for the patent of a cheap method for yielding large amounts of heparin based on pig intestine (which is still today the regular source for UFH and LMWHs). The discovery by Taylor and Moloney made heparin an affordable therapeutic tool and led Connaught Laboratories to abandon the production of the compound they had pioneered (early 1950s). Meanwhile, the introduction of the activated partial thromboplastin time (aPTT) assay and its simplifications provided a simple test of heparin monitoring.[45-47] Furthermore, the incidental *in vitro* and *in vivo* loss of the heparin anticoagulant activity by protamine – a basic protein from salmon sperm that was discovered in a study aimed at finding a "long-acting" heparin – was perceived as the evidence of a potential antidote for heparin.[48] The latter possibility was confirmed in clinical studies and further supported the possibility of a safe use of heparin in clinical practice.[49]

Towards Low-Molecular-Weight Heparin (LMWH)

1. **Chemistry and biochemistry.** The clinical use of heparin had already started when it was found that, at therapeutic concentrations, it behaved as an anticoagulant only in the presence of a plasma component termed at that time "heparin cofactor",[50-52] which has since been identified;[53-55] isolated[56] and renamed antithrombin III, subsequently just antithrombin (AT). At higher than therapeutic concentrations, heparin and heparin-like mucopolysaccharides have an additional effect by catalyzing the inhibition of thrombin by a second inhibitor, the heparin cofactor II.[57] That heparin acts by accelerating the physiologic reaction between AT and thrombin provided crucial hints into the chemistry of heparin fractions and contributed considerably to the progress in the knowledge of the coagulation mechanism. The anticoagulant activity of heparin is primarily related to its ability to bind to AT and accelerate the formation of a molecular complex between AT and the activated forms of several coagulation factors (XII, XI, IX, and X) as well as of plasmin and kallikrein, thereby leading to a conformational change that converts AT into a much more efficient inhibitor of serine proteases. Several groups pioneered those studies (Table 1), and the development of the heparin-sepharose column, which allowed for heparin fractionation, was an important technical advance in this respect.[58] Major concepts that

emerged from the studies of those groups follow: a) In a mixture of heparin and AT in a sucrose gradient, about one-third of the heparin molecules bind to AT and can be separated from the unbound species;[59] b) on a column with immobilized AT, the anticoagulant activity of pharmaceutical-grade heparin (average molecular weight 12,000-15,000 Da) is almost exclusively accounted for by a small fraction of the molecules–those with a high affinity for AT.[60, 61] The remaining molecules (approximately 2/3) have a very limited anticoagulant effect, but still inhibit the activation of prothrombin by FXa,[62, 63] or potentiate the action of high-affinity (low-molecular-weight) fractions;[64] c) while still inhibiting FXa,[60, 65] low-molecular-weight fractions prepared from UFH have progressively less effect on the aPTT, reflecting a less marked inhibition of thrombin (FIIa), d) UHF molecules containing a critical pentasaccharide sequence increase the activity of AT to inactivate FXa, but cannot amplify AT-mediated inactivation of Factors IXa, XIa and IIa (thrombin).[66] Further research showed that the pentasaccharide sequence is randomly distributed along the heparin chains and that approximately 1/3 of UFH and 1/4-1/5 of the chains of LMWHs contain the pentasaccharide sequence. The pentasaccharide was subsequently synthesized,[67] and its anti-anti-FXa activity was characterized.[68, 69] Low-molecular-weight fractions of UFH molecules were then obtained by hydrolytic cleavage and isolated by a variety of techniques, including gel and ultra-filtration, solvent extraction, and enzymatic or thermal depolymerization. The preparation of LMWHs following different patented methods of depolymerization has resulted in the manufacturing of LMWHs with distinctive biochemical, pharmacokinetic and pharmacodynamic profiles (Table 2). Occasional bleeding events due to the contamination of the final product with some anticoagulant principle (e.g. EDTA) have been reported for some of them.[70] *Ad hoc* studies showed the antithrombotic potential and the selectivity of LMWHs on FXa inhibition. On the other hand, the synthesis of a sequence with high-affinity for AT, composed by seven regular heparin-disaccharide units attached to the non-reducing end of the pentasaccharide sequence, helped clarify the observation that, despite the presence of the specific sequence, LMWH molecules lose their anti-thrombin activity when the length decreases significantly.[71, 72] Subsequent observations documented that seven heparin-disaccharide units attached to the pentasaccharide sequence are the shortest synthetic sequence with high-affinity for AT.[73] The AT activity of LMWH was thus found to be proportional to the number of molecules that have at least 12 sugar units at the non-

reducing end of a the pentasaccharide, whereas the anti-FXa activity to be proportional to the number of pentasaccharide sequences it contains.[74] The first pentasaccharide with high affinity for AT was produced in extremely low yield and insufficient purity for the use as a synthetic drug.[75] Total synthesis of highly functionalized pentasaccharides was reported in 1984,[76] and in 1985.[77] After these initial studies, newer analogues of pentasaccharide were synthesized. One of them was fondaparinux.[78] Fondaparinux (MW of 1728 Da) only exhibits anti-FXa activity. As compared to UFH, fondaparinux provides a more predictable dose-response, has a longer half-life, carries a lower risk of severe thrombocytopenia and is not neutralized by protamine. After a successful clinical development, fondaparinux became the first synthetic pentasaccharide available for thromboprophylaxis in patients undergoing orthopedic surgery.[79] As for LMWHs, additional indications have been granted over time (thromboprophylaxis in high-risk abdominal surgery and in medical patients, treatment of deep vein thrombosis [DVT], pulmonary embolism [PE], acute coronary syndromes [ACS] and superficial vein thrombophlebitis).[80]

2. **Mechanism of action of heparin**. Both UFH and LMWHs exert their anticoagulant activity by causing a conformational change in AT. Such change is mediated by the pentasaccharide sequence. Binding of UFH or LMWHs to AT causes a conformational change at its reactive center, which accelerates AT interaction with FXa. Thus, both UFH and LMWHs catalyze the inactivation of FXa by AT. In contrast to its anti-Xa activity, catalysis of AT-mediated inactivation of thrombin requires the formation of a ternary (i.e. heparin–AT–thrombin) complex (Figure 1). This complex can be formed only by heparin fragments ≥5,400 Da or with a chain length >18 saccharides units (including the pentasaccharide sequence).[81] Heparin fragments below such critical chain length (or with mean molecular size < 5,400 Da) inhibit FXa and cause significantly less thrombin inhibition.[82, 83] Likewise, a synthetic pentasaccharide of only 5 monosaccharide units (molecular weight approximately 1,700 Da) contains the domain that binds to AT (but not to heparin cofactor II)[84] and possesses a highly specific *in vitro* activity against FXa, but little AT activity.[66, 69, 78, 85] On the other hand, with increasing chain length, heparins gain a progressively stronger inhibitory capacity against thrombin. The discovery that LMWHs scarcely prolong aPTT (indicating no thrombin inhibition) but are capable of inhibiting FXa, raised the hope that the antithrombotic property (anti-Xa) could be dissociated from the anticoagulant property

(inhibition of thrombin), thus avoiding the bleeding tendency due to UFH. The underlying philosophy is the relevance of inhibiting the cascade system at as early a stage as possible without altering normal hemostasis. With LMWHs, the latter conditions could be fulfilled due to the limited inhibition of thrombin. However, prothrombin is converted to thrombin by the prothrombinase complex (i.e. FXa and FVa adsorbed on a membrane surface).[86] Rather than FXa, FVa is the rate-limiting component in this complex.[87] Studies on Factor V Leiden (after the name of the Dutch city where the mutation was first reported) strongly support this concept. Because of a single amino acid substitution (the specific guanine-to-adenine substitution at nucleotide 1691 [R 506]),[88] the rate of the regulatory breakdown of Factor Va by activated protein C -the natural anticoagulant protein that cleaves and inactivates FVa- is 10-fold slower than normal, resulting in increased thrombin generation.[89, 90] Resistance to the anticoagulant effect of activated protein C has long been documented in thrombosis-prone families.[91] Likewise, heparin molecules large enough to retain some thrombin-blocking action are needed for an effective thrombosis-preventing effect in animal models of stasis thrombosis.[92, 93]

UFH and LMWH: From the bench to the bedside

UFH. After initial studies suggesting that UFH was highly effective in preventing post-operative thrombosis and in treating VTE, in a landmark clinical trial in 1960, Barritt and Jordan first showed that UHF was also highly efficacious in treating pulmonary embolism.[94] Similar to all other settings, UFH was administered intravenously in this study. Indeed, only in the 1970s, large-scale subcutaneous use of low-dose UFH for prophylaxis of postoperative thrombosis was established.[95] This was the result of a study in which Kakkar et al. showed that a prophylactic dose of 5,000 IU UHF given subcutaneously three times daily before and after surgery did not lead to AT depletion and was safe and effective in reducing pulmonary embolism in postoperative patients (-75%), at the expense of a 2.5% increase in wound hematoma.[96] The low-dose subcutaneous heparin regimen led to a more practical, highly cost-effective and attractive approach to prevent DVT, which was soon accepted not only in general surgery.[97] By reviewing the results in more than 16,000 patients from over 70 randomized trials in general, orthopedic and urological surgery, it was evident that the use of perioperative subcutaneous UFH had been able to prevent approximately 50% of all pulmonary emboli and about two thirds of all DVTs, and that twice daily injections were as effective as three times daily.[98] However, treated patients

presented a slightly increased risk of bleeding compared with control groups receiving no heparin. Subsequently, Kakkar et al. also showed that a single daily dose of LMWH was as effective and safe as the standard three-times daily injections of UFH to prevent DVT (in 97 out of 100 patients, no control group in this pioneering study).[99]

LMWHs. Studies with LMWHs have been carried out for all the indications (prevention and treatment of VTE in general and orthopedic surgery; acute coronary syndromes, particularly unstable angina; thrombotic stroke, hemodialysis) in which subcutaneous UFH had been successfully employed to prevent and treat thrombosis (Table 3). These studies have been reviewed in detail.[100, 101] Here, we only consider reports that address the issues raised by Vestraete in his 1990 review whether LMWHs might provide an improvement over and above UFH especially in terms of improved safety. Because the results of single studies will depend on many specific factors – e.g. a special patient population with a specific demography in a specific type of surgery, anaesthesia, dose of study drug and of comparator and the intervals used, adjunctive therapies etc, only meta-analysis results will be quoted in this report.

1. *Meta-analyses of studies on prophylaxis.* The issue whether LMWHs were more efficacious and safer than UFH for DVT prevention has been addressed in two early meta-analyses. By analyzing the results of 52 randomized trials in which LMWH was compared with placebo, dextran or UFH for prophylaxis of DVT in general or orthopedic surgery, superiority in the efficacy of LMWH vs placebo or dextran (odds ratio [OR] 0.31 and 0.44, respectively) and also vs UFH (OR 0.85, p = 0.02) – in the absence of a significant difference in the incidence of major bleeding (LMWH vs UFH) – was documented.[113] An overall benefit for LMWHs over UFH was also found in a second meta-analysis.[114] For all surgical studies, the relative risk (RR) for DVT (LMWH vs HFH) was 0.74 (95% confidence intervals [CI] 0.65-0.86); for pulmonary embolism the RR was 0.43 (95% Cl 0.26-0.72), and for major bleeding it was 0.98 (95% Cl 0.69-1.40). Comparable RRs were observed for general and orthopedic surgery evaluated separately. However, when the analysis for general surgery studies was limited to those studies with strong methodology – as assessed by eight criteria defined in advance – the RR for DVT was 0.91 (95% Cl 0.68-1.23) and for major bleeding 1.32 (95% Cl 0.69-2.56). An additional meta-analysis comparing LMWH with placebo or no treatment, or with UFH showed that the significant reduction in asymptomatic DVT obtained with LMWH versus placebo or no treatment (n = 513; RR 0.28 [95% CI 0.14-0.54]) was associated with a significant reduction in clinical pulmonary embolism (n = 5456; RR 0.25 [0.08-0.79])

and clinical VTE (n = 4890; RR 0.29 [0.11-0.73]), and with a trend towards reduced overall mortality rate.[1] Comparison versus UFH showed a trend in favour of LMWH, with a significant reduction in clinical VTE (P = 0.049); a trend was also found for cancer surgery. All in all, LMWH at doses below 3400 anti-Xa units seemed to be as effective as, and safer than, UFH, while higher doses yielded slightly superior efficacy but increased hemorrhagic risk, including that of major bleeding. A more recent evaluation of only high-quality studies on thromboprophylaxis has recognized that: 1) LMWH is at least as effective as UFH in the prophylaxis of DVT in both general and orthopedic surgery; 2) there is a number of individual trials in orthopedic surgery in which a greater effectiveness has been found particularly in the prevention of proximal thrombosis, but the trend is not significant, and 3) with few exceptions,[115, 116] the overall data argue against improved safety of LMWH as to bleeding.[117]

2. *Meta-analyses of studies on treatment of DVT.* In a meta-analysis of 16 randomized trials with over 2,000 patients, there was a significant reduction in the incidence of thrombus extension (OR 0.51) in favor of LMWH compared to UFH. Despite trends in favor of LMWH, there was no significant difference in such studies as to reduced incidence of major bleeding, total mortality and recurrence of thromboembolism.[120] Hirsh et al.[121] classified studies as level 1 or level 2, according to the degree of blinded assessment. In studies classified as level 1 (i.e. if they were double blinded or if there was blinded assessment of outcome measures), RR for recurrent VTE during the first 15 days and over the entire period of anticoagulant therapy was 0.24, (p = 0.02) and 0.39 (p = 0.006) respectively in favor of LMWH treatment. The RR for major bleeding was 0.42 (p = 0.01), in favor of LMWH. In studies classified as level 2 (i.e. if they did not provide assurance of blinded outcome assessment), no significant differences in the rates of recurrent VTE and of major bleeding were observed. Pooling level 1 and level 2 studies, the RR for overall mortality and mortality in cancer patients was 0.51 (p = 0.01) and 0.33 (p = 0.01) respectively, in favor of LMWH. Thus, the meta-analyses argue for LMWH in the treatment of established DVT as providing greater efficacy, less hemorrhagic effects, and improved convenience over and above UFH. However, the reduced hemorrhagic incidence emerges mainly from one large trial in which the dosage of UFH was exceedingly high.[122] In another large individual trial (over 1,000 patients), there was no difference in incidence of bleeding in the LMWH and UFH groups.[123] A Cochrane Database Systematic review has

addressed the issue whether once daily treatment with LMWH is as effective and safe as twice daily treatment with LMWH. The pooled data (five studies, 1,508 participants included) showed a statistically non-significant difference in recurrent venous thromboembolism between the two treatment regimens (OR 0.82, 95% CI 0.49 to 1.39). A comparison of major hemorrhagic events (OR 0.77, 95% CI 0.40 to 1.45) and mortality (OR 1.14, 95% CI 0.62 to 2.08) also showed a statistically non-significant difference between the two treatment regimens. However, the wide 95% confidence interval implies that there is a possibility that the risk of recurrent VTE might be higher when people are treated once daily.[2]

Overall, in such trials, LMWHs came up as one of a very useful class of drugs in thrombosis treatment and prevention. The greatest advantage of LMWH over UFH was the convenience of the once/twice daily use of subcutaneous injections at a fixed dose (per kg body weight), without any laboratory monitoring. Therefore, LMWHs have replaced UFH as the recommended *treatment for pulmonary embolism*.[124] For *prophylaxis of postoperative DVT*, a single daily subcutaneous injection of one of the various LMWHs provides satisfactory protection and a remarkably low bleeding risk. For the *treatment of DVT*, the decision to treat a person with a once daily regimen of a low-molecular-weight heparin will depend on the evaluated balance between increased convenience and the potential for a lower efficacy/safety ratio. This has allowed large numbers of patients to be treated at home instead of in hospital, a major advantage both for patients and hospitals. Due to distinct biochemical and pharmacological characteristics, each brand of LMWHs should be considered as a distinct entity, and the optimal dose in terms of effectiveness and safety is to be established.

Bleeding tendency and high anti-Xa levels in individual patients: need for monitoring of LMWH*?*

The issue whether clinical effectiveness might be improved by laboratory monitoring to guide dosing of LMWH was first addressed in 1994.[125] The conclusion was that, for prophylaxis, neither UFH nor LMWH have ever been routinely monitored, and that, in the treatment of DVT, clinical trials have shown that LMWH is efficacious and safe without the need for monitoring. However, in 1998, the College of American Pathologists recommended heparin monitoring in some groups of "non-standard" patients (those who are under- or over-weight, children, pregnant patients, and those with renal insufficiency),[126] thus opening a debate.[127-130] In addition to

the groups of patients already identified, routine monitoring for treatment of DVT was felt useful in older patients, those on long-term therapy for malignancy and in those with reduced creatinine clearance.[127, 129, 130] However, the lack of any relationship between the occurrence of major bleeding and high anti-Xa levels in patients in clinical trials where LMWH was monitored and technical limitations inherent to the monitoring have also been emphasized.[128] Later, the issue was re-examined by Hemker.[131] After confirming that monitoring is not necessary in the great majority of patients receiving LMWH for prophylaxis or treatment of DVT, he emphasized that presently the question whether monitoring is useful (e.g. to identify the 30% of patients with VTE who experience a recurrence) cannot be addressed adequately. His argument is that the value of the aPTT -or one of its variants- for detecting the effect of heparin is rather limited and has long been questioned.[132, 133] Although significant, the prolongation of the average aPTT following the administration of heparin varies dramatically from patient to patient, making this test of little use to assess the individual response. On the other hand, by reflecting the concentration of the pentasaccharide, the anti-FXa test documents both heparin molecules with anti-factor Xa activity only and those that are large enough to have anti-thrombin activity. With few exceptions (very low-molecular-weight preparations), the best way to determine active heparin in plasma would be to measure anti-thrombin activity.[134-138] However, although suitable,[139] stable in the individual subject, and directly linked to the risk of thrombosis,[140] the thrombin generating capacity in the normal population is highly variable[139] and of little help in clinical practice.

From UHF to pentasaccharide: lessons learned and conclusions.

According to the concept that a scientific discovery "is seldom made by an individual in isolation but often occurs in a community of scholars and their intellectual history or traditions",[141] milestones in the history of HFH, LMWHs and pentasaccharide have been made possible by the efforts of multidisciplinary teams of investigators (chemists, pharmacologists, biologists and clinicians), and the collaboration and competition between different research groups from the academy and the pharmaceutical industry. Technical advances have greatly facilitated the discovery process. The role of the human factor in the discoveries is also acknowledged: the work of McLean – who discovered heparin while searching for procoagulants in dog liver – changed the focus of Howell's research, pointing him the right direction where to investigate. Finally, research in the area of heparin has provided a paradigm of rigorous science growing in the understanding of the coagulation mechanism. The concept "less thrombin= more bleeding" is supported by a

variety of observations. As in anticoagulation, the capacity to form thrombin is diminished in hemophilia and in other rare bleeding disorders (e.g. FVII, FXI deficiency). The risk of spontaneous or traumatic bleeding is abnormally high in such settings, and it is maximal as the amount of thrombin decreases (e.g. the risk in patients with severe vs those with moderate/mild forms of hemophilia). This argues for thrombin that blood can provide at the site of injury as a major determinant of the tendency to bleed.[142] However, no one-to-one relationship exists between the level of the deficient factor (FVII, FVIII, FIX, and FXI), the underlying genetic defect, and bleeding phenotype.[143-145] Accordingly, rather than measurements of individual factors, global tests that estimate the amount of thrombin that can be formed are preferred in clinical practice. However, a laboratory helps to better recognize a bleeding phenotype only when the test employed mimics the *in vivo* condition. Indeed, in FXI deficiency, thrombin generation is not monitored by the aPTT carried out in platelet-poor plasma (i.e. the usual way it is performed). In such setting, the aPTT reliably measures the amount thrombin formed only if the test is carried out in platelet-rich plasma and contact activation is inhibited.[146] In FXI deficiency, a thrombin generation assay carried out in the presence of platelets strongly differentiates between bleeders and non-bleeders. The key role of platelet tissue factor in hemostasis has long been known[147, 148] and has fostered the concept of cell-mediated coagulation.[149] In addition, upon activation, platelets release factor V and expose procoagulant phospholipids.[150]

In the 1980s, it has been shown that UFH and LMWHs (but not pentasaccharide or heparinoids) were potent *in vitro* inhibitors of platelet aggregation in response to collagen and of platelet adhesion to collagen and to ristocetin – a von Willebrand factor-dependent adhesion process, and the interference with platelet adhesion has been thought crucial for the risk of bleeding in the heparinized patient.[151] More recently, the platelet inhibitory activity of some LMWHs has been reported as ability to reduce platelet /leukocyte interactions.[152] While there was no doubt that the final common effect of any heparin was that less thrombin should appear in plasma, it was postulated that this could be achieved by three independent mechanisms: by increasing the effect of AT (the major effect); by increasing the inhibitory effect of thrombin or heparin cofactor II, and by affecting the activation of blood coagulation on the platelet surface. The obvious consequence was that, at least in theory, it was feasible to dissociate the antithrombotic and hemorrhagic effects of heparin and improve its therapeutic potential.[153] In this respect, the high anti-Xa activity of LMWH would be preferred to prevent, whereas the high anti-thrombin activity of UFH would be best for treating thrombosis. After 25 years of clinical trials in the area,

we know that LMWHs inhibit thrombin generation by multiple pathways; that platelets are an integral part of the clotting system, and that both inhibition of FXa and of thrombin are needed in mediating the antithrombotic effect of UFH and LMWHs. By preventing feedback activation of FV and FVIII,[154, 155] thrombin inhibition is the most important target *in vitro*, although inhibition of FXa confers antithrombotic activity as well.[64] We have also learned that, despite inherent limitations, in certain clinical settings, test systems that estimate thrombin generation *in vivo* should be preferred to older tests.[156] In 12 volunteers who received 9,000 IU of four heparins of different molecular weights, the aPTT was significantly prolonged in only 34% of cases, while the measurement of the endogenous thrombin potential revealed the presence of the drug in 80% of the cases.[134] An anti-FXa test was positive in 98% of the samples in this study, confirming the limitations of a test that only reflects the concentration of the pentasaccharide. Finally, similarly to data from patients with inherited rare bleeding disorders, studies with heparin argue for a scheme of coagulation other than the waterfall model. In such alternative network, the key roles of platelets (through their ability to interact with fibrinogen [aggregation] and von Willebrand Factor [adhesion],[157] and their ability to propagate clotting [by means of blood-borne tissue factor]);[158] of vessel wall-derived tissue factor (through its ability to initiate clotting at the site of vascular injury);[159] and of tissue factor-bearing microparticles (especially in the context of inflammation, cancer and thrombosis);[160] of antithrombin(s) and of feedback inhibitors (proteins C and S, tissue factor pathway inhibitor, etc)[161] are acknowledged. While successful work as to non-anticoagulant indications for heparin is going on (see: Appendix 2), forthcoming work with this model is expected to yield novel information for the understanding of the coagulation mechanism; major hints on patients that make better use of the small amount of thrombin they form than others with similar clinical conditions, and directions to be followed to tailor prevention and treatment in haemostasis and thrombosis.[162]

Practice Points:

- Milestones in the history of unfractionated heparin, Low-Molecular-Weight-Heparins and pentasaccharide have been made possible by the efforts of multidisciplinary teams of investigators, and the collaboration and competition between research groups from the academy and the pharmaceutical industry.
- Research in the area of heparin has provided a paradigm of rigorous science growing in the understanding of the coagulation mechanism.
- In addition to anticoagulation, the concept "less thrombin= more bleeding" is now supported by a variety of observations in hemophilia and in other rare bleeding disorders.
- Like data from patients with rare bleeding disorders, studies with heparin argue for a scheme of coagulation other than the waterfall model in which the key roles of platelets; of vessel wall-derived tissue factor and of tissue factor-bearing microparticles; of antithrombin(s), and of feedback inhibitors (proteins C and S, tissue factor pathway inhibitor, etc) are acknowledged.

Research Agenda:

- Forthcoming work with the alternative network model of coagulation is expected to 1) yield novel information as to why some patients make better use of the small amount of thrombin they form than others with similar clinical conditions, and 2) provide directions to be followed to tailor prevention and treatment in haemostasis and thrombosis.
- Despite inherent limitations, in certain clinical settings, test systems that estimate thrombin generation *in vivo* should be preferred to older tests (e.g. the aPTT).

Appendix

1. The history of the discovery of heparin was written by McLean shortly before his fatal illness in 1957, and this account was published after his death (McLean J: The discovery of heparin. Circulation 1959; 19:75-78).

'Howell gave me the problem of determining the value of the thromboplastic substance of the body. He thought this to be cephalin, obtained from brain, but, of course, knew the thromboplastic material from brain to be a mixture - a crude extract, though a powerful thromboplastic agent. He made this by macerating brain tissue, spreading it on glass panes, drying it over a gas flame in an oven, extracting it in ether, decanting, concentrating the ether extract, and finally by precipitation by alcohol. This precipitate was his thromboplastic substance. He used it in blood clotting experiments. It was kept in a glass vessel with ground glass cover (vaselined), as it was observed that access to air decreased its ability to accelerate clotting. In three months, it was decayed. My problem was to determine what portion of this crude extract was the active accelerator of the clotting process and to that end, to prepare cephalin as pure as possible and determine if it had thromboplastic action. I was also to test the other components of the crude ether-alcohol extract. In my reading of the German chemical literature on phosphatides, I found articles describing extracts of heart and liver secured by a process similar to that for obtaining cephalin from brain. Therefore, the products might be brain and liver cephalin, but were named cuorin (from the heart) and heparphos- phatides (from the liver). I suggested this research programme as a logical supplement to the problem Dr. Howell had assigned to me. He had not known about cuorin or heparphosphatides. I prepared cuorin and heparphosphatides and both were brown, not yellow like cephalin and lacked its fishy like smell. Both had a much less accelerating effect on blood coagulation than cephalin. The more the material was "purified" (ether extract into hot alcohol), the weaker the thromboplastic activity became. The same process of extraction was used for brain, heart and liver. Yet in the brain the end product was almost all cephalin, but in the heart and especially in the liver it was something else which was mixed with cephalin. Many batches were made of both cuorin and heparphosphatide which were tested from time to time to determine whether or not the extract lost its thromboplastic activity as did that of the brain. If I had not saved them, I would probably not have found heparin. This was a fortuitous decision. The various batches were tested down to the point of no thromboplastic activity, but two of those first prepared appeared not only to have lost their thromboplastic activity but actually to retard slightly the coagulation. I had in mind, of course, no thought of an anticoagulant, but the experimental fact was before me; and I retested again and again until I was satisfied that an extract of liver (more than heart) possessed a strong anticoagulant action after its contained cephalin had lost its thromboplastic action.'

2. Future Considerations: Heparin in the Third Millennium

Optimizing the synthesis of the pentasaccharide. The case of worldwide distribution of contaminated heparin in 2007, which caused hundreds of patient deaths in the US, raised concerns over the reliability and safety of animal-sourced heparins.[3] On the other hand, vis-à-vis challenges in product quality control in the preparation process of UFH and LMWHs, easy controls of product quality -in terms of reproduction and reliability- are conceivable for a pure synthetic product -such as pentasaccharide- with a longer half-life and a low IC_{50} value as compared to UHF and LMWHs.[4] A highly efficient approach to synthesize a homogeneous pentasaccharide appeared thus key for clinical applications. Analogues of the pentasaccharide were synthesized, and structure–activity relationship studies revealed that essential sulfate and carboxylate substituents were located at opposite sides of the pentasaccharide molecule.[5] This allowed to improve and optimize the multistep production of fondaparinux. As reviewed in,[6] an effective protecting group strategy for O-sulfation and selective N-sulfation, as well as for stereoselective glycosylation were established. The 1-benzene sulfinyl piperidine/triflic Anhydride -mediated promoter system contributed to coupling disarmed thioglycosides of idouronate with appropriate glucosazide building blocks. The convergent [3+2] coupling approach also improved the use of the difficult to prepare l-iduronic acid building block. Finally, liquid chromatography and nuclear magnetic resonance technology were developed to monitor the process of total fondaparinux synthesis. The optimized synthetic strategy to control the stereochemical configuration and improve the yield of the glycosylation, and the availability of standardized methods to monitor the process of total synthesis of fondaparinux, allowed to overcome current limitations of UFH and LMWHs.

Oral heparin. UFH LMWHs and pantasaccharide need to be administered parenterally. Parenteral administration results in low patient compliance in chronic settings. Oral formulations may be a better option in such cases. In the last decade, three direct oral FXa inhibitors (Rivaroxaban, Apixaban, Edoxaban) have reached the market. Despite pharmacokinetic and pharmacodynamic similarities with LMWHs (see Appendix, Table 1A), their clinical use is limited to certain settings and is not expected to be extended to other major indications of heparin and LMWHs (e.g. pregnancy). Various groups have thus focused their attention on the development of easy to use, non-toxic, effective oral heparin formulations for continued anticoagulant therapy in chronic conditions. The hope that oral heparin complexes[7] or liposome-encapsulated heparin[8] would be available became feasible in the late 1980's. However, due to large molecular weight and high negative charge density, oral bioavailability of heparin was limited and insufficient to provide the

desired therapeutic effects. Common strategies currently used to improve the bioavailability of heparin by oral route are based on improved heparin lipophilicity; heparin protection from the acidic gastric pH; enhanced (gastric) cell-membrane permeabilization, and modification of the tight-junctions. Non-α aminoacids; sodium caprate; polycationic dendron; thiolated polymers poly(acrylic acid)-cysteine and glutathione; N-sulfonato-N,O-carboxymethyl chitosan (SNOCC); or 18-b glycyrrhetinic acid, have been successfully evaluated as oral permeability enhancers for UFH or LMWH conjugated with Deoxycholic acid (DOCA).[9] A significant prolongation of the plasma aPTT and an increase in anti-Xa activity has been reported for some of these approaches.[10,11] Polymeric nanoparticles such as biodegradable, poly-e-caprolactone (PCL) and poly (lactic-co-glycolic acid) (PLGA), and non-biodegradable positively charged polymers (Eudragit RS and RL) have been also successfully evaluated for the oral delivery of heparin.[12] Such nanoparticles are taken up by the M cells of the Peyer's patches of the intestinal tract, the major gateway through which heparin loaded nanoparticles are absorbed. The carrier molecule sodium N-(8 [2-hydroxybenzoyl] amino) caprylate, or SNAC when combined with UHF markedly increases the gastrointestinal absorption of the drug. Following absorption, oral heparin-SNAC appeared to be hemostatically active *in vivo*.[13] An international, multi-center phase III randomized, double-blind double dummy (placebo oral or injection) thromboprophylaxis trial in 2,264 patients with the objective to compare safety and efficacy of two liquid formulations of heparin-SNAC to a standard subcutaneous LMWH regimen (PROTECT trial) in patients undergoing elective hip surgery was carried out.[14] Oral heparin/SNAC solution, low dose 60,000 IU/1.5 g SNAC (ldSNAC) and high dose 90,000 IU/2.25 g SNAC (hdSNAC) were administered trice daily. Oral heparin prophylaxis was initiated 4–6 hours postoperatively and continued through the whole evaluation period (27–30 days), while subcutaneous LMWH (enoxaparin) 30 mg twice daily was initiated 12–24 hours postoperatively and was administered for 10 days followed by an identical subcutaneous placebo regiment (double dummy) for up to a total of 27 – 30 days until the final evaluation. The primary end point was to demonstrate superiority of oral heparin over s.c enoxaparin in reducing the DVT rate as detected by bilateral ascending contrast venography at day 27–30. This study did not meet its primary end point, likely due to a suboptimal dosage form and a poorly tasting liquid formulation. However, it first documented -in a large patient population- that oral heparin can reduce the frequency of postoperative VTE with low frequency of bleeding complications in patients undergoing total hip replacement surgery.[15] No oral formulation of heparin has yet reached the market. Specific open issues concerning the need for protection of heparin from

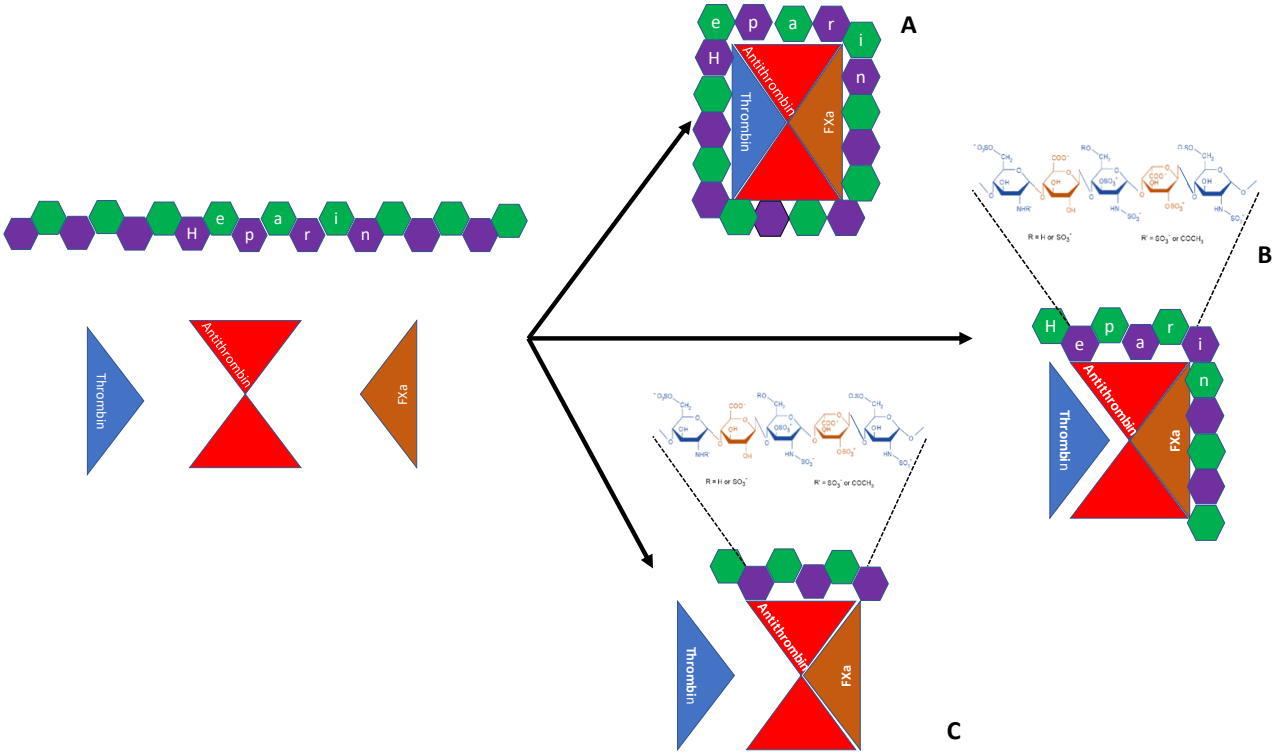
bacterial degradation before its oral absorption; the solubility in a gastric medium (for chitosan) and potential toxicological considerations (for several penetration enhancers) might have hampered the research and development of oral heparin. Newer nanotechnological carriers for oral delivery of heparin might have additional benefits over and above current enhancer strategies and allow for combined approaches. LMWH conjugates with lipids and incorporation of the conjugate into newer nanotechnological carriers is expected to improve bioavailability and help develop future therapeutic options in the area.

Ultra-low molecular weight heparins. The successful process leading to the synthesis and the development of the pentasaccharide (Mr 1728 as he sodium salt), has encouraged the search for less expensive directions to be explored (new heparin depolymerization, chemo-enzymatic processes) to produce and develop ultra-low molecular weight heparins to be employed as drugs. The development of a fully O-methylated O-sulfated Fondaparinux analogue, Idraparinux (once-weekly dosing) was discontinued in phase III trials maybe due to intracranial bleeding. [16] A biotinylated formulation, Idrabiotaparinux, was further clinically investigated.[17] Semuloparin (AV5026) is a ultra-low molecular weight heparin (Mw 2.4 kDa, corresponding to ≈8 disaccharide units), obtained through a modified Enoxaparin depolymerization process that protects the pentasaccharidic sequences.[18] Semuloparin has been successfully evaluated in Phase III/IV trials.[19,20] Finally, using a recombinant version of the heparan biosynthesis enzymes, active oligosaccharides from hexa- up to dodecasaccharides have been obtained by a chemo-enzymatic approach starting from the bacterial polysaccharide K5.[21]

Heparin as a non-anticoagulant agent. Information concerning oxidized AT as a dual inhibitor of coagulation and angiogenesis (Table 2A) combined with a better understanding of the chemistry and biology of carbohydrates -including information on the anti-inflammatory effect of heparin and related compounds-, prompted the search for therapeutic applications of heparin and its derivatives other than the AT/anti-Xa selectivity.[22] In addition to being beneficial in sepsis,[23] non-anticoagulant heparin has been found to be useful in a variety of clinical settings (e.g. acquired immunodeficiency syndrome, adult respiratory distress syndrome, allergic encephalomyelitis, allergic rhinitis, arthritis and asthma) including inhibition of tumor growth and metastasis (Table 2A). In keeping with this, research on glycosaminoglycans and the identification of specific oligosaccharide sequences, has prompted investigators to develop newer drugs with molecular structures that closely resemble parts of the heparin polymeric chain (e.g. selectively O-desulfated heparin; heparintetrasaccharide, pentosan polysulfate, phosphomannopentanosesulfate). Whether

this is the beginning of a new era to move the heparin field forward is unknown so far and deserves to be analysed thoroughly.

Figure 1. Antithrombin-Mediated Inactivation of Thrombin and/or of Factor Xa by heparin fragments of different chain lengths.



Legend to Figure.

The interaction of unfractionated Heparin (UFH) or Low-Molecular-Weight Heparins (LMWH) with antithrombin is mediated by the pentasaccharide sequence of the drugs. Binding of either to antithrombin causes a conformational change at its reactive centre that accelerates its interaction with factor Xa. Consequently, both UHF and LMWH catalyze the inactivation of factor Xa by antithrombin. In contrast to factor Xa inhibition, catalysis of antithrombin-mediated inactivation of thrombin requires the formation of a ternary heparin–antithrombin–thrombin complex. This complex can be formed only by heparin fragments ≥5,400 Da or with a chain length >18 saccharides units (including the pentasaccharide sequence). Heparin fragments below such critical chain length (or with mean molecular size < 5,400 Da) inhibit FXa and cause significantly less thrombin inhibition. The ternary complex formation is depicted in **A;** inhibition of FXa by fragments below the critical chain length is shown in **B.** Interaction of FX alone by pentasaccharide is reported in **C**.

Table 1. Teams that pioneered studies on the mechanism of action of heparin[§].

Group Leader /Study Location	Key Contributions
U. Lindahl University of Uppsala (Sweden)	-Separation of high-activity and low-activity heparin species -Characterization of the AT-binding sequence - Acceleration by heparin of the reactions between AT and thrombin or FXa (ternary complexes)
R.R. Rosenberg Harvard Medical School, Boston (USA)	-Purification and mechanism of action of AT-heparin cofactor -Separation of active and inactive forms of heparin. -Multiple functional domains of the heparin molecule -Effect of heparin and heparin fractions on platelet aggregation
E.A. Johnson National Institute for Biological Standards and Control London (UK)	-Anti-Xa potentiating effect of heparin after subcutaneous injection -Anticoagulant properties of heparin fractionated by affinity chromatography
L.O. Andersson AB Kabi, Stockholm (Sweden)	-Anticoagulant properties of heparin fractionated by affinity chromatography
J. Choay Choay Institute Paris (France)	-Role of the AT-binding pentasaccharide in heparin acceleration of AT-proteinase reactions -The structure of heparin oligosaccharide fragments with high anti-FXa activity containing the minimal AT binding sequence -Synthesis of heparin fragments with high affinity for AT -Anti-Xa active heparin oligosaccharides
B. Casu G. Ronzoni Institute for Chemical and Biochemical Research, Milan (Italy)	-Heparin oligosaccharide fragments with high anti-FXa activity -Structure-activity relationship of heparins

§ See text for the reference(s) of each finding.

Table 2: Commonly used LMWHs: Preparation methods and pharmacodynamic heterogeneity*¥

Generic Name #*+	MW (Da) Average/Range°°	Anti Xa/IIa Ratio	Sulfate/ carboxyl ratio#	Relative potency: Concentrations of each LMWH (anti Xa IU/ml) needed to:		Preparation	
				decrease by 50% thrombin generation	double the lag time preceding thrombin generation	Preparation method°	Treatment/ Major Reagent employed
Enoxaparin	4,500 3,500-5,500	3.9	~2	0.58	0.62	Benzylation followed by alkaline hydrolysis	Alkaline treatment
Tinzaparin	6,500 5,600-7,500	1.6	1.8-2.5	0.28	0.35	Controlled heparinase digestion	Heparinase
Nadroparin	4,300 3,600-5,000	3.3	~2	0.75	0.80	Deaminative cleavage	Nitrous acid
Dalteparin	6,000 3,600-6,400	2.5	2.0-2.5	0.65	0.65	Deaminative cleavage	Nitrous acid
Reviparin	4,400 4,500-5,000	4.2	2.5	-	-	Deaminative cleavage	Nitrous acid
Certoparin	5,400 6,000-6,700	2.4	-	-	-	Deaminative cleavage	Isoamyl nitrite
Parnaparin	5,000 4000-5800	2.3	2.0-2.6	-	-	Oxidative depolymerization	$Cu^{2+} + H_2O_2$
Bemiparin	3,600 3000-4200	9.7	-	-	-	Deaminative cleavage	Nitrous acid

°°*Mw* from monograph in European Pharmacopoeia.

**see: Semin Thromb Hemost. 1999;25 Suppl 3:5-16; Thromb Haemost. 2008;99(5):807-18, and Thromb Haemost. 2012;107(2):201-14 for further details.*

§ Each LMWH agent has its own MW and biological activity profile; thus, LMWHs cannot be used interchangeably (unit-for-unit) with heparin or with each other;

°Different LMWHs are prepared by different depolymerisation methods and have different pharmacodynamic and PK properties;

+ half-life of anti-Xa activity after SC injection varies from 180 min (reviparin) to 275 min (enoxaparin);

#The degree of sulfation and localization of the sulfate residues varies from one to another LMWH and determines the spectrum of activity of the product.

¥Routine monitoring of LMWHs (using anti-Xa activity assay) is not required in clinically stable patients receiving VTE prophylaxis or treatment; it may be considered in certain clinical settings (see text).

Table 3. Clinical settings in which LMWH(s) have been tested^*

Indication	Patient risk status	Setting	LMWH(s) tested						
			Enoxaparin	Nadroparin	Dalteparin	Reviparin	Parnaparin	Bemiparin	Tinzaparin
Prophylaxis in orthopaedic surgery	*No additional risk factors°*	*In-patients*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*
Prophylaxis in major general or gynecological surgery for benign disease or cancer	*Moderate/high risk*	*In-patients*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*
Prophylaxis in medical patients	*Additional risk factors°°*	*In-patients*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*
Prophylaxis of deep vein thrombosis in malignancy (KHORANA score >3)	*high-risk*	*ambulatory*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*	*yes*
Prophylaxis in critically ill patients	*high-risk*	*In-patients*	*Yes*	*Yes*	*Yes*	*no*	*no*	*Yes*	*no*
Prophylaxis of superficial thrombophlebitis	*No additional thromboembolic risk factors*	*Ambulatory*	*Yes*	*Yes*	*no*	*Yes*	*Yes*	*no*	*Yes*
Prophylaxis in ischemic stroke	*high-risk*	*In-patients*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*	*no*	
Prophylaxis in trauma (e.g. spinal cord injury)	*high-risk*	*In-patients*	*Yes*	*Yes*	*Yes*	*Yes*	*no*	*Yes*	*Yes*
Prevention of coagulation in haemodialysis	*No additional thromboembolic risk factors*	*ambulatory*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*
Prophylaxis in pregnancy, puerperium and in high-risk women	*Additional risk factors°°*	*Ambulatory, in-patients*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*
Open urologic surgery; bariatric surgery; major thoracic surgery; coronary artery bypass grafts.	*No additional thromboembolic risk factors*	*In-patients*	*Yes*	*Yes*	*Yes*	*no*	*no*	*no*	*no*
Major vascular surgery, laparoscopy, burn victims	*Additional thromboembolic risk factors*	*In-patients*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*	*no*	*no*
Bridging treatment of VTE after withdrawal of anti-vit-K drugs (surgery or invasive manoeuvres)	*Additional risk factors°°*	*Ambulatory, in-patients*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*
Prophylaxis/treatment of paediatric vein and arterial	*high-risk*	*Ambulatory, in-patients*	*Yes*	*Yes*	*Yes*	*Yes*	*no*	*no*	*Yes*

thrombosis. Prevention of central/peripheral catheter vein thrombosis									
Treatment of acute myocardial infarction	*high-risk*	*In-patients*	*Yes*	*Yes*	*Yes*	*Yes*[§]	*Yes*	*no*	*Yes*
Treatment of acute coronary syndrome	*No additional risk factors*	*In-patients*	*Yes*	*Yes*	*Yes*	*Yes*[§]	*Yes*	*no*	*Yes*
Treatment of acute deep venous thrombosis	*No additional thromboembolic risk factors*	*Ambulatory, in-patients*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*
Treatment of VTE/pulmonary embolism	*No additional thromboembolic risk factors*	*In-patients, ambulatory*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*

^The first randomised trial of heparin was carried out in 1960 (Barritt, D.W. and Jordan, S.C.: Anticoagulant drugs in the treatment of pulmonary embolism; a controlled trial. Lancet, 1, 1309–1312). Patients with pulmonary embolism were randomized to heparin + nicoumalone or to no anticoagulation. Of the 16 patients randomised to anticoagulation none died from pulmonary embolism and there were no non-fatal recurrences. Of the 19 patients randomised to no treatment, five died from pulmonary embolism and there were five non-fatal recurrences.

*See updated ACCP (American College of Chest Physicians); ACC (American College of Cardiology) and AHA (American Heart Association) Guidelines for details.

§ Prevention of acute events in percutaneous transluminal coronary angioplasty (PTCA);

°the presence of additional risk factors reported only in arthroscopic knee surgery patients

°°Bed rest + additional risk factors (history of VTE, sepsis, congestive heart failure, inflammatory bowel disease; acute neurologic disease; severe respiratory disease).

Table 1a.

Direct anti-Xa inhibitors: Comparative pharmacokinetics and pharmacodynamics°

Variable	Apixaban	Rivaroxaban	Edoxaban
Target	Factor Xa	Factor Xa	Factor Xa
Dose (mg/day)	Fixed (2.5-5)	Fixed (15-20)	Fixed (30-60)
Frequency of administration	Twice daily	Once daily	Once daily
Anticoagulation monitoring°	Anti-Xa *(Plasma calibrators needed)* Rotachrom	Anti-Xa *(Plasma calibrators needed,)* Rotachrom	Anti-Xa *(Plasma calibrators needed)* Rotachrom
Plasma concentrations†	470 ng/ml	141-173 ng/ml	303 ng/ml
Vd (L/kg)	21	50	107
T_{max}, hours	3-4	2-4	1-3
Transport proteins	P-gP, BCRP	P-gP, BCRP	P-gP
$T_{1/2}$, hours	12 (8-15)	5-9 (young); 11-13 (elderly)	10-14
Plasma clearance L/hrs)	3	10	21.8
Plasma protein binding	87%	92-95%	55%
Bioavailability	50%	66% without food, ~100% with food	62%
Elimination Kinetics	Biphasic	First-order	Biphasic
Liver metabolism	CYP3A4 (25%)	CYP3A4 (~66%)	CYP3A4 (<4%)
Renal Elimination § *With normal renal function*	27%	66% (half as inactive metabolite)	35-50%
Antidote°	Andexanet Alfa*	Andexanet Alfa*	Andexanet Alfa*
Absorption with H2B/PPI	No effect	No effect	No effect
Gastro-intestinal tolerability	Good	Good	Good
Effect of Body weight	Exposure: -30% increase in subjects <50 kg -30% decrease in subjects >120 kg	Exposure: -25% increase in subjects <50 kg -25%decrease in subjects >120 kg	Exposure: increase in subjects <60 kg
Effect of age	AUC: 32% increase in subjects ≥65 yrs	AUC: 50% increase in subjects ≥65 yrs	None
Effect of food	No effect (Intake with food discouraged)	Mean AUC increases to ≈40% (Intake with food mandatory)	No effect (Intake with food: no official recommendation)
Effect of gender	18% higher exposure in females	None	None

In pregnancy	Contraindicated	Contraindicated	Contraindicated
Interactions with drugs	Strong P-gp and CYP3A4 inhibitors and inducers	Strong P-gp and CYP3A4 inhibitors and inducers	Strong P-gp inhibitors

Abbreviations: CYP=cytochrome P450; P-gP=P glycoprotein; BCRP=breast cancer resistance protein. Vd=Volume of distribution

* (r-Antidote, PRT064445; Portola Pharmaceuticals), recombinant truncated enzymatically inactive factor Xa which reverses the anticoagulant action of factor Xa inhibitors.

§ Frequent laboratory monitoring needed if creatinine clearance is 20-50 mL/min). DOACs are contraindicated if creatinine clearance is < 20 mg/dl.

[†] Maximum concentration.

° Anti-Xa is the monitoring system employed for LMWHs too

°See: Blood Rev. 2017 Jul;31(4):193-203 for further details on this issue.

Table 2A. Future Considerations and Perspectives: The use of heparin as a non-anticoagulant agent.

Clinical setting	Suggested readings
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Suggested readings for the Appendix 2
Future Considerations: Heparin in the Third Millennium

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