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Comparison of Plasma Prothrombin and Factor VII and Urine Prothrombin F1 Concentrations in Patients on Long-Term Warfarin Therapy and Those in the Initial Phase

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Control of warfarin anticoagulation during the initial phase of therapy is difficult and empirically based. Plasma and urine samples were obtained from normal controls, patients under stable anticoagulation, and patients in the initial phase of anticoagulation. Total plasma prothrombin, des-carboxy (non-adsorbable with barium chloride) prothrombin, and native (total minus non-adsorbable) prothrombin were quantitated using Echis carinatus venom activation. Functional plasma factor VII (VII) was measured using a one-stage clotting assay. Total and des-carboxy urine prothrombin F1 (F1) were measured by ELISA. All urine F1 in normals and both anticoagulated groups was adsorbed by barium chloride. Plasma des-carboxy prothrombin concentration was similar for the two anticoagulated groups and did not correlate with 1/INR. Native prothrombin correlated with 1/INR in both the stable (r = 0.76) and initial phase (r = 0.74) groups. For any given INR, the subjects on stable anticoagulation had lower native prothrombin concentrations than the initial phase patients. Functional factor VII concentration also correlated significantly with 1/INR in both the stable (r = 0.64) and initial phase (r = 0.76) patients. Unlike native prothrombin, VII concentrations did not vary between the two cohorts for any given INR. Previous studies indicate that native prothrombin is a superior predictor of both hemorrhagic and thromboembolic complications during warfarin therapy. Our findings indicate that VII, and not prothrombin, may be the predominant factor monitored by the INR. This further supports the need to reevaluate the usefulness of the INR in the monitoring of warfarin therapy during the initial phase. Am. J. Hematol. 57:193–199, 1998.

Key words: warfarin; prothrombin; anticoagulation

INTRODUCTION

Warfarin and similar oral anticoagulants inhibit the vitamin K-dependent gamma-carboxylation of glutamic acid residues on factors II, VII, IX, and X and proteins C and S. The resulting under- or des-carboxylated forms are incapable of participating in calcium-dependent activation and are either retained or partially or completely released into the circulation via species- and factor-specific mechanisms [1,2].

The prothrombin time, first proposed by Quick in 1935 [3], is a means of monitoring and regulating therapy with vitamin K antagonists and an expression of in vitro extrinsic coagulation. Its greatest advantages are the relative simplicity and rapidity with which it can be performed. However, a multitude of factors can alter the time to a fibrin clot including several coagulant molecules and inhibitors, calcium, pH, and the means of handling and expediency with which the test is performed [4]. The prothrombin time's relationship to each individual factor and the subsequent correlation with in vivo coagulation is unclear. The INR (International Normalized Ratio) is calculated by dividing the patient's prothrombin time by a control prothrombin time and raising the quotient to a power termed the ISI (International

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Sensitivity Index). The ISI is provided by the manufacturer and depends on the activity of the tissue thromboplastin used in the clotting assay. Reporting of the prothrombin time as an INR minimizes the variability between institutions secondary to heterogeneity in tissue thromboplastins but does not effect any of the other in vitro shortcomings outlined above.

Ten to 20% of patients treated with warfarin develop a hemorrhagic or thromboembolic complication [5–8] during their course of therapy. In the initial phase of anticoagulation (i.e., the first weeks of therapy), the INR is exquisitely labile and its relationship to the plasma concentration of vitamin K-dependent coagulants is complex [9,10]. The risk of hemorrhage per 100 patient months is increased between 2–7 times in the first 3 months of therapy compared to patients on warfarin for greater than 1 year [6–8]. Several previous studies indicate that the plasma concentration of native (i.e., functionally carboxylated) prothrombin may be a more accurate indicator of the likelihood of thromboembolic and hemorrhagic complications associated with warfarin therapy [9–13]. Therefore, we measured the concentration of multiple fragments of prothrombin in both the plasma and the urine to evaluate potentially superior markers for the monitoring of warfarin anticoagulation.

There are various methods available for the measurement of native prothrombin and its des-carboxy or undercarboxylated derivatives [5,14–16]. By varying the target region, immunologic methods can be sensitive to the native prothrombin [17], the des-carboxylated derivative [18], or both. Activity measurements depend on the conversion of prothrombin to thrombin and its subsequent activity on a chromogenic substrate or fibrinogen. If prothrombin activation is performed using calcium-dependent physiologic activators such as tissue thromboplastin or factor Xa, the reaction measures only the native prothrombin. In contrast, the use of prothrombin activating snake venoms indiscriminately quantitates both the native and des-carboxy forms of prothrombin. Native prothrombin is selectively adsorbed by insoluble, barium and calcium salts, allowing its separation from des-carboxy forms. The molecules can then be separately quantitated using either the Echis venom or an ELISA with a non-selective antibody.

Prothrombin fragment 1 (F1) is the amino-terminal region of the prothrombin molecule that includes the Gla-residues necessary for calcium-dependent activation. Fragment 1 + 2 is released from prothrombin upon cleavage by the prothrombinase complex and has been studied as a potential marker of in vivo coagulation in hypercoagulable states [19] and in oral anticoagulant therapy [20–22]. Fragment 1 is liberated from fragment 1 + 2 by thrombin, is present within the urine of healthy controls, and may be increased in hypercoagulable states [19,23]. Measurement of urine fragment 1 offers promise as an alternative means of monitoring oral anticoagulation or detecting hypercoagulable states.

MATERIALS AND METHODS

Plasma samples from anticoagulated subjects were obtained from the George Washington University Hospital Laboratory on the day of collection and frozen at −50°C. All ''stable'' anticoagulated samples were drawn during outpatient visits to the clinics of the George Washington University Medical Center. All ''initial phase'' anticoagulated samples were obtained from inpatients at the George Washington University Hospital. Twenty-one of the thirty initial phase patients were receiving heparin in addition to warfarin. Hospitalized patients concurrently receiving thrombolytic therapy were not included. Normal control plasmas were obtained from healthy volunteers, centrifuged at 2,000*g* for 15 min and stored at −50°C. All samples were collected by venipuncture into a vacutainer containing 1/10 volume of 3.8% sodium citrate (Becton-Dickson, San Jose, CA). Standard plasma was obtained from George King Laboratories (Overland Park, KS). Normal control urines were obtained from healthy volunteers and stored at −50°C. Initial phase patient urine samples were obtained within 3 hr of blood sampling. Stable patient urine samples were obtained from patients seen at the clinics of the George Washington University Department of Hematology and Oncology.

Prothrombin Time

Prothrombin times were performed by the George Washington University Hospital Laboratory on the date of collection. The INR was calculated based on standard plasma and the manufacturer's reported ISI.

Barium Adsorption

Barium adsorption was performed by adding one part 10% BaCl to nine parts undiluted plasma or urine and centrifuging at 10,000*g* for 2 min.

Echis Assay

Sample plasma (100 μL) diluted 1:100 in 0.02M Tris-0.15M NaCl buffer, pH 7.2, was added to a microtiter plate well (Fisher, Pittsburgh, PA). Then 50 μL of 0.025 mg/ml Echis venom (Sigma, St. Louis, MO) diluted in Tris was added and the plate was gently shaken. At 10 min, 50 μL of 0.5 mM S-2238 (Chromogenix, Franklin, OH) was added and the plate was read kinetically at 405 nm for 2 min (Molecular Devices, Palo Alto, CA).

ELISA

Prothrombin and F1 were measured by an enzyme-linked immunosorbent assay (ELISA) sandwich method with anti-human prothrombin (Dako, Carpinteria, CA) that non-selectively binds fully- and des-carboxylated forms of prothrombin and F1. Urinary F1 was measured in arbitrary units. Urine creatinine was quantitated using

TABLE I. Plasma INR and Prothrombin Concentrations (% of Standard)*

	INR	Native II	Des-carboxy II	Total II
Normal (n = 40)		104.5 ± 12.3	6.19 ± 2.16	110.1 ± 25.16
Initial phase (n = 40)	2.16 ± 1.07	65.05 ± 27.69 (r = 0.74)[a]	14.07 ± 5.77 (r = 0.15)[a]	79.11 ± 30.20 (r = 0.70)[a]
Stable (n = 40)	2.13 ± 0.58	36.56 ± 14.23 (r = 0.64)[a]	12.80 ± 6.43 (r = 0.10)[a]	49.35 ± 14.24 (r = 0.65)[a]

*Normal concentration of total II = 108 ± 19 μg/mL.
[a]r values represent correlation with 1/INR.

a Kodak Ektachem DT60 (Eastman Kodak, Rochester, NY) and the Kodak Ektachem DTSC module. F1 results are given relative to a single ''reference'' urine in units per mg creatinine.

Factor VII Assay

Sample plasma (25 μL) diluted 1:5 in 0.02M Tris-0.15M NaCl buffer, pH 7.2, was added to 25 μL of Factor VII deficient plasma (George King Laboratory). Equal volumes of tissue thromboplastin (100 μL) and 0.025M $CaCl_2$ were added and the clotting time was measured using an ST4 clot timer (American Bioproducts, Parsippany, NJ).

ANALYSIS

As previously reported [9,15] the ELISA and Echis assays were highly correlated (data not shown). The plasma prothrombin data presented below was obtained using the Echis assay to measure total prothrombin. Native prothrombin was calculated as the difference between the total prothrombin and the non-adsorbable (i.e., des-carboxy) prothrombin. The urine F1 data was obtained using the ELISA. The relationships between INR and both native prothrombin and functional factor VII approximated rectangular hyperbolas. Therefore, the data was analyzed and presented using the inverse of the INR.

RESULTS

Plasma

Stably anticoagulated patients show a substantial decrease in the native prothrombin as compared to normals (Table I). When the stable patients are compared with patients in the initial phase of anticoagulation, significant differences exist. Despite a similar mean and distribution of INR between the anticoagulated cohorts, the amount of both native (Fig. 1a, b) and total prothrombin differs significantly (Table I). The average des-carboxy prothrombin was similar between the two groups and no relationship existed between des-carboxy prothrombin and 1/INR in either group.

The relationship between 1/INR and native prothrombin is seen in Figure 2. The concentration of native prothrombin correlates significantly with 1/INR in each anticoagulated cohort. For any given INR, stably anticoagulated patients have lower native prothrombin concentrations than initial phase subjects with the same INR. No significant differences were noted between initial phase patients receiving concurrent heparin and warfarin therapy and those receiving only warfarin (data not shown).

The concentration of factor VII also correlated significantly with 1/INR in both anticoagulated cohorts (Table II). In contrast to prothrombin, there was not a significant difference between the two groups in the concentration of factor VII for any given INR (Fig. 3).

Urine

The urine concentration of creatinine was higher in controls than in either anticoagulated group. All urine F1 in stably anticoagulated patients, patients in the initial phase of anticoagulation, and controls was adsorbable with barium chloride. The concentration of urine F1 per mg creatinine in the stably anticoagulated patients was much lower than in the non-anticoagulated controls. In contrast, urine F1 per mg creatinine among the unstably anticoagulated patients was higher than in the controls (Table III).

DISCUSSION

Despite concerted efforts over the span of decades to standardize the prothrombin time, oral anticoagulation exists in the absence of definitive clinical trials demonstrating the most efficacious regimen for induction, maintenance, and monitoring of therapy [24,25]. Accepted practice in the in-patient setting begins with 5 days of combined heparin and warfarin therapy. Typically, the first few doses of warfarin are empirically based and subsequent doses rely on daily prothrombin times. Hemorrhage and thromboembolism are the primary adverse events associated with excessive and inadequate anticoagulation, respectively, and are related to multiple risk factors including age, intensity of anticoagulation, and the presence of co-morbidities [6–8,26,27].

Based on previous findings [5,11–13], the plasma con-

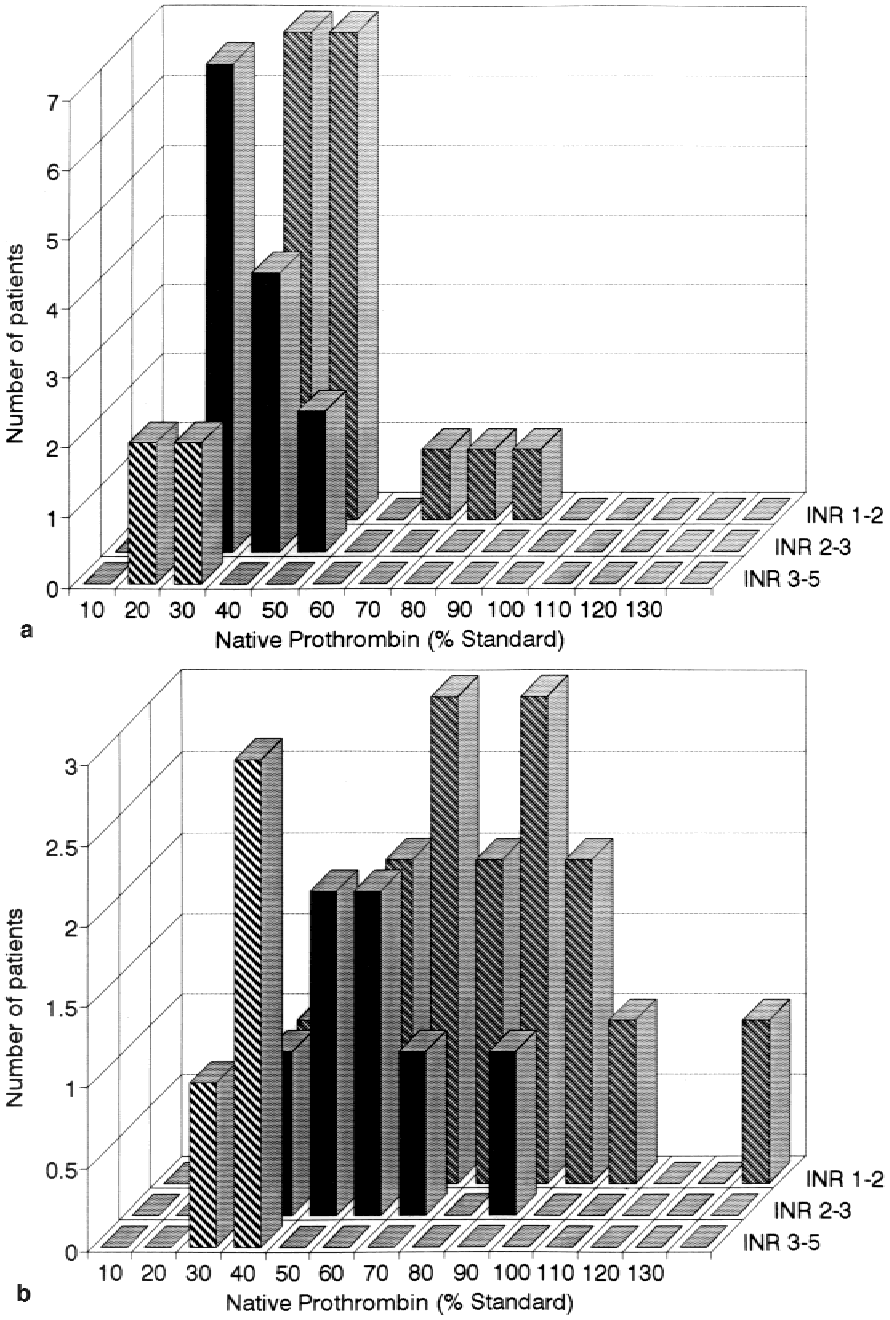

Fig. 1. Distribution of native prothrombin concentration for stable (a) and initial phase (b) patients stratified by INR. For each range of INR, a lower concentration of native prothrombin is noted in stably anticoagulated patients as compared to those in the initial phase.

centration of native prothrombin may be superior to the INR as a predictor of both hemorrhage and thromboembolism in patients on warfarin. Furie et al. [11] conducted a randomized prospective trial comparing the incidence of adverse events when warfarin therapy was regulated within either a prothrombin time index (patient prothrombin time/control prothrombin time) of 1.5–2.0 or a concentration of native prothrombin between 12–24 μg/ml (normal = 108 ± 19 μg/mL). Overall, the group monitored using the native prothrombin had 85% fewer combined hemorrhagic and thromboembolic complications. Thirty years earlier, Sise et al. [12] studied a large group of patients on stable anticoagulation and recommended the regulation of prothrombin concentration between 12–25% using a one-stage factor assay in place of the Quick time. Kornberg et al. [13] compared the predictive values of native prothrombin and INR in the monitoring of warfarin prophylaxis following total hip arthroplasty. They found significant correlations between the development of both hemorrhagic and thromboembolic complications and the concentration of native prothrombin. No such correlation existed between those ad-

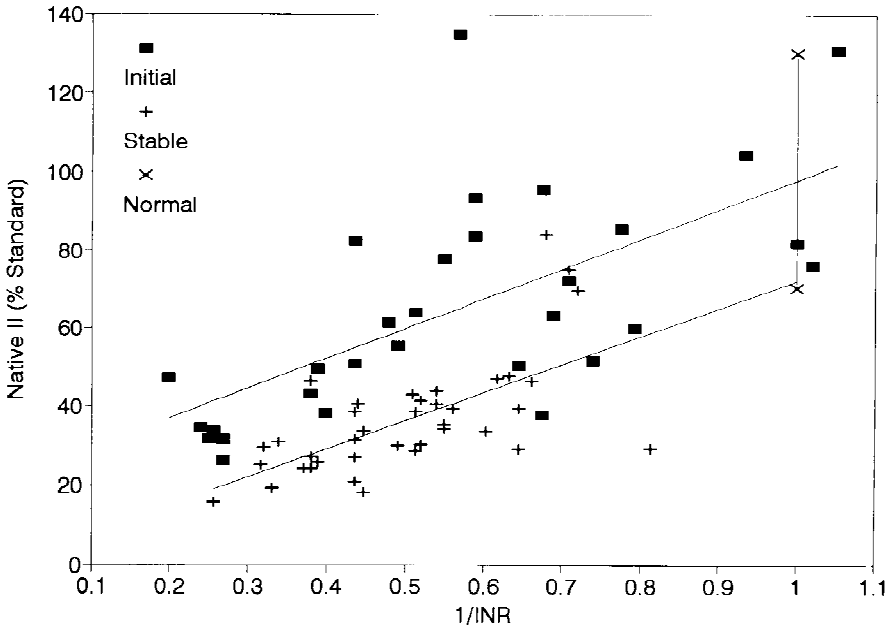

Fig. 2. Relationship between native prothrombin and 1/INR in stable and initial phase patients. Significant correlation exists in both patient populations. For any given INR, the concentration of native prothrombin among stably anticoagulated patients is significantly lower than among patients in the initial phase.

TABLE II. Comparison of Factor VII Concentrations (% of Standards)*

	INR	Functional Factor VII
Initial phase (n = 40)	2.16 ± 1.09	24.55 ± 19.60 (r = 0.72)[a]
Stable (n = 40)	2.27 ± 1.55	23.97 ± 12.75 (r = 0.64)[a]

*Normal concentration of factor VII = 0.5 ± 0.13 μg/mL.
[a]r values represent correlation with 1/INR.

verse events and the INR. The relationship between native prothrombin concentration and anticoagulation is further supported by in vitro experiments demonstrating that above very low levels of factors VII, IX, or X, thrombin formation is solely dependent on the concentration of functional prothrombin [14]. Therefore, the difference in native prothrombin between stable and initial phase patients may indicate a disparity in in vivo anticoagulation between patients from each group with similar INR.

The decrease in native prothrombin is dictated by immutable pharmacokinetic principles. If the half life of plasma prothrombin is 80 hr [28], it will take 1 week to reach the therapeutic range of 25% native prothrombin assuming: (1) there is instant and total inhibition of gamma carboxylation, (2) no previously formed active prothrombin is released into the circulation, and (3) complete and free exchange exists between the intravascular and extravascular compartments with a similar prothrombin half-life in both compartments. Sise et al. [12] measured functional prothrombin concentrations daily after a single dose of 300 mg of intravenous warfarin in five patients who had received no previous anticoagulation. Only after 5 days did all patients have prothrombin concentrations below 40%. Upon initiating warfarin therapy, the INR reaches a therapeutic value significantly faster than the concentration of native prothrombin. O'Reilly et al. [29] measured the ''prothrombin complex'' concentration using a saline dilution curve after a single loading dose of 1.5 mg/kg of warfarin. The patient plasma concentrations decreased to 37 ± 6% of normal by 24 hr and 24 ± 4% of normal by 36 hr.

The INR represents an undefined relationship between multiple coagulation factors and the in vitro milieu. Factor VII has the shortest half-life of the vitamin K-dependent coagulation factors ($t_{1/2}$ = 6 hr). Therefore, a rapid fall in VII concentration appeared to us as the most likely explanation for the precipitous rise in INR after initiating warfarin therapy. As Figure 3 indicates, no appreciable difference exists between the two patient cohorts in the relationship between factor VII concentration and INR despite the difference in concentrations of native prothrombin. Therefore, the INR during the initial phase reflects a combination of factor concentrations different from those present under stable anticoagulation and may depend primarily on factor VII.

The above findings seem to indicate that upon completion of heparin therapy, the patient with a therapeutic INR is left inadequately anticoagulated and susceptible to further thromboembolic phenomena. Yet, the literature indicates a significantly increased incidence of bleeding in the initial period of anticoagulation. Two commonly accepted explanations have been offered [6,7]. The first is that doses are adjusted more frequently and the INR is often erratic during the first few months of therapy. This is an attractive explanation as it implies that more vigilant screening and expertise in control will minimize ad-

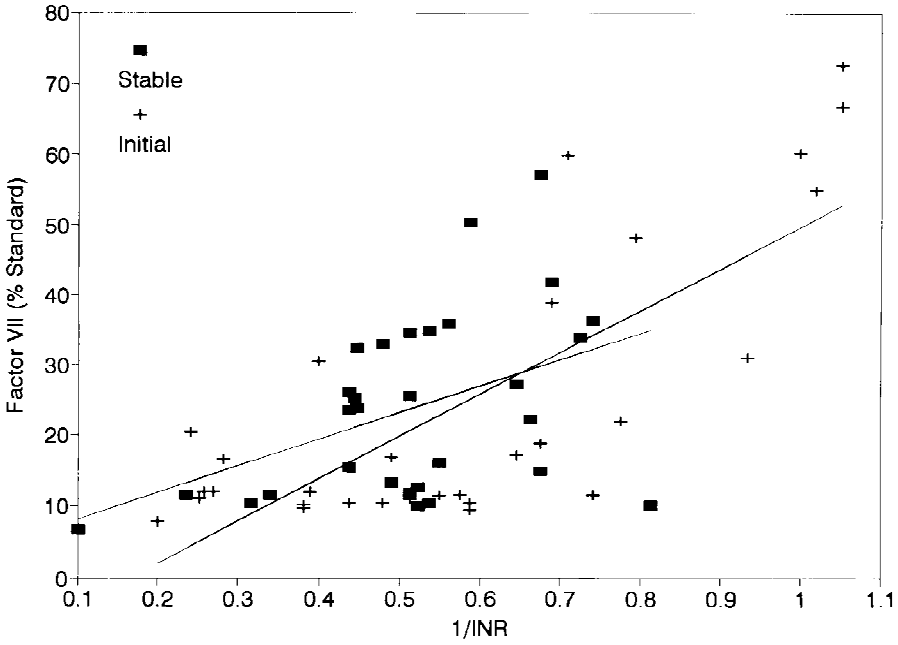

Fig. 3. Relationship between functional factor VII and 1/INR in stable and initial phase patients. A significant correlation exists in both patient populations. No significant difference in factor VII concentration is noted between patient populations for any given INR.

TABLE III. Comparison of Urine F1 Concentrations

	Number	INR	Urine F1 (units)[a]	Urine Cr (mg/dl)	Urine F1/Cr (units/mg/dl)[b]
Normal	18		115 ± 42	116 ± 84	1.53 ± 1.13
Stable	8	2.16 ± 0.87	69 ± 34	69 ± 38	1.21 ± 0.75
Inital phase	44	2.08 ± 1.03	137 ± 56	73 ± 41	2.42 ± 1.49

[a]Percent of a single reference urine.
[b]Values were calculated individually and then averaged.

verse events. Assuming that outpatient warfarin therapy is monitored every 2 weeks, a patient with an INR of 2.0 at their previous check-up who presents with an INR of 4.0 may have a significantly decreased concentration of all vitamin-K dependent coagulation factors. However, in the patient beginning warfarin therapy in the hospital, an increase in INR from 2.0 to 4.0 over a 24-hr period reflects a significant change in factor VII concentration with only a small change in native prothrombin. It is important to note that the natural history of patients with congenital factor VII deficiency is variable. Typically, even a very low level of functional VII will prevent spontaneous bleeding. In fact, a level of only 10–20% of normal is considered adequate to assure normal hemostasis at the time of major surgery [30,31].

The second explanation for the increased incidence of hemorrhage in the first few months of warfarin therapy is that patients with a high risk for hemorrhage (i.e., those with a subclinical lesion or hemorrhagic diathesis) will bleed during the initial period of anticoagulation. This is consistent with several studies indicating an increased likelihood of hemorrhage in anticoagulated patients with multiple comorbidities [6–8,26]. If this is the case, the only means for decreasing the incidence of hemorrhage in the initial phase may be more aggressive screening of patients before starting anticoagulation. Those patients without a bleeding predisposition may, in fact, be inadequately anticoagulated for the days to weeks after the completion of heparin therapy and before the concentration of native prothrombin falls within the therapeutic range. Whether the potential benefits of prolonging heparin therapy until the patient achieves a therapeutic concentration of prothrombin outweigh the risks is uncertain.

A number of possibilities exist to explain the discrepancy between urine F1 concentrations in the control and anticoagulated cohorts. The control samples were obtained from younger, healthy volunteers. The relative decrease in creatinine clearance and muscle mass with age probably account for the higher concentration of creatinine found in the control group. F1, with a molecular weight of approximately 22,000, is easily filtered at the glomerulus [32]. Whether reabsorption or secretion occurs distal to the glomerulus is under investigation [33]. The decrease in F1 per mg creatinine in the stably anticoagulated patients may represent a response to anticoagulation. With the down-regulation of prothrombin cleavage, less F1 + 2 is liberated and subsequently less F1 appears in the urine. The concentration of urine prothrombin fragments may be increased in states where prothrombin cleavage is up-regulated (e.g., acute thrombus formation) [19]. This may explain the elevated F1 per mg creatinine found in unstably anticoagulated patients. Therefore, a system for monitoring warfarin therapy using the concentration of urine F1 would depend on both the patient's renal function and the presence of acute clot formation and degradation. The inability of

des-carboxylated prothrombin molecules to become activated and release F1 + 2 in the circulation and periphery may explain the absence of des-carboxy F1 from all urine samples. On the other hand, secretory mechanisms specific for fully carboxylated moieties may exist within the nephron, which explains the presence of only native F1 in urine [33].

The significant correlation between 1/INR and native prothrombin indicates that the INR, though perhaps not optimal, may be an adequate approach to the monitoring of long-term warfarin therapy. This is not the case among patients in the initial phase when a therapeutic INR is not predictive of in vivo anticoagulation. A more appropriate means of initiating therapy would begin with 1–2 weeks of an estimated therapeutic dose of warfarin before acquiring an initial INR. Because the patient's body habits, age, diet, hepatic function, and other medications all may effect the response to warfarin, estimating the proper dose is extremely difficult. Therefore, the monitoring of oral anticoagulation using an ELISA or Echis venom-based assay may facilitate the clinician's role in the initial phase and decrease the incidence of adverse events associated with both excessive and inadequate warfarin therapy.

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