Differential inhibition of thrombin generation by vitamin K antagonists alone and associated with low-molecular-weight heparin

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Summary
Vitamin K antagonists (VKA) treatment starts with co-administration of low-molecular-weight heparin (LMWH). The anticoagulation induced by the two drugs is still not well determined. In the present study we used thrombin generation assay to evaluate the hypo-coagulation induced by treatment with VKA and by the combination of VKA with LMWH. Tissue factor triggered thrombin generation in platelet-poor plasma was assessed in samples from 15 healthy volunteers, 97 samples from patients treated with VKA and 41 samples from patients receiving enoxaparin and VKA. Patients were classified according to international normalised ratio (INR) level (<2, 2–3 and >3). In plasma samples from patients treated with VKA having INR 2–3 the inhibition of thrombin generation reached 50% compared to controls. In samples with INR>3 this inhibition was 80%. In samples from patients receiving both LMWH and VKA, thrombin generation was significantly decreased compared to the controls and VKA group. In samples with an INR 2–3 obtained from patients treated with LMWH and VKA, the inhibition of thrombin generation was similar to that observed in samples with an INR>3 obtained from VKA treated patients. Thrombin generation assay is sensitive to detect the global the anticoagulant effect produced by the association of LMWH and VKA. For equal INR dual anticoagulant treatment induces significantly more profound inhibition of thrombin generation compared to treatment with VKA alone. The clinical relevance of this observation merits to be studied in prospective studies in patients with defined indications of anticoagulant therapy.

Keywords
Oral anticoagulants, vitamin K antagonists, bridging treatment, LMWH, thrombin generation

Introduction
Vitamin K antagonists (VKA) are the cornerstone for long-term treatment of venous thromboembolism (VTE) and prevention of thrombosis in patients with prosthetic heart valves or atrial fibrillation (). Their antithrombotic effect requires the reduction of prothrombin levels, the half-life of which is about 72 hours (h). Concomitant administration of therapeutic doses of unfractionated heparin (UFH) or low-molecular-weight heparin (LMWH) with VKA is recommended during initiation of oral anticoagulant treatment and until the international normalised ratio (INR) reaches the therapeutic levels (2–3) for at least two consecutive days (1). Similarly, bridging therapy with UFH or LMWH is suggested during temporary interruption of VKA after surgical or invasive procedures in patients who are in intermediate or high thrombotic risk and receiving long-term anticoagulant treatment (1–3). Clinical studies in hospitalized patients treated with VKA do not show any excessive bleeding rate when doses are adapted according to INR, even during the first five days of co-administration of LMWH (4). Nevertheless, in real-life clinical practice the duration of co-administration of LMWH and VKA is often longer and the bleeding risk during the initiation of VKA treatment is somewhat higher compared to that reported in clinical trials (5).
The degree of anticoagulation induced by the combined treatment with UFH or LMWH and VKA is not known since the available clotting tests are not sensitive to the effect of both VKA and heparin. The INR, based on prothrombin time (PT), explores in vitro the extrinsic clotting pathway and adequately reflects the anticoagulation induced by VKA but it is not sensitive to the anticoagulant activity of heparins. Activated partial thromboplastin time (aPTT), explores the intrinsic clotting pathway and is sensitive to UFH. However, aPTT is hardly influenced by therapeutic doses of LMWHs and although it increases during treatment with VKA, it is less sensitive than INR for the monitoring of this treatment.

The monitoring of the anticoagulant effect induced by co-administration of VKA and LMWH could be useful in some patients who are in high bleeding or thrombotic risk such as elderly or unstable patients, pregnant women or patients hospitalised in intensive care units. The lack of an appropriate assay is a significant drawback for optimization of the combined anticoagulant treatment with VKA and LMWH.

Thrombin generation assay performed in the presence of low tissue factor (TF) concentration is sensitive to the anticoagulant effect of LMWHs, and VKA (6, 7). The aim of the present study was to explore the effect of the association of LMWH and VKA treatment on TF triggered thrombin generation. In the first part of the study, we evaluated thrombin generation profile of plasma samples with different INR levels spiked with increasing concentrations of enoxaparin. In the second part, we assessed thrombin generation in plasma samples from patients treated with VKA alone or associated with LMWH. We demonstrate that the association of VKA treatment with LMWH induces a more profound inhibition of thrombin generation compared to VKA treatment alone.

Materials and methods
Blood samples
Blood samples from patients were collected according to standardised procedure for routine coagulation tests. Blood was drawn by the antecubital vein with atraumatic vein puncture into siliconised BD Vacutainer tubes (Becton Dickinson, Meylan, France) containing 1 vol/9 vol of buffered trisodium citrate (3.8%). Platelet-poor plasma (PPP) was prepared after twice centrifugation of citrated whole blood for 15 minutes (min) at 2,000 x g at room temperature. PPP for thrombin generation assessment was aliquoted and frozen at –80°C until assayed.

Baseline coagulation tests
PT was performed on fresh PPP using human thromboplastin (Thromborel® S, Dade Behring; Marburg, Germany). Anti-Xa activity was measured using the one-step amidolytic assay (Coamatic® Heparin from Chrombogenix, Milan, Italy) calibrated against the international standard provided by the manufacturer. Plasma samples assessed for anti-Xa activity were obtained four hours after the last subcutaneous injection of LMWH. Both PT and plasma anti-Xa activity were assessed using the STA-R analyzer (Diagnostica Stago Asnières France) and the INR was calculated by the software of the instrument.

Thrombin generation assay
Reagents
Bovine serum albumin (BSA) and Tris-HCl were obtained from Sigma laboratories (St. Louis, MO, USA). Recombinant Human Tissue Factor Thromboplastin (Dade Innovin®) was provided by Dade Behring (Marburg, Germany). This reagent was reconstituted by addition of 5 ml NaCl 0.9%, and subsequently diluted in Hepes buffer; pH 7.35 (containing 20 mM Hepes, 140 mM NaCl and 5 mg/ml BSA). Thrombin calibrator (Thrombinoscope® b.v.) was obtained by Biodis/Diagnostica Stago (Singes, France). The fluorogenic substrate Z-Gly-Gly-Arg-AMC was obtained from Bachem (Bubendorf, Switzerland). Synthetic phospholipids (DOPS/DOPE/DOPC reconstituted according to manufacturer’s instructions in a ratio 1:1:1.25) were obtained from Avanti Polar Lipids InC (Albaster, AL, USA). Prothrombin deficient plasma was from Diagnostica Stago (Asnières, France).

Thrombin generation assay
Thrombin generation was studied according to the assay described by Hemker et al. (8). In each well of a micro-plate, 80 µl PPP were mixed with 20 µl solution containing diluted recombinant human TF Thromboplastin (1:1,000 final dilution in plasma) and synthetic phospholipids (4 µM final concentration in plasma). Thrombin generation assessed in the presence of the above concentrations of TF and synthetic phospholipids has been previously standardised (8). Thrombin generation curves were corrected for substrate consumption and inner filter fluorescence effects using a Thrombin Calibrator®. At each cycle of the test, a patient’s plasma sample (80 µl), studied in duplicate, spiked with Thrombin Calibrator (20 µl) was running in parallel with each tested plasma sample supplemented with the calibrator. Thrombin generation was initiated by adding the triggering solution (20 µl) containing CaCl2, (16.7 mM final concentration) and the fluorogenic substrate (417 µM final concentration). A plate reader fluorometer (Fluoroscan Ascent®, ThermoLabSystems, Helsinki, Finland) and the appropriate software (Calibrated Automated Thrombogram® b.v., Maastricht, The Netherlands) were used for thrombin generation assessment.

The following parameters of thrombogram were analysed: (a) the lag-time of thrombin generation, (b) the time to reach maximum concentration of thrombin (ttPeak), (c) the maximum concentration of thrombin (Peak), (d) the endogenous thrombin potential (ETP) and (e) the mean rate index (MRI) of the propagation phase of thrombin generation calculated by the formula: Peak/ (ttPeak – lag-time) and expressed in nM/min. Inter-individual and intra-assay variability were assessed in healthy individuals and have been published elsewhere (9).

In-vitro study
Pool PPP from healthy volunteers (n=15) yielding INR=1, and pool PPP from patients treated with VKA yielding INR=2 (n=5), INR=3 (n=5), INR=5 (n=5), INR=8 (n=3) and INR=13 (n=3) were supplemented in vitro with saline (control) or increasing concentrations of enoxaparin (0.25; 0.5; 0.8 and 1 anti-Xa IU/ml). Subsequently, plasma samples were assessed for thrombin generation as described above.

In a separate experiment, normal plasma and plasma with INR 2 supplemented with increasing concentrations of enoxapar-
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**Results**

**In-vitro inhibition of thrombin generation by LMWH and VKA**

In the control group, the lag-time of thrombin generation was 1.5 ± 0.03 min, the tPeak was 4 ± 0.03 min, the MRI of the phase of thrombin generation was 151 ± 2.4 nM/min, and the Peak of thrombin was 303 ± 5 nM and the ETP was 1,690 ± 289 nM×min.

INR was almost linearly correlated with the prolongation of the lag-time and tPeak. An hyperbolical pattern of reduction of MRI, Peak and ETP was observed at INR values increasing from 1 to 13. The reduction of these parameters of thrombin generation was similar and for this reason, only ETP will be analysed. When INR was 2 or 3, the reduction of ETP was 50% compared to the control experiment. When INR was higher than 5, the inhibition of thrombin generation was more than 80%. In plasma samples with INR 2 the addition of enoxaparin at concentrations ranging from 0.25 to 0.8 anti-Xa IU/ml resulted in almost complete abrogation of thrombin generation (70% to 80% inhibition compared to the control experiment). When INR was lower than 2, the addition of enoxaparin 0.25 anti-Xa IU/ml reduced the ETP by 50%.

In plasma with INR 2 prothrombin level was 30%. One hour after triggering coagulation which was almost completely consumed (residual prothrombin <1%). In the presence of enoxaparin 0.5 anti-Xa IU/ml the residual prothrombin was 10%. Residual prothrombin was 18% and 26% when enoxaparin concentration was 0.8 and 1 anti-Xa IU/ml, respectively. A similar increase of residual prothrombin was found in serum prepared from normal plasma supplemented with enoxaparin.

**Thrombin generation in patients treated with VKA alone or associated with LMWH**

Characteristics of patients and results of routine coagulation tests

Patients from the two groups had no significant differences regarding age and sex. The INR was significantly higher in the samples from the VKA group compared to those from LMWH/VKA group.

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**Table 1: Basic characteristics of patients from whom plasma samples were assessed for thrombin generation.** Values are mean ± SD. *p<0.05 compared to VKA group.

<table>
<thead>
<tr>
<th></th>
<th>VKA group (n=97)</th>
<th>LMWH/VKA group (n=41)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>69.5 ±13.4 years (range: 39–92)</td>
<td>68.1 ±15.2 years (range: 18–88)</td>
</tr>
<tr>
<td><strong>Sex (m/f)</strong></td>
<td>44/53</td>
<td>14/27</td>
</tr>
<tr>
<td><strong>INR</strong></td>
<td>3.38 ±1.9 (range: 0.96–12.2)</td>
<td>2.39 ±1.9 (range: 0.97–10)</td>
</tr>
<tr>
<td>&lt;2</td>
<td>26 (26.8%)</td>
<td>22 (53.7%)*</td>
</tr>
<tr>
<td>2–3</td>
<td>25 (25.8%)</td>
<td>12 (29.3%)</td>
</tr>
<tr>
<td>&gt;3</td>
<td>46 (47.4%)</td>
<td>7 (17.1%)*</td>
</tr>
<tr>
<td><strong>Mean anti-Xa activity in plasma (anti-Xa IU/ml)</strong></td>
<td>-</td>
<td>0.67 ± 0.32 (range: 0.1 – 1.27)</td>
</tr>
</tbody>
</table>

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) or standard deviation (SD) as indicated. No previous data on the effect of combined LMWH and VKA treatment on thrombin generation were available. Thus, the sample size of the studied groups was not calculated upon an assumed difference of the two treatments (VKA vs. LMWH and VKA) but it was determined by feasibility reasons. The inter-individual variability of thrombin generation assay was the only parameter which was taken in consideration at the initial planning of the study. Statistical comparison was done using the unpaired Student’s t-test to control significance of the differences between the studied groups of patients. Unpaired Student’s t-test was also used to control differences between the subgroups of patients. Pearson correlation coefficient and regression analysis were used. Differences were considered as significant when p<0.05. Statistical analysis was performed with the SPSS software for Windows (SPSS Inc; release 10.0.1, Chicago, IL, USA).
Compared to VKA group, significantly more samples in the LMWH/VKA group had INR values lower than 2 (26.8% vs. 53.7%; p<0.005) and significantly less samples had INR values >3 (47.4% vs. 17.1%; p<0.05). Plasma samples from the LMWH/VKA group were obtained from patients receiving therapeutic doses of enoxaparin. The mean anti-Xa activity in plasma, measured 4 h after the last subcutaneous injection, was 0.67 ± 0.32 anti-Xa IU/ml (range 0.1 to 1.27 anti-Xa IU/ml). The aPTT ratio was similar between the two studied groups (data not shown).

**Thrombin generation in VKA group**

Treatment with VKA induced a significant prolongation of both lag-time and ttpPeak (5.8 ± 0.3 min and 8 ± 0.9 min, respectively) compared to the control group (1.5 ± 0.3 and 4 ± 0.3 respectively; p<0.001). In addition, VKA treatment significantly reduced the MRI (58.2 ± 3.7 nM/min), the Peak (122 ± 7 nM) and the ETP (643 ± 35 nMxmin) compared to control (162 ± 24 nM/min, 303 ± 57 nM, 1690 ± 289 nMxmin respectively; p<0.05) (Table 2). Similarly to the experiment in vitro lag-time and ttpPeak were almost linearly correlated to INR. ETP plotted against INR showed an hyperbolical pattern of decrease. The decrease of ETP reached a plateau when INR values were higher than 5.

In the subgroup of samples with INR<2 the lag time and ttpPeak were prolonged by 1.6– and 1.3-fold, respectively, compared to the control group (p<0.005). In this case ETP values was decreased by about 35% compared to the control group (p<0.005).

In the subgroup of plasma samples with INR values between 2 and 3, the lag-time and ttpPeak were prolonged by two- to three-fold. The ETP decreased by 65%, compared to the control group (p<0.001).

In the subgroup of plasma samples with INR values higher than 3, the lag-time and the ttpPeak were prolonged by three- and four-fold, respectively. The ETP was decreased by 75% compared to the control group (p<0.001).

**Influence of LMWH and VKA co-administration on thrombin generation**

In LMWH/VKA group the lag-time was 8.1 ± 1 min, the ttpPeak was 15.4 ± 1.9 min, the MRI was 36.6 ± 7 nM/min, the Peak was 88 ± 17 nM and the ETP was 518 ± 104. All these parameters were significantly decreased compared to either the control group or the VKA group. In LMWH/VKA group INR values did not correlate with any parameter of the thrombogram. In contrast, inhibition of thrombin generation was inversely correlated to the plasma anti-Xa activity. Pearson correlation coefficient between anti-Xa activity in plasma and each parameter of thrombogram were as follows: r= 0.38 for lag-time, r=0.5 for ttpPeak, r=-0.7 for MRI, r=-0.72 for Peak and r=-0.7 for ETP.

The analysis of the subgroups defined according to the INR level showed that for the same INR range the co-administration of LMWH resulted in more profound inhibition of thrombin generation as compared to that induced by VKA monotherapy. As shown in Figure 1, the inhibition of thrombin generation in LMWH/VKA subgroup with INR<2 was similar to that observed in samples from VKA subgroup with an INR 2 to 3. Samples from the LMWH/VKA subgroup with INR 2 to 3 had an inhibition of thrombin generation similar to that observed in VKA group with INR>3.

In samples with INR<2 the inhibition of thrombin generation was higher than 40% when anti-Xa activity in plasma was between 0.6 and 0.8 anti-Xa IU/ml. Of note, in four samples out of 41 (9.7%) ETP was within normal values although the anti-Xa activity measured in plasma was 0.1, 0.39, 0.5 and 0.6 anti-Xa IU/ml in each sample. Higher concentrations of anti-Xa activity were associated with a complete inhibition of thrombin generation. In samples with INR values between 2 and 3 thrombin generation was completely abrogated when the levels of the anti-Xa activity were equal of higher than 0.6 anti-Xa IU/ml (Fig. 2).

Thrombin generation was completely suppressed in only four of the 97 plasma samples from the VKA group (4%) obtained from patients with a mean age of 78 ± 3 years having INR=7 ± 3.

In contrast, thrombin generation was completely suppressed in 19 samples out of 41 (49%) from the LMWH/VKA group. These 19 samples compared to the samples from the same group in which thrombin generation was not completely inhibited, had

**Table 2: Thrombogram parameters in controls and in plasma samples from patients receiving VKA alone or VKA and LMWH. Values are mean ± SEM.**

<table>
<thead>
<tr>
<th>Thrombogram parameters</th>
<th>Control group n=15</th>
<th>VKA group n=97</th>
<th>LMWH/VKA group n=41</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag-time (min)</td>
<td>1.5 ± 0.3</td>
<td>5.8 ± 3.2</td>
<td>8.1 ± 7.8*</td>
</tr>
<tr>
<td>ttpPeak (min)</td>
<td>4 ± 0.3</td>
<td>8 ± 3.6</td>
<td>15.4 ± 12.3*</td>
</tr>
<tr>
<td>MRI (nM/min)</td>
<td>162 ± 24</td>
<td>58.2 ± 37*</td>
<td>36.6 ± 37*</td>
</tr>
<tr>
<td>Peak (nM)</td>
<td>303 ± 57</td>
<td>122 ± 69*</td>
<td>88 ± 113*</td>
</tr>
<tr>
<td>ETP (nMxmin)</td>
<td>1690 ± 289</td>
<td>643 ± 346*</td>
<td>518 ± 669*</td>
</tr>
</tbody>
</table>

*p<0.01; VKA group versus control group. *p<0.05; LMWH/VKA group versus VKA group.

**Figure 1: Endogenous thrombin potential in the control group (dark bars) and in plasma samples obtained from patients treated with LMWH/VKA (shadowed bars) and VKA alone (open bars) stratified according to INR values. Bars represent mean ± SEM. p<0.05 when compared VKA versus LMWH/VKA in each subgroup of INR for each parameter of thrombogram and versus control group. * p>0.05.**
higher INR values (3 ± 2 vs. 1.8 ± 0.8; p<0,05) and anti-Xa activity (0.8 ± 0.1 anti-Xa IU/ml vs. 0.47 ± 0.2 anti-Xa IU/ml; p<0,05). They were obtained from patients with similar age (68 ± 12 years vs. 67 ± 18 years) who were receiving significantly higher daily doses of enoxaparin (14,315 anti-Xa IU/day vs. 11,272 anti-Xa IU/day; p<0,05).

Discussion

The inhibition of thrombin generation is less than 35% at INR values lower than 2, is 40% to 70% at therapeutic levels of INR (2 to 3), and it is higher than 80% when INR is equal or higher to 5 which is related to high bleeding risk (10–12). This observation is in accordance with previous studies demonstrating that in VKA-treated patients having INR within the therapeutic range, the concentration of prothrombin F1+2 in plasma is almost halved compared to non-treated controls (13–16). Similar findings have been also reported by Altman et al who found that when INR values are within the therapeutic range, thrombin generation is reduced by about 50% (17).

The combination of LMWH and VKA enhances the inhibition of thrombin generation compared to VKA alone. The addition in vitro of enoxaparin in plasma samples with different INR values led to an enhancement of inhibition of TF-triggered thrombin generation and prevented prothrombin activation. An earlier study from our group showed that after TF pathway activation, enoxaparin induces a decrease of prothrombin activation in a similar extent as the synthetic pentasaccharide (an antithrombin-dependent specific inhibitor of factor Xa) whereas hirudin (a specific inhibitor of thrombin) does not hamper prothrombin consumption (18). Thus the decrease of thrombin generation induced by enoxaparin reflects the reduced prothrombin activation because of inhibition of factor Xa rather than the inhibition of generated thrombin by the anti-IIa activity of enoxaparin. A similar pattern of inhibition of thrombin generation was observed when thrombogram was assessed in plasma samples from patients receiving therapeutic doses of enoxaparin and VKA.

The degree of hypo-coagulation in plasma samples from enoxaparin-treated patients and VKA cannot be predicted by the INR values. The correlation of thrombin generation inhibition with the anti-Xa activity in plasma was found to be weak though significant showing that this effect is produced mainly by the enoxaparin chains which catalyse the antithrombin dependent inhibition of FXa (19). However, thrombin generation is driven by clotting factors which are activated by factor Xa (i.e. factors VII and IX) and is downregulated also by tissue factor pathway inhibitor (TFPI) which, in addition to antithrombin is influenced by treatment with enoxaparin (20–24). Thus, the observed inhibition of thrombin generation most probably is produced by the effect of enoxaparin of several steps of thrombin generation process rather than on the unique inhibition of factor Xa. In addition, anti-Xa activity in plasma cannot adequately predict the inhibition of thrombin generation. In about 10% of patients receiving enoxaparin and VKA, thrombin generation was within normal range although anti-Xa activity in plasma was significant. There is increasing evidence in favor of some biological resistance to the anticoagulant effect of LMWHs detected by global clotting assays. According to the employed method the frequency of non-responders to LMWH raises up to 10% of treated patients (25, 26). Whereas global assays such as thrombin generation are more sensitive than the specific measurement of anti-Xa to evaluate this phenomenon merits to be investigated.

Thrombin generation assay triggered by low TF concentration is sensitive to the global anticoagulant effect produced by both VKA and LMWH treatment. The ex-vivo study showed that in the subgroup with INR values lower than 2, the presence of at least 0.5 anti-Xa IU/ml enoxaparin was sufficient to reduce by 50% thrombin generation; i.e. in a similar degree to that ob-

![Figure 2: Endogenous thrombin potential (ETP) plotted against anti-Xa activity of plasma samples stratified according to the INR values and obtained from LMWH/VKA-treated patients. The shadowed zone corresponds to the normal range of ETP.](image-url)
served in patients receiving only VKA with INR values within the therapeutic range. To the best of our knowledge, this is the first study providing laboratory evidence that the anticoagulation induced by therapeutic doses of LMWH at the instauration of VKA treatment is equivalent in terms of thrombin generation inhibition, to that obtained by VKA anticoagulation with an INR within the therapeutic range. In-vitro studies have shown that enoxaparin, at concentrations ranging from 0.5 to 0.8 anti-Xa IU/ml inhibits by about 50% to 70% thrombin generation and prothrombin activation (27). In healthy volunteers therapeutic doses of enoxaparin induce about 50% inhibition of thrombin generation (25). However, this observation has not been adequately confirmed in patients treated with enoxaparin. Our study cannot address this question since the addition of a group of patients suffering by atrial fibrillation and treated only with enoxaparin without any exposure to VKA was not feasible. This limitation of the study does not allow to estimate the impact of sole enoxaparin treatment on thrombin generation in patients with similar underlying disease as our patients.

Enoxaparin treatment has an additional inhibitory effect on thrombin generation to that produced by VKA. Noteworthy, in samples from patients receiving VKA and LMWH the presence of anti-Xa activity equal or higher than 0.6 anti-Xa IU/ml resulted in complete suppression of thrombin generation even when the INR was within the therapeutic range. It appears that in these conditions the therapeutic window of enoxaparin, in terms of thrombin generation inhibition, becomes rather narrow. The present study was not designed to correlate laboratory findings to clinical outcomes. Thus the clinical relevance of these observations in the prediction of the bleeding risk in patients receiving VKA and LMWH needs to be evaluated in a prospective study. Patients treated with VKA requiring bridging therapy with LMWH represent an appropriate clinical model for such a study, since the degree of hypercoagulability is influenced by the surgical procedure.

Recent studies report an increase of the number of patients who receive combined treatment with LMWH and VKA in outpatient (28, 29). Bridging therapy is becoming a common practice for patients with prosthetic heart valves or atrial fibrillation who are hospitalised for surgical procedures (2, 3). Early hospital discharge of surgical patients is widely applied and it is expected that in real-life clinical practice a non-negligible percentage of out-patients is treated with LMWH and VKA association. In patients, treated at home or in non-hospital institutions the duration of dual treatment with LMWH and VKA is significantly longer compared to hospitalised ones and the bleeding risk seems to be higher (5). Weight adjustment of enoxaparin is effective and safe. However, a recent report shows that approximately 40% of patients treated with enoxaparin for acute coronary syndromes receive excess or lower-than-recommended dose and this is related to increased bleeding risk or mortality (30). Probably in these situations a more careful management of dual anti-coagulant treatment is needed. This approach might also apply in patients receiving bridging therapy while VKA effect persists and LMWH treatment is initiated. This might be a field where thrombin generation assessment should be prospectively evaluated as a tool for the estimation of the global anticoagulation produced by LMWH and VKA treatment.

The present study was not designed to assess the evolution of inhibition of thrombin generation during the initiation of VKA treatment, but it detects the combined effect of VKA and LMWH treatment in one time point. Moreover, the clinical relevance of thrombin generation profile in patients treated with VKA alone or associated with LMWH cannot be evaluated, and for this reason the clinical interpretation of our findings should be cautious.

The present study is the first one demonstrating that co-administration of VKA and enoxaparin has a higher antithrombotic effect compared to treatment with VKA alone. This effect cannot be predicted by either INR or anti-Xa activity levels in plasma but it can be evaluated by TF-triggered thrombin generation assay.

Our study raises a question regarding the influence of vitamin K-dependent clotting factors on thrombin generation profile in both VKA and VKA/LMWH-treated patients. In newly anticoagulated patients higher levels of clotting factors might be expected, and this could explain discrepancies of ETP inhibition.

What is known about this topic?
- To this purpose we employed minimal tissue factor triggered thrombin generation assay, which has been shown to be sensitive to the anticoagulant effect of either low-molecular-weight heparin (LMWH) or vitamin K antagonist (VKA) treatment. This has been demonstrated by previous studies published by Hemker’s group, Altman’s group and our group also.
- Until today it is not known if association of VKA and LMWH treatments induces more profound hypocoagulability compared to each treatment alone.
- The results from the available laboratory test routinely used for the monitoring of these treatments (prothrombin time for VKA and anti-Xa activity for LMWHs) do not show such an interaction, but this is a misleading approach since each one of these test is “mono-specific” for VKA or LMWH, respectively. Consequently information on this topic is missing.

What does this paper add?
- The present study is the first, at the best of our knowledge, providing both in-vitro and ex-vivo evidence showing that co-administration of LMWH and VKA (at situations simulating the initiation of VKA treatment or bridging therapy) induces significantly more important hypocoagulation compared to VKA monotherapy.
- The present study is a “modelisation” of the combined anticoagulant effect of LMWH and VKA treatment on thrombin generation. It was not designed to evaluate the clinical relevance of this finding, thus extrapolation of the data in clinical practice should be very cautious.
- The findings from our work can be used for the design of trials focusing to study the profile of thrombin generation inhibition during co-administration of LMWH and VKA in homogenous population of patients and to assess its clinical relevance.
for the same INR. Probably newly and chronically anticoagulated patients are not directly comparable and this has to be investigated in future studies.

In conclusion, the present study shows that TF-triggered thrombin generation assay is a tool for the evaluation of the global anticoagulant effect produced by VKA and LMWH treatment. LMWH treatment induces an additional inhibition of thrombin generation to that produced by treatment with VKA which results in a significant narrowing of the therapeutic window of LMWH. If this effect on thrombin generation has any impact on the bleeding risk of patients who receive dual anticoagulant treatment which can be predicted by thrombin generation assay has to be studied.

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References