Relationship between international normalized ratio values, vitamin K-dependent clotting factor levels and in vivo prothrombin activation during the early and steady phases of oral anticoagulant treatment

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**Background and Objectives.** In vitro studies have shown that the rate of prothrombin activation is linearly related to the concentration of factor II (FII) in the assay system, suggesting a key role of prothrombin levels in the expression of the antithrombotic activity of oral anticoagulant treatment (OAT). We investigated the in vivo relationship between prothrombin activation and vitamin K-dependent clotting factor levels during the early and steady phases of OAT in patients and in healthy volunteers.

**Design and Methods.** The changes in international normalized ratio (INR) and in the plasma levels of FVII, FX, FII, protein C (PC) and prothrombin fragment 1.2 (F1+2) induced by OAT were monitored over 9 days in 10 patients – not on heparin – starting warfarin after heart valve replacement (HVR) and in 9 healthy volunteers submitted to an 8-day course of warfarin treatment. FII and F1+2 plasma levels were also measured in 100 patients on stable oral anticoagulant treatment with INRs ranging from 1.2 to 6.84.

**Results.** Because HVR patients had subnormal FVII, FX and FII levels after surgery, INR values > 2.0 were attained already 24 hours after the first warfarin dose. In healthy volunteers, INR values greater than 2.0 were first observed after 72 hours. Nadir levels of FVII, PC, FX and FII were reached between 40 and 88 hours in HVR patients and between 72 and 192 hours in healthy volunteers. The FII apparent half-disappearance time (t2/2) was 99 hours in HVR patients and 115 hours in healthy volunteers (p = ns). In HVR patients there was no normalization of initially elevated F1+2 levels until day 7 with an apparent t2/2 of 132 hours. In healthy volunteers, a decrease to subnormal F1+2 levels was observed by day 8 of treatment (apparent t2/2 = 107 hours). In both HVR patients and healthy volunteers, FII and PC levels were independent predictors of the changes in F1+2 levels (p = 0.0001). In patients on stable OAT, only FII levels were independent predictors of the variation in F1+2 levels (p = 0.0001).

**Interpretation and Conclusions.** During the early phase of oral anticoagulant treatment in vivo prothrombin activation is a function of the balance between FII and PC levels and is not significantly prevented until nadir levels of FII are obtained. This provides an explanation for the requirement of overlapping heparin and oral anticoagulant treatment for at least 48 hours after the achievement of therapeutic INR values in patients with thromboembolic diseases. In addition, in vivo prothrombin activation is a function of FII levels rather than INR values also in patients on stable oral anticoagulant treatment. ©2002, Ferrata Storti Foundation

Key words: oral anticoagulation, INR, factor II, protein C activity, prothrombin fragment 1.2, early phase of anticoagulation.

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clotting factors with a longer half-life. In vivo prothrombin activation can be monitored by measuring the levels of prothrombin fragment 1+2 (F1+2), which is released from factor II by the action of the prothrombinase complex. However, the relationship between the decrease in vitamin K-dependent clotting factors and the resultant degree of in vivo prothrombin activation has not been extensively investigated either during the early or the steady phase of oral anticoagulant treatment. To evaluate such a relationship, we measured F1+2 levels in patients and in healthy volunteers submitted to oral anticoagulant treatment without concomitant heparin therapy. In addition, we also explored the association between factor II, protein C and F1+2 levels in a group of patients on stable oral anticoagulation.

Design and Methods

After obtaining informed consent, 10 patients submitted to surgery for heart valve replacement (6 women and 4 men, mean age 63±14 years, 5 mitral valve, 3 aortic valve, 2 mitro-aortic valve) and free of surgical complications were enrolled in the study. They each received 5 mg of warfarin on the first or second postoperative day with later dosages adjusted based on INR values obtained daily.

Nine healthy subjects (6 men, 3 women, mean age 38±6 years) agreed to a protocol involving assumption of warfarin (10 mg/day) for 3 days, with the following dosages adjusted based upon daily INR measurements for a total of 8 days.

One hundred patients (48 F, 52 M, mean age 69±11 yrs) on oral anticoagulant treatment for at least 3 months with warfarin (n = 68) or acenocoumarol (n = 32) because of: atrial fibrillation (n = 46), peripheral artery occlusive disease (n = 14), venous thromboembolism (n = 24) or heart valve prosthesis (n =16) attending our anticoagulation clinic agreed to allow their plasma to be evaluated for factor II:C and F1+2 content.

Plasma samples were selected to span a wide range of INR values. Patients with lupus anticoagulants were not included in this series. The study was approved by the Ethical Committee of our Institution.

Laboratory parameters

Blood samples (5 mL) were taken from the patients until day 9 of treatment, for a total of 15 samples, and from the healthy subjects until day 15 (seven days after interruption of anticoagulant treatment, for a total of 15 samples). Blood was collected into vacutainer tubes (Becton-Dickinson) containing tri-sodium citrate (0.19 M) and, after centrifugation, platelet-free plasma was immediately used to test INR values (Recombiplastin, Instrumentation Laboratory, Milan, Italy, ISI = 0.90). Plasma aliquots (0.4 mL) were snap frozen with methanol and dry ice and stored at -70°C until assayed for clotting factors and F1+2 levels. The coagulant activity of factor VII, X and II was measured in one-stage assays using the above mentioned thromboplastin reagent and immunodepleted plasmas from Stago (Genevilliers, France). The anticoagulant activity of protein C was measured by a commercial reagent (STA Protein C clotting, Stago). Calibration curves were obtained using dilutions of normal pooled plasma collected from 40 apparently healthy subjects (20 men, 20 women) and stored at -70°C. F1+2 levels were measured by a commercially available ELISA (Dade-Behring), using an ELISA reader (Titertek Multiscan). In a population of 60 apparently healthy subjects (30 women and 30 men, mean age 40.8±12.2 years) F1+2 levels were 0.500±0.17 nM (range: 0.27-0.92 nM).

Statistical analysis

For descriptive purposes, mean±SD or 95% confidence limits of the mean are reported for the laboratory parameters Mean±SE or median and interquartile ranges are reported in the figures. Statistical analysis was conducted on log-transformed data to approach normal distributions. One-way analysis of variance for repeated measures was separately used in HVR patients and in healthy volunteers to evaluate the significance of the changes for each of the laboratory parameters. Post-hoc comparisons were carried out with the Student’s t test for paired data applying the Bonferroni’s correction. Apparent half-disappearance times were extrapolated by linear regression analysis using measurements obtained until 48 hours (INR, FVII, PC), 88 hours (FX, FII) and 147 hours (F1+2) in patients and until 72 hours (INR, FVII, PC) and 120 hours (FX, FII, F1+2) in healthy volunteers. Differences between HRV patients and healthy volunteers were evaluated by analysis of variance or the t test for unpaired data. The independent effects of vitamin K-dependent clotting factors and of other parameters on the variation in F1+2 levels was explored in the generalized linear model. One-way analysis of variance was used to estimate differences in INR, FII, PC and F1+2 values in patients on stable oral anticoagulant treatment grouped according to tertiles of either INR, PC or FII distribution.
Results

As expected because of the disturbances of coagulation associated with cardiopulmonary bypass surgery,\textsuperscript{7,8} the baseline levels of vitamin K-dependent clotting factors and F1+2 differed significantly in patients and healthy volunteers beginning oral anticoagulant treatment. Thus FVII, FX, PC and FII levels were lower and INR and F1+2 levels higher in patients than in healthy volunteers (Table 1).

Figure 1 shows the changes observed in INR and F1+2 in patients and in healthy volunteers after the initiation of oral anticoagulant treatment. In HVR patients a significant increase in INR was already observed after 24 hours (\(p = 0.025\)), with the highest values recorded by day 2 of treatment. Initially elevated F1+2 levels (\(p < 0.0001\) versus the control population) did not decrease significantly until day 4 (88 h), with normalization of plasma F1+2 (0.68±0.26 nM) at 160 hours (Figure 1, left panel). In healthy volunteers, significant daily increments in INR were observed up to 96 hours (\(p < 0.02\)), but values greater than 2.0 were only attained after 72 hours of treatment. Average INR values greater than 2.0 were maintained for 24 hours and then gradually increased until day 7 after the interruption of anticoagulant treatment. FII levels were still significantly lower than baseline (80%±7%, \(p = 0.001\)) 7 days after the interruption of anticoagulant treatment (Figure 2, right panel).

Table 1 shows average baseline and individual nadir levels and apparent half-disappearance times \((t/2)\) of the variables evaluated in HVR patients and healthy volunteers.

The time interval required to attain an average INR value of 2.0 was three-fold longer in healthy volunteers than in HVR patients.
volunteers than in HVR patients (p = 0.01). FVII and PC disappeared more rapidly in patients than in healthy volunteers (p ≤ 0.03), while no significant difference was observed for the disappearance rate of FX, FII and F1+2 (Table 1).

Thus, both in patients and in healthy volunteers a significant reduction in F1+2 levels was temporally associated with the attainment of nadir levels of factor II (40% or lower), which followed the observation of therapeutic INR values by 48-72 hours.

The relationship between the changes in F1+2 levels and in vitamin K-dependent factors was explored in a generalized linear model including F1+2 levels as the dependent variable and vitamin K-dependent factors and time as predictors. Of the vitamin K-dependent clotting factors, only FII (p = 0.0001) and PC (p = 0.0001) were independent predictors of F1+2 levels, both in HVR patients and healthy volunteers. In HVR patients, FII (r partial = 0.377) and PC (r partial = -0.349) explained 31.3% of the variation in F1+2 levels, with time (r partial = -0.601) contributing an additional 11.8%. In healthy volunteers, FII (r partial = 0.413) and PC (r partial = -0.327) explained 25.9% of the variation in F1+2 levels. Neither in patients nor in healthy volunteers were the changes in INR predictive of the variation in F1+2 levels.

The relationship between INR, FII, PC and F1+2 levels was also examined in patients on stable oral anticoagulant treatment. To this purpose, F1+2 levels were related to the tertiles of the INR, FII and PC distributions. Tertiles included INR values lower than 2.40, between 2.40 and 3.69, and greater than 3.75; FII levels lower than 22%, between 22% and 30%, and greater than 30%; PC levels lower than 14%, between 14% and 22%, and greater than 22%. When compared according to the INR distribution, PC levels were not significantly different in the lower and intermediate tertiles, while they were lower in the higher tertile of the INR distribution (p = 0.0001, Figure 3, upper panels). At variance, there was a good overall agreement between INR values and FII levels, which differed significantly according to the tertiles of the INR distribution (p = 0.0001). F1+2 levels were similar in the lower and intermediate tertiles of the INR distribution and in the corresponding tertiles of the PC distribution, while they were lower in the presence of low PC and high INR values (p ≤ 0.042). At variance, F1+2 levels were lower in the first tertile (p = 0.001) and higher in the third tertile (p = 0.04) than in the intermediate tertile of the FII distribution (Figure 3, lower panels). In a generalized linear model including gender, age, indication for anticoagulant treatment, INR, PC and FII levels as predictors, 24% of the variation in F1+2 levels was explained by FII levels only (p = 0.0001).

Discussion
In this study, we first examined the relationship between in vivo prothrombin activation, reflected by F1+2 levels, and the decrease in vitamin K-dependent factors in patients and in volunteers beginning oral anticoagulant therapy without heparin treatment. The rate of thrombin generation is obviously dependent on the initial stimulus and the efficiency of natural anticoagulant mechanisms. Patients submitted to heart valve replacement surgery had higher F1+2 levels than healthy volunteers, despite their lower levels of vitamin K-dependent clotting factors. A significant reduction...
In F1+2 levels was first observed 88 and 120 hours after the initiation of anticoagulant treatment in patients and in healthy volunteers, respectively. In both groups of subjects, the decrease in F1+2 levels was not associated with the achievement of therapeutic INR values (>2.0), but it was related with the changes in the plasma levels of FII, following the attainment of therapeutic INRs by 24-48 hours. F1+2 from the circulation has been estimated to be cleared from the circulation in about 90 min. In vitro experiments have clearly shown that the rate of thrombin generation is linearly related to the factor II concentration, consistent with the Km for the prothrombinase complex. This predicts that in vivo, the decrease in F1+2 levels would strictly follow the decrease in factor II levels upon initiation of oral anticoagulant treatment. In our series, we observed a statistically significant association between FII and F1+2 levels, confirming a causal relationship between circulating FII and in vivo prothrombin activation.

In addition we also identified a negative relationship between PC and F1+2 levels, confirming the relevance of the protein C system in modulating in vivo prothrombin activation during the early phase of oral anticoagulant treatment. To our knowledge, the changes in F1+2 levels during the early phase of oral anticoagulant treatment have been explored, but less extensively, in three papers. In one paper, the initiation of oral anticoagulants in two subjects who did not exhibit protein C deficiency led to a paradoxical increase in F1+2 levels during the first day of therapy. In 10 healthy volunteers treated with a high- or a low-intensity warfarin regimen for only 4 days, Kyrle et al. observed a similar 20% to 30% decrease in F1+2 levels by 72 hours independent of the greater reduction in both FII and protein C levels obtained with the high-intensity regimen. A third study evaluated INR, factor II and F1+2 levels in the initial phase of oral anticoagulant therapy in 39 patients following aortic or mitral valve replace-

Figure 3. Relationship between INR, factor II, PC and F1+2 levels in patients on stable oral anticoagulant treatment. Upper panels: INR, PC and FII levels according to tertiles of the INR distribution. Lower panels: F1+2 values according to tertiles of the INR, PC and FII distribution. P values refer to significance of the difference versus the preceding tertile.
The authors observed that despite the INR being in the therapeutic range, FII and F1+2 levels remained high, suggesting their superior in the monitoring of the early phase of oral anticoagulation. Our data provide a biochemical basis for the requirement of prolonging heparin therapy for at least 2 days after reaching therapeutic INR values, because of the possibility that high INR values may reflect a decrease in FVII and PC, but not yet FII and hence F1+2 levels. The relationship between INR and F1+2 levels in patients on stable oral anticoagulant treatment has been addressed in many studies. With few exceptions, reduction or near-normalization of F1+2 levels at a low intensity of anticoagulation was reported in these studies; however, no further decrease in F1+2 levels was observed in some studies for INR values greater than 4.0.

High INR values may not necessarily reflect a profound depletion of factor II, and, to our knowledge, no study has evaluated the relationship of F1+2 levels with factor II levels in such patients. In patients on stable oral anticoagulation with a wide range of INR values, we found a better agreement of F1+2 levels with factor II levels than with INR or PC values.

Although the strength of the association might be somehow impaired by additional parameters, these data suggest that FII levels may reflect the true in vivo degree of anticoagulation better than INR values not only during the early phase of oral anticoagulant treatment, but also in patients on stable anticoagulation. With the introduction of portable prothrombin time monitors that monitor patients' self-adjustment of the oral anticoagulant dose is becoming popular in some European countries and in the US.

The process of INR calibration aims to obtain comparable sensitivity of thromboplastin reagents to factor II levels, but discrepant sensitivities of thromboplastin reagents to the different vitamin K-dependent factors explored by the prothrombin time may be responsible for uncertainty in true INR values. Taken together these data are a strong indicator that monitoring FII levels in patients on oral anticoagulant treatment may represent a good alternative to INR monitoring, reflecting in vivo antithrombotic potential more closely.

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ADA, LC, AF and SVD contributed to the design of the study, analyzed the data and prepared the manuscript. PDV and EP performed the clotting assays reported in this study.

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