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ORIGINAL ARTICLE

During warfarin induction, the Fiix-prothrombin time reflects the anticoagulation level better than the standard prothrombin time

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Essentials

- Fiix-prothrombin time (PT) monitoring of warfarin measuring factor (F) II and X, is effective.
- Plasma obtained during warfarin induction and stable phase in Fiix-trial was assayed.
- Fiix-PT stabilized anticoagulation earlier than monitoring with traditional PT-INR.
- FVII had little effect on thrombin generation that was mainly determined by FII and FX.

Summary. *Background:* The prothrombin time (PT) is equally prolonged by reduction of each of the vitamin K-dependent (VKD) factors (F) II, VII and X. The Fiix-PT is only affected by FII and FX, the main contributors to thrombin generation (TG). *Objective:* To test the hypothesis that variability in warfarin anticoagulation is reduced early during monitoring with the normalized PT-ratio calculated from Fiix-PT (Fiix-International Normalized Ratio [INR]) compared with traditional PT-INR monitoring. Also, that because of its insensitivity to FVII, Fiix-PT more accurately reflects TG when Fiix-INR and PT-INR are discrepant. *Methods:* Samples from Fiix-trial participants monitored with either Fiix-PT or PT were used. VKD coagulation factors and TG were measured in samples from 40 patients during stable anticoagulation and in serial samples obtained from 26 patients during warfarin induction. TG was assessed in relation to selective reduction in single VKD factors. *Results:* During Fiix-warfarin induction full anticoagulation measured as FII or FX activity was achieved at a similar rate to that with PT-warfarin but subsequently stabilized better. Fiix-INR but not PT-INR mirrored total TG during initiation. During induction, FII (R^2 = 0.66) and FX (R^2 = 0.52) correlated better with TG and with a steeper slope than did FIX (R^2 = 0.37) and in particular FVII (R^2 = 0.21). *In vitro*, FII and FX were the main determinants of TG at concentrations observed during VKA anticoagulation, whereas FVII and FIX had little influence. *Conclusions:* Fiix-PT monitoring reduces anticoagulation variability, suggesting that monitoring FVII has a limited role during VKA management. TG is better reflected by Fiix-PT.

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Introduction

During standard anticoagulation with warfarin or other vitamin K antagonists (VKA), dosage is adjusted according to either the Quick prothrombin time (PT) [1] or less commonly the prothrombin proconvertin time (P&P, Owren's PT) [2]. These tests are interchangeable for the purpose of managing VKA and their results can be reported as a standardized PT ratio, the international normalized ratio (INR) [3,4]. Both tests are equally affected by independent decreases in each of three vitamin K-dependent (VKD) coagulation factors (F) (i.e. II, VII and X). However, prior studies suggest that it is mainly reduced FII activity [5–8] or the combined reduction in FII and FX activity [6,9] that reduces thrombin generation and clot formation or prevents intravascular coagulation during VKA administration. This suggests that monitoring FVII may not be needed and may even confound VKA management [9]. It also suggests that alternative monitoring methods should be sought. Some investigators have suggested monitoring with global coagulation assays, such as automated thrombin generation (TG) assays that indicate clot

formability[10–12], but to our knowledge their usefulness for this purpose has not been prospectively shown and other options may also be viable.

The new Fiix-PT differs from the traditional PT in being affected only by reduced FII and FX activity in the test sample [9]. In the recent Fiix-trial, monitoring of warfarin with Fiix-PT in comparison with standard monitoring with PT stabilized anticoagulation, reduced dose adjustments and led to a 46% non-inferior reduction in thromboembolism (TE) that was significant in the long term without increasing bleeding [13,14]. However, insufficient numbers of warfarin-naïve patients were enrolled in the trial to assess clinical outcome during induction. As severe FVII deficiency that would not be evident by Fiix-PT during warfarin induction could induce hemorrhage, the level of anticoagulation during induction must be determined.

We hypothesized that by managing warfarin based on the Fiix-PT normalized ratio (Fiix-INR, Fiix-NR), variability in anticoagulation during induction would be reduced early and without causing severe depression of FVII activity when compared with standard PT-INR monitoring. We also hypothesized that TG is more accurately reflected by the Fiix-PT when discrepant Fiix-INR and PT-INR are present because of the insensitivity of Fiix-PT to FVII. Using consecutively obtained frozen plasma samples drawn during the first weeks of warfarin intake from randomly selected participants in both arms of the Fiix-trial, we tested the hypotheses by measuring coagulant activity of FII, FVII, FIX, FX and automated TG. Treatment levels in patients on stable long-term warfarin anticoagulation were determined for reference.

Materials and methods

Trial overview

The Fiix-trial was a single-center double-blind randomized clinical trial conducted at our institution that compared the outcome of patients on warfarin monitored with the Fiix-PT with a control population managed with standard Quick PT (INR) [13]. In short, 1148 patients were randomized to monitoring either with Fiix-PT (Fiix arm) or PT (control arm). Results of both tests were reported as a blinded 'research INR' (R-INR) to the anticoagulation dosing center. The trial was conducted in accordance with the Helsinki declaration and the protocol was approved by the National Bioethics Committee of Iceland (VSNb2011040019/03.15) and the Data Protection Agency of Iceland (2011040560AMK/-). Written informed consent was obtained from all participants. The current study is a subgroup study of the Fiix trial specially testing consecutive frozen samples from naïve patients starting warfarin and samples from patients on stable warfarin treatment selected from a pool of 1085 available citrated plasma samples that were obtained from 715 participating patients (438 men and 277 women, average age 71.4 years) during a single study month. All samples were stored frozen at −7 °C.

Warfarin dosing

Using our routine warfarin initiation protocol, patients under the age of 65–70 years starting on warfarin received a daily dose of 6 mg for the first 3 days, followed by adjusted doses based on a reported blinded research INR (Fiix-INR or PT-INR depending on randomization). No loading dose was administered. In older patients a lower daily dose of 4 mg was administered during the first 3 days. If patients showed only a minor or no INR response after the initial 3 days, the dose was increased by 50% and in the case of an excessive response, the dose was reduced by 50%. When patients had reached full anticoagulation (two R-INRs within treatment range) they were switched to software-assisted dosing using the DAWN anticoagulation software (4-S, Milnthorpe, UK) [15].

Samples

Blood was drawn into 3.2% sodium citrate Vacuette® tubes (Greiner, Kremsmünster, Austria), centrifuged at 2000 *g* for 15 min and the supernatant platelet-poor plasma (PPP) used for INR assaying. After measuring the Fiix-INR and PT-INR, the samples were aliquoted and stored at −70 °C for further testing.

Coagulation instruments, reagents and assays

STA-Néoplastine Cl plus (rabbit brain thromboplastin + calcium chloride) and STA-Owren Koller buffer were obtained from Diagnostica Stago® (Asnieres, France.) Triniclot activated partial thromboplastin time (APTT) reagent for FIX assay was obtained from Trinity Biotech Plc , Southern Cross, Bray, UK. Factor-deficient PPP for the factor assays and FII and FX double immunodepleted PPP (Fiix depleted plasma) were obtained from Haemotologic Technologies Inc. (Essex Junction, Vermont, USA). The Fiix-depleted plasma was aliquoted in 1-mL tubes and frozen at −70 °C. Normal PPP for experiments was obtained from George-King Biomedical (Overland Park, Kansas, USA).

All assays were clotting assays performed on a STA-R evolution coagulation analyzer (Diagnostica Stago®). Owren-Koller buffer was used as a diluent. The Fiix-INR assay uses 80 μL of diluted patient sample (1 : 7 in buffer) mixed with 25 μL of Fiix-depleted plasma, with an incubation time of 240 s. Coagulation is then activated by adding 80 μL of the STA-Néoplastine CI plus. The Quick PT-INR assay uses 50 μL of undiluted patient sample with an incubation time of 240 s. Coagulation is activated by adding 100 μL of the STA-Néoplastine CI plus. The INR was calculated using the locally calibrated international sensitivity index (ISI) based on DEKS

standards (Danish Institute for External Quality Assurance for Laboratories in Health Care) [4]. Both the Fiix-INR and PT-INR were measured in both groups, but only the relevant INR (Fiix-INR for test group and PT-INR for controls) results were reported to dosing staff as a blinded R-INR ('research-INR').

All the coagulation factor assays use 50 μL of diluted patient sample (1 : 10) and 50 μL of plasma deficient in the corresponding coagulation factors. All coagulation factor assays have an incubation time of 240 s. Assays for FII, FVII and FX use 100 μL of STA-Néoplastine CI plus as an activator and FIX uses an APTT-based assay using 50 μL of Triniclot and 50 μL of $CaCl_2$.

Thrombin generation

TG was measured in quadruplicate in citrated plasma as a calibrated automated thrombogram using the Fluoroskan Ascent™ microplate fluorometer (Thermo Fisher Scientific, Microplate Instrumentation, Vantaa, Finland). The PPP reagent contained 4 μmol/L phospholipids and 5 pmol/L human tissue factor. The Thrombin Calibrator and FluCa kit (Fluo-Substrate) was from Thrombinoscope B.V. (Maastricht, the Netherlands). Normal thrombin generation was assessed in citrated samples from 20 healthy non-anticoagulated individuals for reference.

Data analysis

In graphs showing temporal relationships, test results from patients starting treatment were summarized graphically by showing either the mean or mean ± one standard error of the mean (SEM) of pooled consecutive 3-day intervals, or as described in the text. The GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA) was used for statistical calculations. The chi-squared test was used to compare categorical data. Non-linear regression was calculated. A P-value of < 0.05 was considered statistically significant.

Results

Coagulation factors during stable anticoagulation

In order to determine stable treatment ranges for VKD factor activity and thrombin generation during the therapeutic normalized ratio anticoagulation range of 2–3, FII, FVII, FIX and FX activity and automated thrombin generation were measured in 20 samples obtained from as many patients with stable anticoagulation in each warfarin monitoring arm and results were pooled (n = 40). All had been shown to have Fiix-INR or PT-INR constantly within the range 2–3 for over 10 months by serial measurements. The mean (95% distribution; range) stable treatment per cent activity range was as follows: FII 27 (15–40; 18–40), FVII 48 (18–79; 23–89), FIX 61 (32–89; 36–85) and FX 15 (11–19; 10–22). TG during stable anticoagulation was as follows: lag time 5.8 (2.5–9.0; 3.4–10) min, peak TG 94 (35–154; 36–159) nM, endogenous thrombin potential (ETP) 504 (246–763; 300–788) nM*min, compared with 2.6 (1.6–3.6; 2–3.7), 294 (160–441; 138–465) and 1563 (1089–2173; 1086–2177) nM*min, respectively, in 20 healthy subjects. Thus, the mean peak TG and mean ETP during stable anticoagulation were about 33% of the mean TG observed in healthy subjects.

INR variation and vitamin K-dependent factor activity levels during Fiix-warfarin or PT-warfarin induction

Frozen serial samples drawn from 26 warfarin-naïve patients during their first 7 weeks (days 1–36) of treatment were available for factor assays. During the 36-day initiation period, 64 and 76 monitoring tests were carried out in 12 and 14 patients in the Fiix-PT and PT arms, respectively. The median (IQR) number of tests was six (5–7) and seven (5–8), respectively. As the number of available samples on each day was limited, the results were pooled into 12 consecutive 3-day intervals (Fig. 1). In patients starting on warfarin, mean Fiix-INR ≥ 2 was reached on day 11 in both groups. However, the PT-INR suggested full anticoagulation on day 8 in both groups.

FII and FX reached their treatment ranges (as defined above), 15–40% and 11–19%, respectively, faster with Fiix-warfarin. They remained mainly within the range from day 11 to day 36 (Fig. 1) in the Fiix-monitoring group, with FX having particularly little variation, whereas larger variation in FII and FX was present during PT-warfarin monitoring from day 11 onwards.

FVII levels were variable. The day-8 higher rise in PT-INR in the Fiix-PT group shown in Fig. 1(A) is mainly a reflection of a faster fall in FVII activity with Fiix-warfarin management because FX is similar in both groups and reduction in FII lags behind. The mean FVII level decreased to a nadir of 20% in the Fiix group compared with a nadir of 30% in the PT monitoring group on day 11, with the lowest observed FVII absolute values being 4% and 6%, respectively. The lowest FII and FX activities were 15% and 10% in the Fiix-group and 9% and 6% in the PT group, respectively.

Fiix-INR and PT-INR in relation to thrombin generation during warfarin induction

Thrombin generation, Fiix-INR and PT-INR could all be assessed in 67 frozen serial samples obtained from 15 patients (eight in the Fiix group and seven in the PT group) during warfarin initiation on days 4–27 and the results from the two monitoring arms were pooled (Fig. 2). The graph shows pooled three-day results (days 4–6 = day 5, days 7–9 = day 8, etc.) comparing how the normalized ratios align with the ETP at different stages

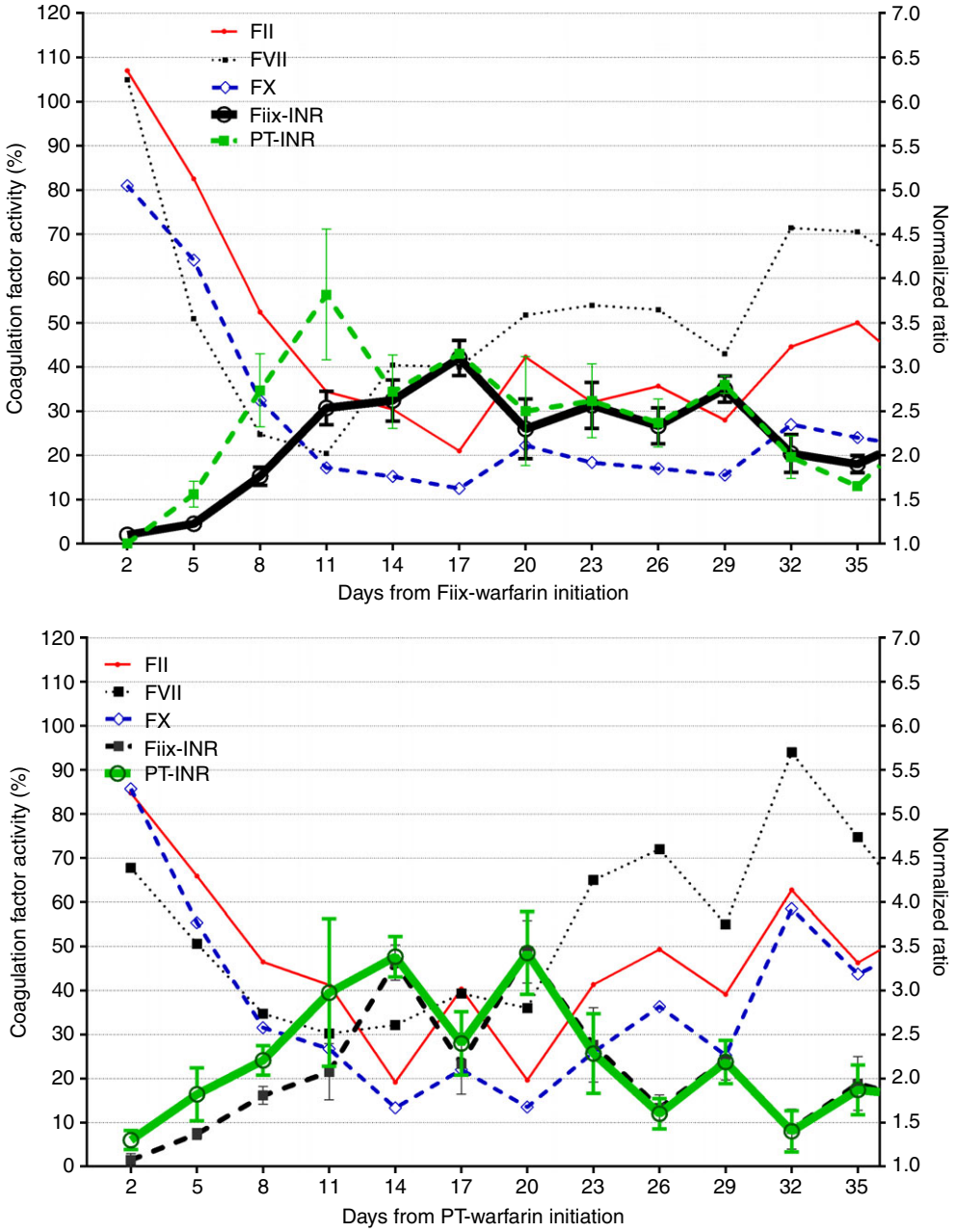

Fig. 1. Anticoagulation during first 5 weeks of warfarin induction. Measurements of Fiix-INR, PT-INR and factor (F) II, VII and X activity carried out at 3-day consecutive intervals (1–3, 4–6, etc.) were pooled and means are shown, and for normalized ratios ± 1 SEM is also shown. Results obtained during Fiix-INR monitoring (12 patients) are shown in the upper panel and those with PT-INR monitoring (14 patients) in the lower panel. Results with both Fiix-INR and PT-INR are shown for both groups but the bold line indicates the monitoring test results in each group. The shaded area reflects the INR target range of 2–3. INR, International Normalized Ratio; PT, prothrombin time; SEM, standard error of the mean.

of initiation. During days 4–27, the Fiix-INR was a reciprocal image of the ETP, whereas the PT-INR (lower panel) did not mirror thrombin generation as well on days 5, 8 and 11. Figure 3 demonstrates that in relation to Fiix-INR, divergent high PT-INR results are observed sporadically with the PT-INR and progressively divergent results occur once the TG decreases below the treatment range. Thus, a high PT-INR tends to overestimate the reduction in TG that is present.

Thrombin generation in relation to normalized ratios and vitamin K-dependent factor activities in patient samples ex vivo

The left-sided panels of Fig. 4 show that when TG (ETP) is measured in samples obtained from patients during induction of warfarin, FII ($R^2 = 0.66$) and FX ($R^2 = 0.52$) reflect the ETP better than do FVII ($R^2 = 0.21$) and FIX ($R^2 = 0.37$), the latter two having

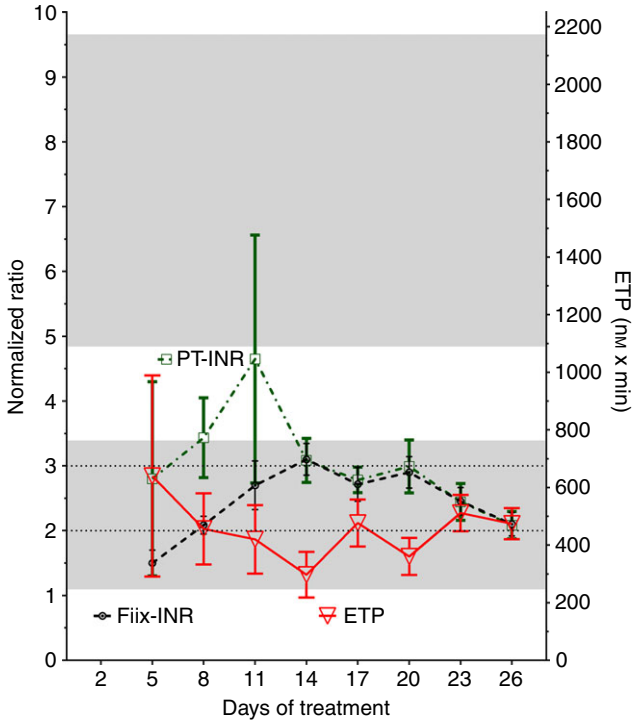

Fig. 2. Fiix-INR and PT-INR in relation to thrombin generation during warfarin initiation. TG shown as ETP, Fiix-INR and PT-INR could all be measured in consecutive samples obtained from 15 individuals during days 4–27. Results of ETP and normalized ratios measured during eight 3-day consecutive intervals (4–6, 7–9 etc.) were pooled and are shown as means ± 1 SEM. The lower shaded area shows the expected stable treatment 95% distribution for ETP and the upper shaded area represents the expected values in healthy non-anticoagulated subjects. SEM, standard error of the mean.

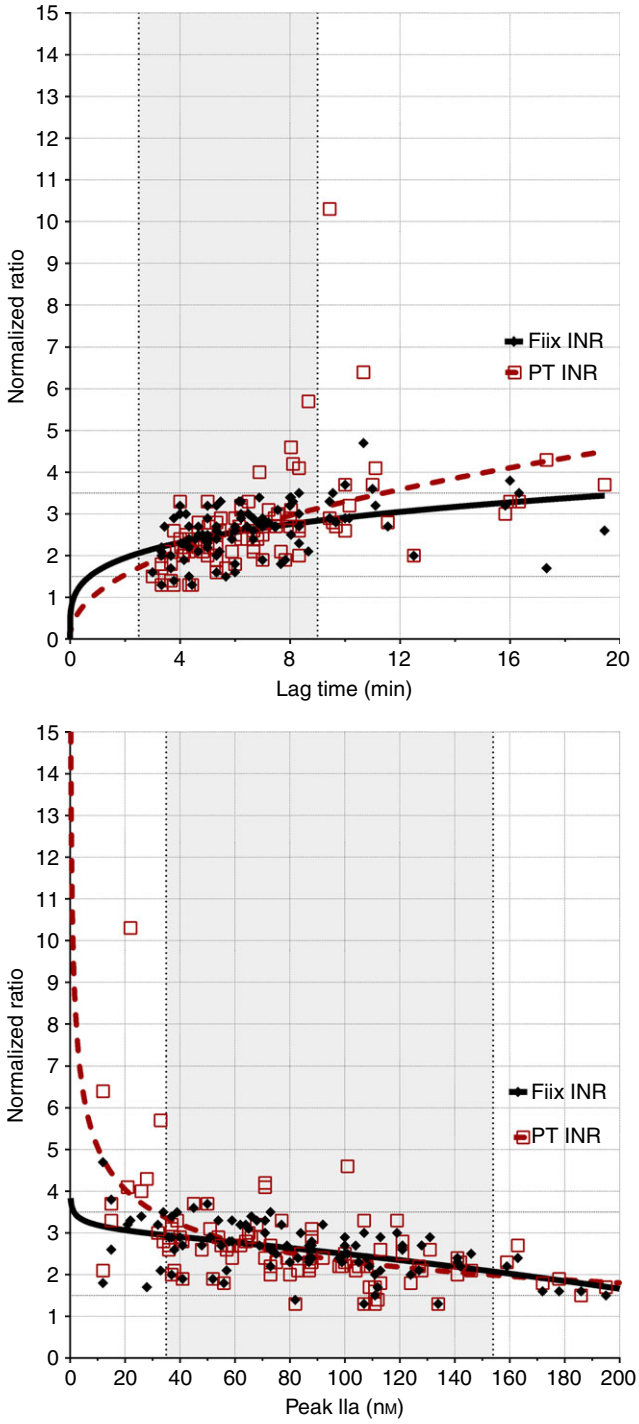

Fig. 3. Fiix-INR and PT-INR in relation to thrombin generation during warfarin induction. The variables were measured in 57 samples obtained during warfarin induction and in 40 patient samples obtained during stable treatment. The shaded areas reflect the treatment range for TG (the lag-time, peak TG and ETP) observed during stable warfarin anticoagulation with INR 2–3. INR, International Normalized Ratio; PT, prothrombin time; TG, thrombin generation; ETP, endogenous thrombin potential.

flatter curves and wider scatter. In samples obtained during the stable phase, anticoagulation factors II (R^2 = 0.55), X (R = 0.42) and VII (R^2 = 0.55) appear to reflect the ETP with a steep slope (Fig. 4; right-sided panels), whereas FIX shows little relationship with ETP (R^2 = 0.13). Note that during the stable phase FVII activity was mainly above 30%, considerably higher than during the initiation period. Also, note the very narrow FX activity range during the stable phase.

Thrombin generation in relation to individual vitamin K-dependent factor activities in vitro

As the ETP in patient samples reflects simultaneous alterations (to varying degrees) of the different VKD factors that have very different half-lives, we also carried out 'clean' experiments measuring the influence of progressively reducing the activity of individual VKD factors, one at a time. These experiments shown in Fig. 5 demonstrate that FII and FX influence peak thrombin generation and ETP at factor activity levels similar to those observed during warfarin management. In contrast, FVII and FIX do not influence thrombin generation to a significant degree at activity levels corresponding to initiation and in particular during stable-phase warfarin treatment.

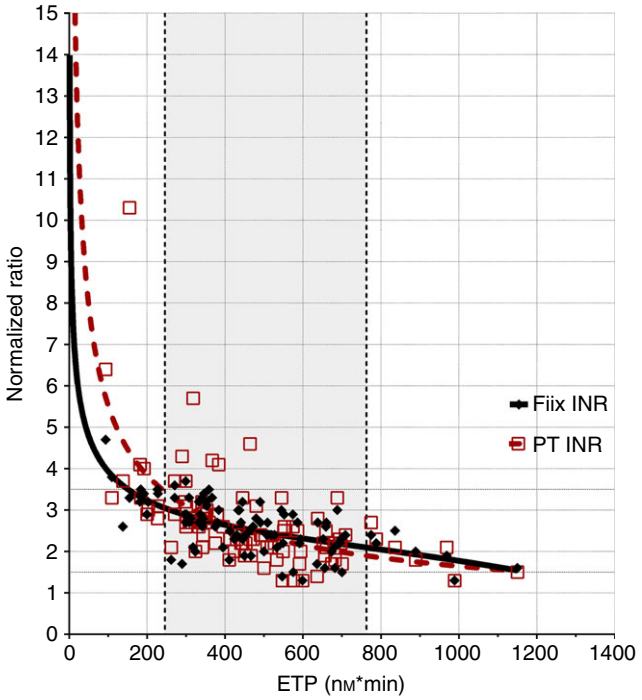

Fig. 3. continued.

Discussion

Our results demonstrate that warfarin anticoagulation, quantified as reduced FII or FX, develops at a similar rate during Fiix-warfarin initiation to that with standard PT-warfarin initiation and without severe FVII deficiency occurring. Once achieved, anticoagulation, measured as normalized ratios, FII or FX, varies less when Fiix-PT guides the dosing. TG is also more accurately reflected by the Fiix-PT. Importantly, during the stable phase FVII and FIX remain at relatively high levels that would be considered safe and not expected to cause clinical bleeding.

During warfarin initiation, the very short half-life of FVII (4–6 h) causes its faster decline than that of FII and FX, leading to an early prolongation of the PT-INR that is not reflected by the Fiix-PT. Traditionally, this early PT prolongation is not considered reflective of an antithrombotic effect until two PT-INRs a few days apart fall within the therapeutic range [16–18] because reduction in the longer half-life of FII, FIX and FX activities lags behind by a few days. It is felt that FII, FIX and FX must be reduced as well in order to achieve a complete antithrombotic effect [5,6,9,17]. Actually, the role of FVII and FIX in the antithrombotic effect of VKA is questionable because experiments suggest that the antithrombotic effect is foremost induced by lowered FII activity [5,6] or by a joint effect of reduced FII and FX, the latter described previously in our thromboelastometric experiments [9] and supported by prior studies [5,6] and our current *in vitro* thrombin generation experiment. Those results demonstrate only a minor influence of FVII and FIX alone on fibrin clot formation or TG at the factor activity levels that are observed during controlled warfarin treatment. To understand VKA action, it is essential to realize that moderately reduced FVII alone causing marked prolongations of the PT-INR has only minimal influence on TG. Thrombin generation will only be compromised at very low (i.e. supratherapeutic) FVII (and FIX) levels.

With our dose-management method, the anticoagulant effect of warfarin measured as reduced FII and FX developed somewhat slower than has been previously reported based on the PT [17] but it should be kept in mind that initiation practices vary [19]. However, the anticoagulant effect stabilized faster during Fiix-PT monitoring than with PT monitoring, probably as a consequence of more precise dosing of warfarin when reduced FVII does not influence the monitoring test. Importantly, a dangerous reduction in FVII did not occur more frequently during Fiix-INR monitoring. In addition to the Fiix-INR improving anticoagulation stability early, it also reflects thrombin generation more accurately than the PT-INR does during periods when fast reductions in the short half-life of FVII occur, but reduction in the longer half-life of FII and FX lags behind. In other words, Fiix-INR and PT-INR discrepancies will occur for a few days whenever significant dose adjustments are made. When anticoagulation becomes stable there is little difference. The strong dependence of the PT-INR on FVII reductions at activity levels that are insufficiently reduced to affect TG adversely also explains why the association of the PT-INR with TG has been widely variable in previous studies [10].

In the last decade new oral anticoagulants have been approved for use in patients with venous thromboembolism and atrial fibrillation. These new agents need less monitoring than traditionally managed warfarin and have shown promise in large multicenter clinical trials [20–27]. The current study and the clinical results of the Fiix-trial [13,28] suggest that improvements in anticoagulation management and outcome can also be achieved with warfarin (i.e. by replacing PT-INR dosing with Fiix-INR dosing). First, it is easier to dose VKA patients monitored with the Fiix-PT, as evident by less need for dose adjustment [13]. The likely explanation is the absence of a confounding influence of a fluctuating FVII on the Fiix test result and dosing decisions in patients. Interestingly, as shown in the current paper, in our cohort FVII varied no more when it was not measured than when it was. In other words, instead of the dosing staff trying to chase FVII during PT-INR monitoring, during Fiix-INR monitoring FVII chases the Fiix-INR. Secondly, sporadic very high PT-INRs caused by rapid fluctuations in FVII do not occur with the Fiix-PT, which therefore more accurately reflects thrombin generation, and this could translate into better management with improved clinical

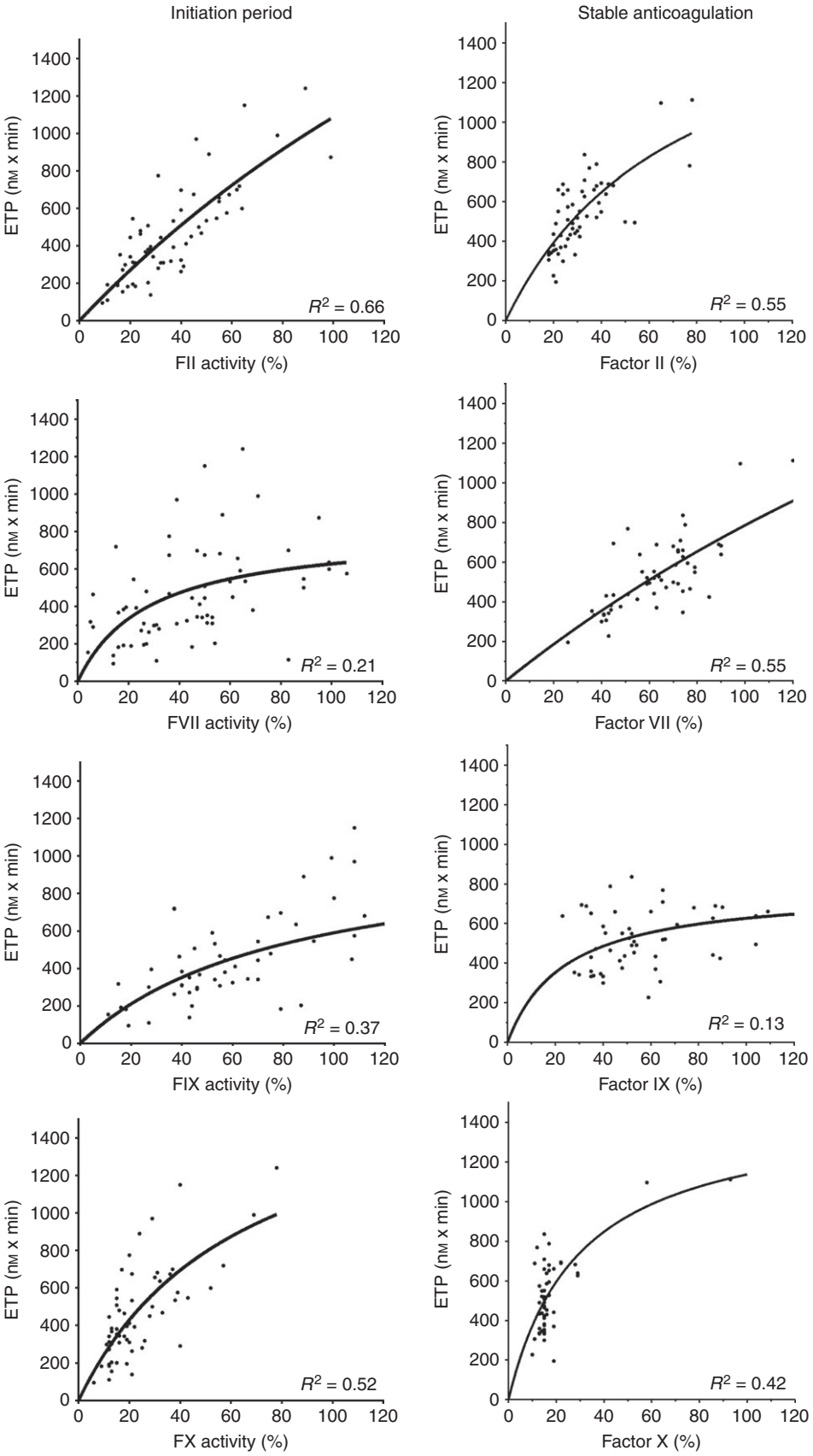

Fig 4. Correlation of thrombin generation with vitamin K-dependent coagulation factors in samples from patients initiating warfarin or during stable warfarin therapy. Thrombin generation is shown as endogenous thrombin potential (ETP).

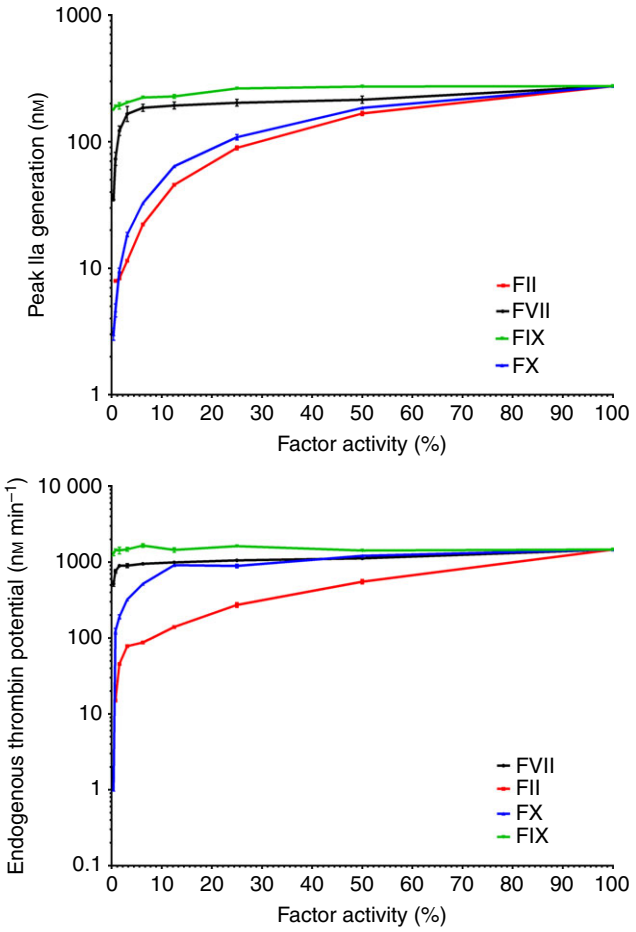

Fig. 5. Thrombin generation in relation to each individual vitamin K factor activity *in vitro*. Progressive serial dilutions were made by mixing single-factor-deficient plasmas with normal plasma. Peak thrombin generation and endogenous thrombin potential (ETP) are shown for each factor. Means ± 1 standard error of the mean (SEM) of quadruplicate experiments are shown.

outcome. Third, as the new Fiix-PT more accurately reflects the anticoagulant effect than the PT does, once the Fiix-INR reaches 2.0 or higher concomitantly administered fast-acting anticoagulants can be immediately discontinued. Fourth, the clinical Fiix-trial demonstrated that the dose-management improvements with Fiix-PT-monitoring translated into higher time within target range (TTR), lower INR variability and a 59% (RR 0.41) statistically non-inferior reduction in long-term thromboembolic event rates compared with well-managed PT-warfarin. [13,28] Fifth, because of earlier stabilization, Fiix-warfarin may be more suitable for short-term anticoagulation than is PT-warfarin.

Some limitations of the current study need to be addressed. As we only had a limited number of samples, drawn at varying time-points during warfarin induction in different patients, available for serial clotting factor measurements and TG assays, a certain variance in results is to be expected. Also, the number of evaluable samples at each time-point varied (Fig. 1). We did not use a warfarin loading dose and, therefore, we can only claim that an improvement occurred during initiation with the Fiix-PT in patients managed with a low-dose initiation approach [19]. Finally, it is possible that even further stabilization of warfarin dosing could be achieved if a Fiix-PT-specific dosing protocol had existed.

In conclusion, monitoring warfarin induction with the Fiix-PT, which is affected only by reduced FII and FX, stabilizes anticoagulation better than standard PT-INR monitoring during treatment induction and without causing more severe reduction in FVII. The Fiix-PT also reflects thrombin generation better than the PT during warfarin induction.

Addendum

P. I. Jonsson, MS student, collected test samples, performed laboratory assays, analyzed data, and wrote the first draft of the manuscript. L. R. Letertre performed laboratory thrombin generation assays, analyzed data, and reviewed the manuscript. S. J. Juliusson collected and analyzed clinical data, and edited the manuscript. B. R. Gudmundsdottir designed the protocol, supervised laboratory work, and edited the manuscript. C. W. Francis edited the manuscript. P. T. Onundarson designed the protocol, supervised analysis, and co-wrote the manuscript.

Disclosure of Conflict of Interests

P. T. Onundarson and B. R. Gudmundsdottir have been granted a patent on Fiix prothrombin time. The Fiix prothrombin time is an invention of P. T. Onundarson and B. R. Gudmundsdottir.

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