

APPENDIX B

Pharmacological Study oligosaccharide (2004)
Final Report

COMPARATIVE *IN VITRO* STUDY OF THE EFFECT OF LMWH
(ENOXAPARIN) AND OLIGOSACCHARIDES ON FACTOR VIIa
GENERATION AND PROTHROMBIN ACTIVATION AFTER
TRIGGERING TISSUE FACTOR CLOTTING PATHWAY IN HUMAN
WHOLE BLOOD

Beginning of investigation : October 2004
End of investigation : December 2004
Draft Report : December 9th, 2004
Preliminary Report : December 2004
Final Report : February 2005

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1. Summary

It has been shown that FVIIa plays an important role in arterial thrombogenesis and increased levels of FVIIa have been observed in several hypercoagulable states. Thus, the inhibition of FVIIa generation seems to have a special interest in the design of the antithrombotic treatment. Increased levels of prothrombin fragments 1+2 (F₁₊₂) assigning increased thrombin generation have also been associated to thrombotic events occurrence. In a previous study, we have shown that the synthetic pentasaccharide, a LMWH (enoxaparin) and unfractionated heparin significantly inhibited FVIIa generation and prothrombin activation during *in vitro* clotting of human plasma. In this study, we propose to evaluate *in vitro* the effect of 12 synthetic oligosaccharides and a LMWH (Enoxaparin) on the generation of either FVIIa or F₁₊₂ after triggering tissue factor pathway in human normal plasma and whole blood, respectively.

Oligosaccharides concentrations ranging between 2.5 and 10 µg/ml significantly inhibit FVIIa generation 60 min after triggering coagulation. Interestingly, we have not observed a clear concentration depending effect on the inhibition of FVIIa generation. However, decreasing the oligosaccharides concentration to 1.25 and 0.625 µg/ml, the inhibitory effect on FVIIa generation also decreases. A dose effect is observed for some compounds. At higher concentrations there is plateau and no further inhibitory effect observed.

The potency of the inhibitory effect on prothrombin activation allows us to classify the oligosaccharides in three different groups: weakly active (15), moderately active (1, 2, 4, 5, 6, 8, 10 and 11) and highly active (9, 12,13 and 14). The oligosaccharide n°14 presents the highest effect on both FVIIa generation and prothrombin activation.

In conclusion, based on the activity of these blindly studied oligosaccharides and regarding the parameters (FVIIa generation, prothrombin activation) we determined their relative potency and we classified the most active product at the lowest concentration as follow: 14>13>12>10=9=11>8=6=5=4=2=1>15.

2. Aim of the study

It has been shown that FVIIa plays an important role in arterial thrombogenesis leading to the concept that FVIIa is an important target for an antithrombotic strategy. The clinical relevance of this factor is of great interest. The tissue factor (TF) pathway is preponderant *in vivo* in the initiation of blood coagulation in normal haemostasis and in thrombotic states [1]. Factor VII (FVII) is a serine protease zymogene which is converted by limited proteolysis into activated factor VII (FVIIa) - a two chain serine protease. In steady state conditions, traces of FVIIa are present in normal plasma whereas FVIIa levels increase in thrombotic states [2, 3]. The blood coagulation process is initiated when cryptic TF is exposed to circulating blood and binds to plasma factor VIIa triggering the thrombin generation process [4]. Thrombin generation can be described as occurring in two consecutive phases (reviewed in 5 and 6). In the initiation phase, FVIIa/TF complex activates factor IX (FIX) and factor X (FX) [7, 8]. The free FXa initially produced generates picomolar amounts of thrombin which induce an initial platelet activation. Additionally, FXa cleaves FIX and generates FIX α , which is also the intermediate product following the proteolytic cleavage of FIX by either FVIIa/TF complex or FXIa [9, 10]. The propagation phase is characterized by further activation of platelets by thrombin, which also activates FVIII, FV and FXI. Formation of the enzymatic complex (intrinsic tenase), composed by FIXa, FVIIIa, negatively charged phospholipids and calcium ions leads to further activation of FX, which forms in the presence of FVa, phospholipids and calcium ions the prothrombinase complex. Prothrombinase is the activator of prothrombin (FII) and induces a burst of thrombin generation. During the coagulation process, feedback activation of FVII zymogene is induced principally by FXa, FIXa and FVIIa/TF complex as well as by FXIa and FXIIa [11, 12, 13]. After triggering the TF pathway, the contribution of FX, FIX and FXI to the activation of FVII was significantly more important than that of any other serine protease of blood coagulation [14].

Since the amount of generated serine-protease is amplified at each step downstream of the coagulation process, inhibition of the generation and the activity of serine proteases located at the initial steps of blood coagulation may result in a decrease of thrombin generation. FXa is located at a critical point of the coagulation process, but upstream as compared to thrombin.

In a previous study, we have shown that the synthetic pentasaccharide, a LMWH (Enoxaparin) and unfractionated heparin significantly inhibited FVIIa generation and prothrombin activation during *in vitro* clotting of human plasma [15]. More recently, we demonstrated that the synthetic pentasaccharide (as well as Enoxaparin) enhances the

antithrombin (AT)-mediated inhibition of FXa and FIXa, resulting in a significant reduction of FVIIa generation [^{xiv}]. Inhibition of FIXa by the synthetic pentasaccharide (fondaparinux) has also been recently shown by the group of J Weitz [^{xvi}].

The inhibition of FVIIa generation seems to have a special interest in the design of the antithrombotic treatment for the following reasons:

- Recently, it has been shown that FVIIa is in competition with the zymogene FVII which acts as an inhibitor of thrombin generation. An increase of FVIIa formation rate saturates the available TF molecules and accelerates thrombin generation [^{xvii}]. This has been confirmed by clinical and experimental data using recombinant FVIIa.
- Increased levels of FVIIa have been observed in several hypercoagulable states.
- Enoxaparin administration in patients with unstable angina (with high plasma FVIIa and prothrombin F₁₊₂ levels) leads to rapid reduction (about 60%) of both FVIIa and F₁₊₂ [^{xviii}].
- Phases II and III clinical trials showed that specific inhibitors of FVIIa (i.e. NAPc₂ and ASIS) have an important antithrombotic effect.

The molecular structure of FVIIa is known with a better understanding of its catalytic domain [^{xxi}]. Studies published by Rao et al have shown that FVIIa /TF complexes are inhibited by heparin in the presence of AT (22; 23). In a previous study, our group has demonstrated that Enoxaparin and Pentasaccharide are able to inhibit *in vitro* generation of FVIIa [^{xv}]. More recently, we have shown the inhibition of FVIIa generation and prothrombin activation in unstable angina patients treated with Enoxaparin [^{xx}]. Consequently, the evaluation of the inhibitory potential of this class of antithrombotic drugs on the regulation of FVIIa generation is of special interest.

All these data lead us to study more precisely the possible relationship between the structure of various components of Enoxaparin and their potential effect on FVIIa generation.

We propose to compare *in vitro* the effect of 12 synthetic oligosaccharides and a gold standard LMWH (Enoxaparin) in two sets of experiments one on the generation of FVIIa and the second on prothrombin activation through F₁₊₂ measurement after triggering TF pathway in human normal plasma and whole blood, respectively.

3. Materials and methods

3.1 Compounds

12 oligosaccharides (powder) and Enoxaparin coted (1, 2, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14 and 15) were blindly provided by Aventis (the identity of each product is unknown). The weight of each product was not mentioned. Thus, we have determined the weight of each oligosaccharides as explained in table I.

Human recombinant TF (RecombiPlasTin, HemosIL™) was obtained from Instrumentation Laboratory (Italy), CaCl₂ 0.025M was from Diagnostica Stago (France), EDTA, CaCl₂, TRIS HCl were from Sigma (France).

3.2 Blood samples

Blood was drawn from 3 healthy donors, with no history of thrombotic or haemorrhagic disease, and who have not taken any medication within two weeks prior to the study. Blood samples were collected into siliconized Vacutainer tubes (Becton Dickinson, Meylan, France) containing 1:9 volume of buffered trisodium citrate (3.8%).

3.3 Experimental protocol

3.3.1 Kinetics of inhibition of FVIIa generation

3.3.1.1 Tissue factor pathway activation

Factor VIIa levels were measured in serum prepared after TF pathway activation as follows: to one volume of normal human PPP (pool from our laboratory), thawed at 37°C, is added one volume of diluted (1:250) non calcified thromboplastin (TP). After a 3 min incubation period, coagulation is triggered by adding one volume of CaCl₂ solution (0.025 M). The procedure is performed in plastic (polystyrene) tubes to avoid intrinsic pathway activation.

Normal PPP was spiked with increasing concentrations of studied oligosaccharides (0.625, 1.25, 2.5, 5 and 10 µg/ml) or Enoxaparin or saline (control). The concentration of the studied compounds was expressed on gravimetric basis.

3.3.1.2 inhibition of FVIIa generation

In previous studies, we have shown that the level of FVIIa generated one hour after triggering coagulation was significantly decreased and well correlated with the final concentration of unfractionated heparin, LMWH and Pentasaccharide. Thus, the inhibition of FVIIa generation is studied as follows: 60 min after CaCl₂ addition the clot is discarded by winding it onto a plastic spatula and the remaining liquid is immediately aliquoted and frozen at -20°C. FVIIa levels were determined with the one-stage clotting assay using recombinant thromboplastin (TF1-218) truncated to interact only with FVIIa (Staclot FVIIa-rTF; Diagnostica Stago, Asnières, France), with clotting times determined by chronometric method. The Star4[®] (Stago) was used to perform the clotting time determination.

3.3.2 Kinetics of inhibition of F₁₊₂ generation

To study the kinetics of prothrombin F₁₊₂ generation after TF activation in whole blood, we developed an original method [xv]. Briefly, 100 µl of normal citrated whole blood were mixed with 10 µl of saline (control) or various concentrations of studied oligosaccharides. They were mixed with 25 µl of diluted RecombiPlasTin. The final concentration of thromboplastin used in the plasma was 1:3200. Then, coagulation was triggered by adding CaCl₂ (0.1 M). The experimental procedure was performed in plastic (polystyrene) tubes at 37°C. Before (t₀) and at various intervals after CaCl₂ addition (t₃, t₆, t₉, t₁₅ and t₆₀ min), 50 µl of serum were mixed with 200 µl of buffer containing 0.1 M NaCl, 0.05 M TRIS HCL, 1% (v:v) BSA and 100 mM EDTA (pH 7.4). After quenching, samples were centrifuged during 10 min at 2500g and the sera were frozen at -20°C until F₁₊₂ dosage.

Prothrombin F₁₊₂ levels in serum were determined using the commercially available ELISA kit (Enzygnost F₁₊₂micro, Dade-Behring, Germany Marburg).

The following parameters were analyzed:

- i) Lag time (Lt, min), arbitrary chosen, corresponds to the time at which F₁₊₂ level reach 10 nM.
- ii) maximal velocity of prothrombin F₁₊₂ activation (Vmax, ΔnM/min).
- iii) maximal level of prothrombin F₁₊₂ levels in the serum (Cmax, nM).

3.4 Statistical analysis

Results are reported as mean ± SD. A student's t test was used to assess the statistical significance. Statistical significance was accepted for p values < 0.05

4. Results

4.1 inhibition of FVIIa generation

In the first series of experiments and in the presence of 2.5 µg/ml final concentration all the products significantly inhibit FVIIa generation determined by measuring the amount of FVIIa present in the serum obtained 60 min after triggering coagulation. At this concentration the oligosaccharides n° 12, 13 and 14 seem more active than the remaining ones. Besides oligosaccharide n°15, increasing the oligosaccharide concentrations (5 and 10 µg/ml) did not lead to further significant decrease of FVIIa generated level (figure 1). However, decreasing the oligosaccharide concentrations (1.25 and 0.625 µg/ml) significantly reduce the inhibitory effect on FVIIa generation (figure 2).

Comparing the inhibitory effect on FVIIa generation at the lowest concentration used (0.625µg/ml) the oligosaccharides could be classified as follow 14>13>12>10, 9>11>15.

4.2 Kinetics of inhibition of F₁₊₂ generation

The effect of each oligosaccharide at different concentrations (2.5, 5 and 10 µg/ml) on the kinetics of F₁₊₂ generation was reported in table II and figure 3. The inhibition of prothrombin activation in the presence of each oligosaccharide was evaluated by calculating the lag-time (Lt) prolongation and the percentage of inhibition of either Vmax and Cmax compared to the control (Figure 3). Following these parameters, we can classify the studied oligosaccharides in three different groups: weak activity (n°15), moderate activity (n°1, 2, 4, 5, 6, 8, 10 and 11) and high activity (n°9, 12, 13 and 14). The oligosaccharides n°9, 10, 11, 13 and 14 significantly decrease the Cmax whether the others have a limited, if any, effect on the Cmax.

Oligosaccharide	1	2	3	4	5	6	7	8	9	10	11	12	
1	2.670	2.694	2.731	2.685	2.711	2.700	2.600	2.664	2.673	2.716	2.624	2.671	2.611
2	2.660	2.684	2.726	2.680	2.706	2.690	2.590	2.658	2.663	2.706	2.624	2.667	2.606
3	10	10	5	5	5	10	10	5	10	10	10	5	5
4	20	20	10	10	10	20	20	10	20	20	20	10	10

Table I. Weight of studied oligosaccharides. *The oligosaccharides were dissolved in 500 µl of saline and diluted to obtain a final concentration of 100 µg/ml. Thus, oligosaccharides labelled 1,2,6,8,10,11 and 12 were diluted at 1:200 and remaining oligosaccharides were diluted at 1:100.*

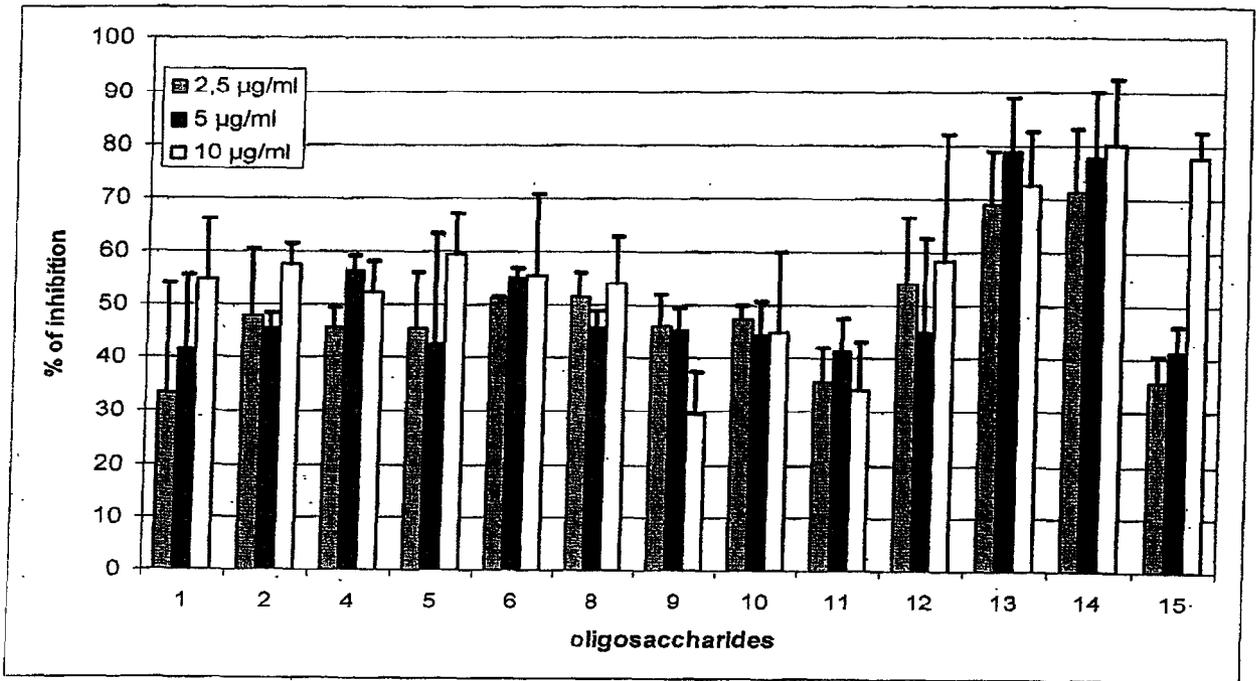


Figure 1. Percentage of FVIIa inhibition in the presence of different oligosaccharides at high concentrations (2.5, 5 and 10 µg/ml). Mean ± SD (n=3).

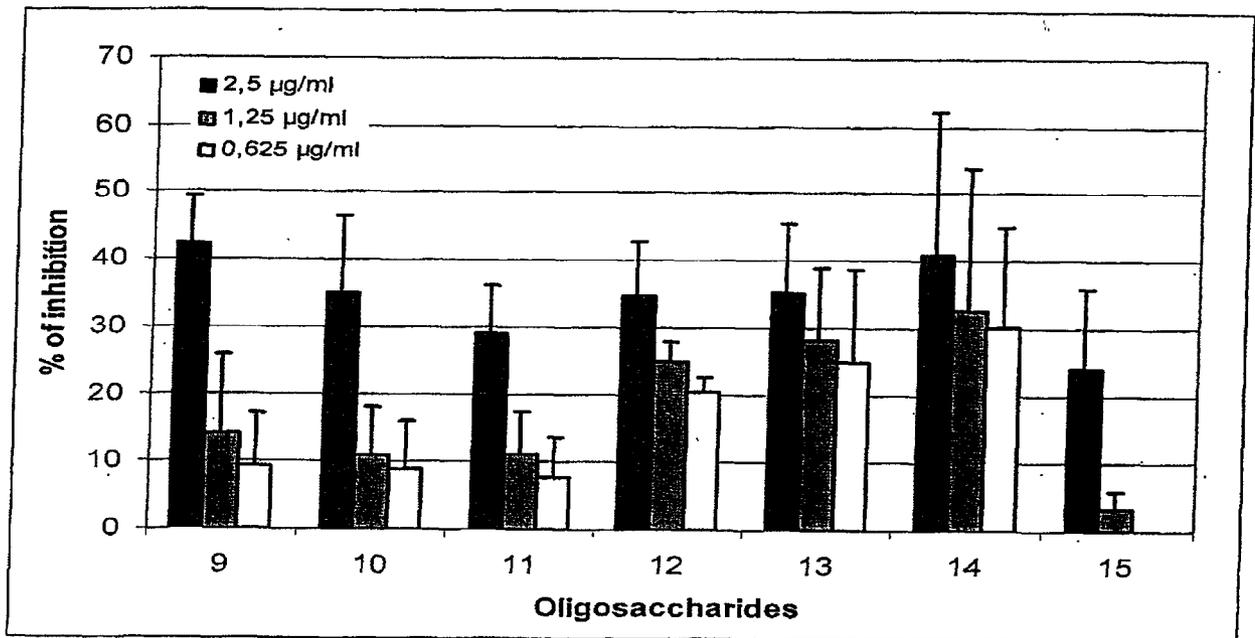


Figure 2. Percentage of inhibition of FVIIa in the presence of different oligosaccharides at low concentrations (2.5, 1.25 and 0.625 µg/ml). Mean ± SD (n=3).

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oligosaccharides	Concentration	Lt (min)	Vmax (nM/min)	Cmax (nM)
control	0 µg/ml	3.10 ± 0.2	112 ± 36	1066 ± 107
1	2.5 µg/ml	3.10 ± 0.2	75 ± 20*	996 ± 339
	5 µg/ml	3.23 ± 0.3	68 ± 25*	929 ± 255
	10 µg/ml	3.60 ± 0.4	59 ± 6*	989 ± 371
2	2.5 µg/ml	3,37 ± 0.5	67 ± 7*	814 ± 152
	5 µg/ml	3,77 ± 0.6	64 ± 17*	805 ± 101
	10 µg/ml	5,07 ± 1.5	47 ± 23*	830 ± 202
4	2.5 µg/ml	3,87 ± 0.6	71 ± 14*	875 ± 181
	5 µg/ml	4,27 ± 0.6*	52 ± 16*	839 ± 318
	10 µg/ml	6,17 ± 0.8*	27 ± 26*	728 ± 200*
5	2.5 µg/ml	3,10 ± 0,14	56 ± 1*	730 ± 141
	5 µg/ml	3,50 ± 0,71	62 ± 7*	766 ± 122
	10 µg/ml	4,10 ± 1,27	51 ± 14*	749 ± 132
6	2.5 µg/ml	3,40 ± 0,14	72 ± 29	761 ± 170
	5 µg/ml	3,40 ± 0,14	84 ± 28	867 ± 320
	10 µg/ml	3,75 ± 0,35	35 ± 22*	690 ± 186
8	2.5 µg/ml	4,17 ± 0,29*	73 ± 12*	915 ± 178
	5 µg/ml	4,63 ± 0,51*	66 ± 18*	846 ± 246
	10 µg/ml	5,17 ± 0,76*	50 ± 12*	843 ± 224
9	2.5 µg/ml	6,50 ± 2,50*	53 ± 26*	882 ± 160
	5 µg/ml	8,00 ± 1,73*	5 ± 2*	456 ± 142*
	10 µg/ml	10,00 ± 2,00*	2 ± 0*	168 ± 76*
10	2.5 µg/ml	5,50 ± 0,87*	63 ± 29*	864 ± 201
	5 µg/ml	6,37 ± 1,70*	45 ± 35*	775 ± 233
	10 µg/ml	7,83 ± 2,02*	20 ± 9*	639 ± 193*
11	2.5 µg/ml	4,83 ± 1,26*	66 ± 24*	759 ± 158
	5 µg/ml	6,00 ± 0,50*	46 ± 39*	756 ± 205*
	10 µg/ml	8,83 ± 2,02*	14 ± 8*	553 ± 172*
12	2.5 µg/ml	8,50 ± 3,54*	39 ± 52*	1014 ± 55
	5 µg/ml	9,75 ± 4,60*	13 ± 15*	983 ± 305
	10 µg/ml	12,00 ± 4,24*	6 ± 6*	681 ± 433
13	2.5 µg/ml	7,75 ± 1,77*	44 ± 45*	993 ± 224
	5 µg/ml	9,50 ± 2,12*	11 ± 12*	593 ± 278
	10 µg/ml	11,00 ± 1,41*	4 ± 2*	305 ± 289*
15	2.5 µg/ml	3,00 ± 0,00	118 ± 21	1565 ± 24
	5 µg/ml	3,40 ± 0,14	114 ± 13	1565 ± 56
	10 µg/ml	5,25 ± 1,77	59 ± 35*	1218 ± 207
14	2.5 µg/ml	6,25 ± 0,35*	18 ± 18*	820 ± 301
	5 µg/ml	11,50 ± 6,36*	8 ± 9*	946 ± 344
	10 µg/ml	15,00 ± 7,07*	2 ± 1*	352 ± 302*

Table II. Effect of different oligosaccharides on prothrombin activation. *Mean ± SD
p<0.05 (*n*=3).**

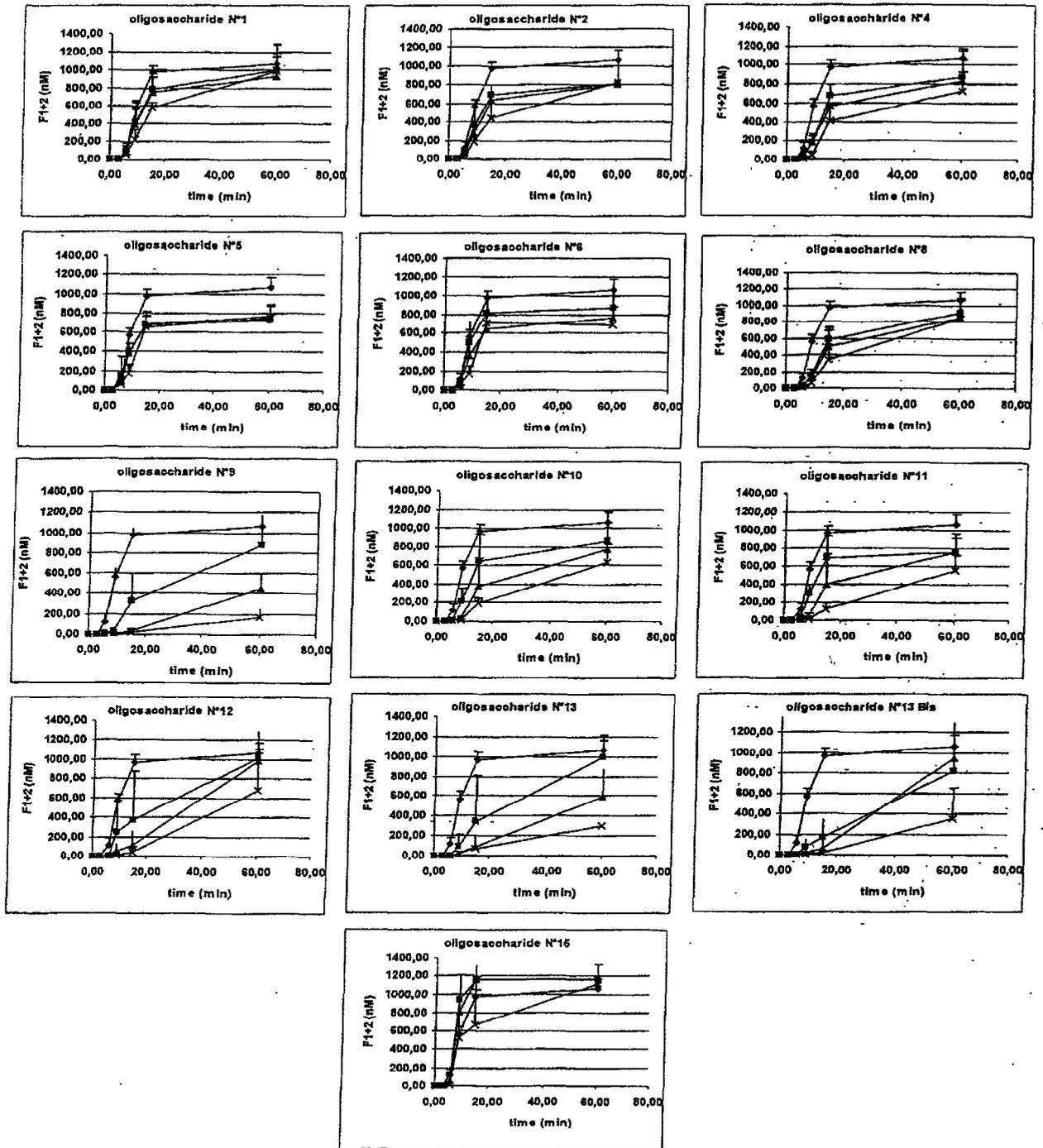


Figure 3: Prothrombin activation Kinetics in the absence (\diamond) and the presence of 2.5 $\mu\text{g/ml}$ (\blacksquare), 5 $\mu\text{g/ml}$ (\blacktriangle) and 10 $\mu\text{g/ml}$ (\times) of different oligosaccharides. *Mean \pm SD* ($n=3$).

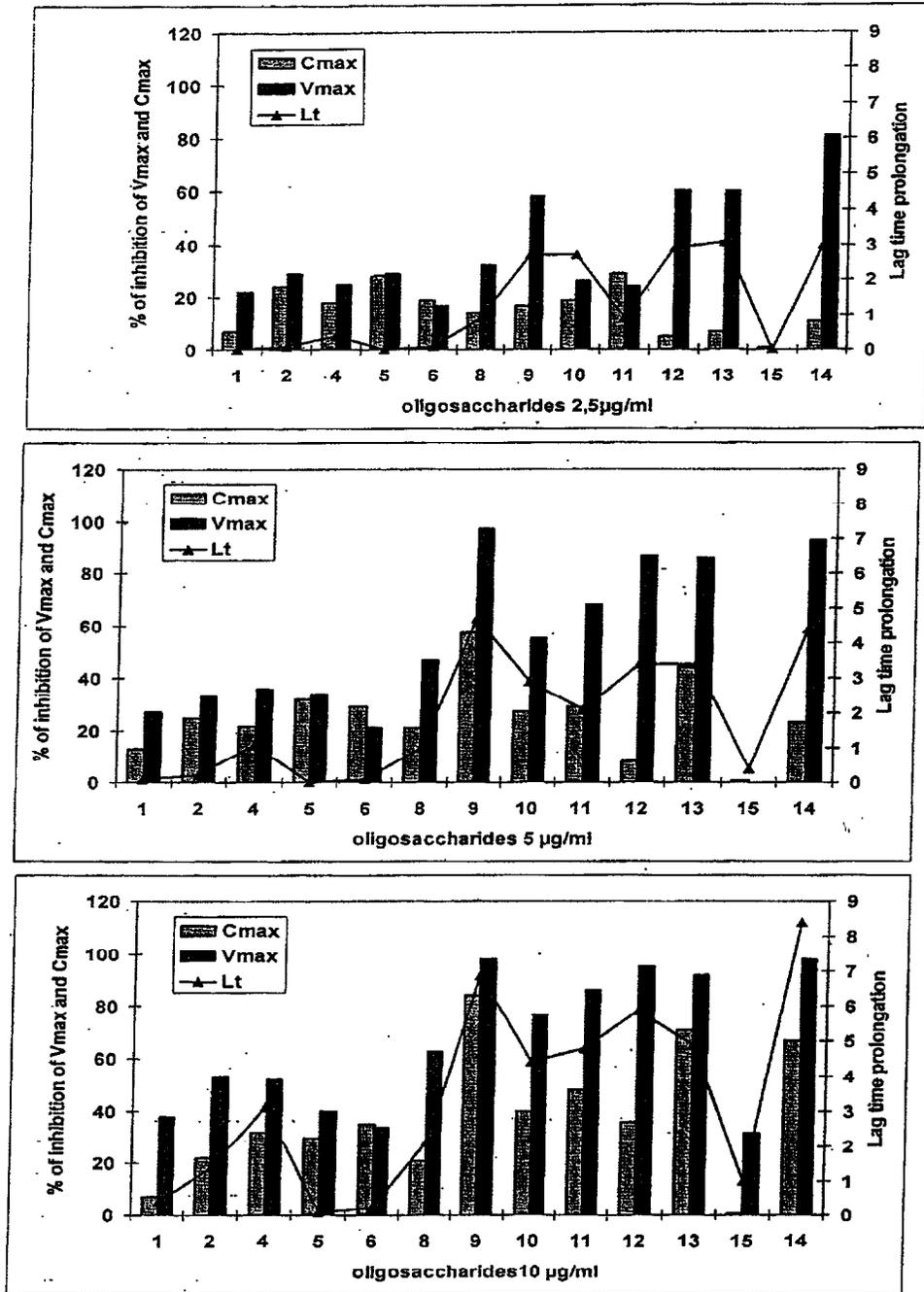


Figure 4. Lag time prolongation and percentage of inhibition of Vmax and Cmax of prothrombin activation induced by the different oligosaccharides. The lag time, Vmax and C max of the control were 3.1 ± 0.2 min, 110 ± 20 nM/min, and 1066 ± 107 nM, respectively ($n=3$)

5. Discussion

The level of factor VIIa in normal plasma is about 3 ± 0.1 ng/ml. Previous studies performed by our group have shown that triggering the extrinsic clotting pathway lead to rapid generation of FVIIa. One hour after the recalcification of normal platelet poor plasma (PPP) the FVIIa generation is stabilized and the amounts of FVIIa are dramatically increased compared to PPP level [xv]. In the present study, the addition of these different oligosaccharides to a normal PPP before clotting at the concentration of $2.5 \mu\text{g/ml}$ induced a significant reduction of FVIIa levels as compared to control. Interestingly, the inhibitory effect on FVIIa generation was not dose-dependent. Thus, no significant differences were observed between the different oligosaccharides used at concentrations greater than $2.5 \mu\text{g/ml}$. However, the oligosaccharides n° 12, 13 and 14 seemed more active than the others for inhibiting FVIIa generation. In a previous study we have shown that low concentration of Enoxaparin is sufficient to significantly decrease FVIIa generation. Furthermore, increasing Enoxaparin concentrations did not further increase this inhibitory effect [xv].

In order to classify more these studied oligosaccharides, we have compared the effect on FVIIa level of lower concentrations (1.25 and $0.625 \mu\text{g/ml}$) of selected oligosaccharides n° 9, 10, 11, 12, 13, 14 and 15. At these concentration a more clear concentration dependent effect on FVIIa generation was observed. The comparison of the studied compounds, on the basis of their effect at the lowest studied concentration ($0.625 \mu\text{g/ml}$) yielding a detectable inhibitory effect on FVIIa generation, allows to classify them as follows: $14 > 13 > 12 > 10 = 9 > 11 > 15$.

In addition to their impact on FVIIa generation, we have also studied the potential inhibitory effect of the different oligosaccharides on whole blood prothrombin activation. At the lowest concentration ($2,5 \mu\text{g/ml}$) all the products, except n°15, reduce the V_{max} of prothrombin activation rate. However, a very limited, if any, effect on the C_{max} of prothrombin fragments 1+2 generation was observed. For several compounds, a greater inhibitory effect on all the prothrombin activation parameters was obtained after increasing oligosaccharide concentrations. The most substantial effect is exerted on the prolongation of the lag-time. Thus, the classification of the oligosaccharides regarding their effect on prothrombin activation is: $14 > 13 = 12 = 9 > 10 = 11 > 15$. It is some what similar to that proposed for the FVIIa generation.

Finally, combining the oligosaccharides inhibitory activity on all the studied parameters (FVIIa generation, prothrombin activation), we observe that the most active products at lowest concentration are successively from the most to the less active $14 \geq 13 > 12 > 10 = 9 = 11 > 8 = 6 = 5 = 4 = 2 = 1 > 15$.

6. Conclusion and perspectives

We have already shown that during human plasma clotting, initiated by activation of TF pathway with minutes amount of TF, synthetic Pentasaccharide, Enoxaparin and UFH, *via* their anti-Xa activity, downregulate FVIIa generation by inhibiting both generation and activity of FIXa and FXa in a completely AT-dependent manner. In addition, Pentasaccharide inhibits also FVIIa activity in an AT-dependent manner as well [15].

These results could help to interpret the data presented herein and to design perspectives of the present work.

Thus, the studied oligosaccharides inhibit FVIIa generation in a variable extent. If they possess the pentasaccharide domain, we could assume that their effect is principally AT-dependent. We also expect, but this has to be proven, that some of them probably have an AT-dependent or even a direct effect on FVIIa activity as well. The observed differences are significant and they could be related to the different affinity of the compounds for AT leading to different inhibition of FXa and FIXa and down-regulation of the respective feed-back activation of FVII.

However, it must be clarified if this difference of AT affinity has an impact on the inhibition of FVIIa activity and which is its influence on the final product of TF pathway activation, i.e. thrombin generation.

For a complete description and comparison of the oligosaccharides effect on the blood coagulation process, the following studies are proposed:

1. Further analysis of the results for the different oligosaccharides taking into consideration their specific anti-Xa activity.
2. Direct influence of the compounds on FVIIa activity. Determination of AT or TFPI (Tissue factor Pathway Inhibitor) dependency for this effect.
3. Effect on the compounds on thrombin generation after TF pathway activation in PRP (Platelet Rich Plasma)
4. Effect on the compounds on FVIIa activity and generation in PPP selectively depleted in various coagulation factors (X, IX, XI...)

7. Raw Data

FVIIa ng/ml

	2,5 µg/ml	5 µg/ml	10 µg/ml
control	17,70	17,70	17,70
1	11,80	10,30	8,10
5	9,60	10,20	7,10
2	9,20	9,60	7,50
6	8,60	7,40	7,90
4	9,60	7,70	8,50
8	8,60	9,60	8,10
9	9,50	9,70	12,40
10	9,30	9,80	9,70
11	11,30	10,40	11,60
12	8,10	9,70	7,40
13	5,50	3,70	4,80
14	5,10	3,90	3,50
15	11,40	10,40	3,90

Table 1. Concentrations of FVIIa (ng/ml) generated 60 min after triggering coagulation in the absence (control) and the presence of the different oligosaccharides at different concentrations. (raw data used for figure 1)

FVIIa ng/ml

	2,5 µg/ml	1,25 µg/ml	0,625 µg/ml
	27,00	27,00	27,00
	15,60	23,20	25,02
	16,90	24,01	24,60
	18,50	25,13	24,88
	17,61	21,34	20,83
	17,46	19,41	20,27
	15,92	18,14	18,84
	20,50	26,00	27,00

Table2. Concentrations of FVIIa (ng/ml) generated 60 min after triggering coagulation in the absence (control) and the presence of the different oligosaccharides at different concentrations. (raw data used for figure 2)

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