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LETTERS TO THE EDITORS

Hemophilia, rodentophilia and humanity

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See also Giangrande PLF. Treatment of hemophilia: recombinant factors only? Yes. *J Thromb Haemost* 2003; **1**: 214–5; Mannucci P.M. Treatment of hemophilia: recombinant factors only? Yes. *J Thromb Haemost* 2003; **1**: 216–7.

Dear Sir,

PM Mannucci's scholarly letter [1] discussing plasma vs. recombinant clotting factors could not be more timely. For the past several years, we have tried to enlist collaboration in methods to purify and increase the yield of plasma proteins, particularly coagulation factors, but the official recommendations of the National Haemophilia Foundation and the World Federation of Hemophilia are to utilize recombinant products in the treatment of hemophilia, and the world follows those recommendations; two-thirds of all factor (F) VIII sales are of recombinant products [2]. New plasma derivatives would thus seem outdated and unwanted, even though, as Mannucci points out, access to such clotting factors is not available to 80% of the world's population.

We have reported that simply increasing the citrate content of plasma produces a linear increase in cryoprecipitate, yielding about 100% FVIII at 10–12% citrate. This 'supercryoprecipitate' is a cost-effective alternative to recombinant FVIII techniques, which could provide worldwide availability of the clotting factor, and can be used in a blood bank or during the manufacture of FVIII concentrates [3].

As I recently wrote [4], purification of therapeutic proteins by monoclonal antibodies is empirically and intellectually exciting, but scientifically unsound and even dangerous, especially in light of the abundance of 'natural' FVIII and FIX in human plasma. Once antibodies bind to antigens during the chromatographic procedures used to purify the clotting factors, the energy released by the binding causes atomic disruptions that result in conformational changes and molecular reorganization. The protein may still function but it is not 'natural' and can be expected to elicit undesirable effects; shortened half-life, antigenic potential and elicitation of 'infusion reactions.' In fact, such reactions seem to be so commonplace now that the mild term 'adverse reaction' implies the acceptability of such issues as anaphylaxis and serum sickness. If, as Mannucci mentions, new infectious agents remain a risk, it is hard to believe that we are willing to accept proteins for intravenous injections that are produced in the ovarian cells of Chinese hamsters and purified on proteins (monoclonal antibodies) made in cells (malignant ones, at that) in mice. Of course, potential new infectious agents are much more likely in recombinant products and must be made as safe as plasma proteins.

While Mannucci rightly discusses the newest blood-transmitted virus, West Nile, attention also should be given to the hundreds of other common, non-enveloped enteroviruses, rhinoviruses and picornaviruses (associated with the common cold), and the infectivity of some pathogens such as norovirus, which has been plaguing cruise ship passengers.

If medicine continues its present vogue of utilizing mouse-made monoclonal antibodies for a wide range of disease, the trace quantities of those proteins found in so many products may well cause what are euphemistically called 'adverse reactions'. It took more than 10 years to gain interest in using detergents to inactivate lipophilic viruses (the solvent tri-*n*-butyl phosphate [TNBP] is not really needed) [5]. It has taken at least that long to encourage interest in the use of iodine as a second disinfectant; an added measure of safety for any therapeutic protein. By admixing iodinated polystyrene-divinylbenzene anion exchange resin with the base (non-iodinated) resin in a 'salt and pepper' arrangement, plasma flows over the ion-exchange column, inactivating bacteria and viruses and capturing bound and unbound iodine. No known pathogens, not even prions, are truly resistant to iodine sterilization (cascade iodination) [6].

Since more plasma is being obtained to produce gammaglobulin for intravenous use, we should then take advantage of this opportunity to obtain greater quantities of natural human FVIII. We should not ignore these opportunities to provide safe, cost-effective hemophilia treatments to the 80% of the world's population that cannot afford recombinant products, but also to the wealthier nations, who cannot afford more adverse reactions and escalating treatment costs.

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Anticoagulation with warfarin downregulates inflammation

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Dear Sir

It is now apparent that a dynamic interaction may exist between the two processes of coagulation and inflammation. There is considerable evidence supporting a role for inflammation predisposing to blood clotting [1,2]. However, this study investigates the possibility that the coagulation system can directly effect regulation of inflammation.

We have previously demonstrated, in an epidemiological study, that D-dimer and C-reactive protein (CRP) are correlated, and postulated that fibrin turnover (as measured by D-dimer) may be a determinant of inflammation and CRP in the population [3]. Roumen-Klappe *et al*. suggested that a rise in CRP shown during the acute phase of deep vein thrombosis (DVT) is a direct result of the thrombotic event rather than a causative factor [4], and *in vitro* studies by Robson *et al*. have demonstrated that fibrin degradation products including D-dimer have the effect of increasing production and secretion of the inflammatory mediators interleukin (IL)-1 and IL-6 from monocytes [5]. These studies suggest that increased turnover within the

Table 1 Summary of Results

		Median	Mean change from baseline	Lower 95% CI for mean change	Upper 95% CI for mean change	*P*
INR	Day 0	0.9	–			
	Day 15	2.4	+1.67	+1.23	+2.1	<0.001
	Day 29	2.2	+1.57	+0.97	+2.16	<0.001
D-dimer	Day 0	58	–			
($ng\,mL^{-1}$)	Day 15	33	−49.07	−76.61	−21.53	0.002
	Day 29	39	−30.73	−62.63	+1.17	0.058
CRP	Day 0	2.3	–			
($mg\,L^{-1}$)	Day 15	0.8	−1.97	−3.83	−0.12	0.038
	Day 29	1.4	−0.15	−2.90	+2.16	0.909
IL-6	Day 0	2.9	–			
($pg\,mL^{-1}$)	Day 15	1.6	−0.84	−1.70	+0.01	0.053
	Day 29	2.8	−0.04	−0.99	+0.92	0.931

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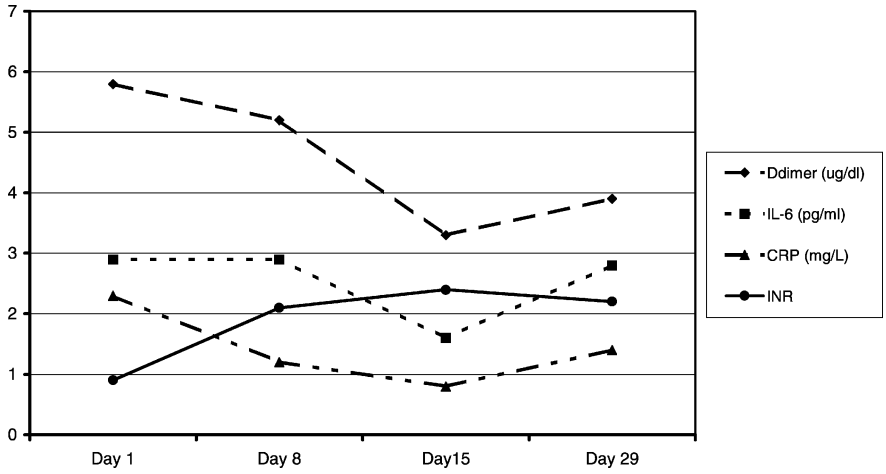

Fig. 1. Comparative median values of INR, D-dimer, CRP and IL-6.

coagulation system could effect upregulation of the inflammatory system. Conversely, Downing *et al*. have shown that low-dose low molecular weight heparins (LMWH) possesses anti-inflammatory properties distinct from its anticoagulant properties [6].

The aim of our study was to determine whether down-regulation of inflammation might also be achieved by oral anticoagulation, as a result of reduced activation of coagulation.

The study subjects consisted of 15 patients (10 male, five female; mean age 61 years, range 48–75 years) referred for elective outpatient anticoagulation for stroke prophylaxis. None had any history of recent thromboembolic events. All were commenced on Warfarin using a standardized 5-mg loading regime with venous International Normalized Ratio (INR) checked at day 5, then at weeks 1, 2, and 4 [7].

Citrated plasma samples were either processed immediately (INR) or stored in 1-mL aliquots at -70°C until analysis. INR was assessed using Manchester PT Reagent on an AMAX CS-190. CRP was assayed using a high-sensitivity assay on the Prospec Nephelometer (Dade Behring, Liederbach, Germany). ELISA was used to measure D-dimer (Biopool AB, Umea, Sweden) and IL-6 (R&D System Europe, Abington, UK).

Changes in parameter value from baseline were calculated and assessed using Student's *t*-test.

Our results demonstrate at day 15 a mean reduction of $49.07\,\mathrm{ng\,mL^{-1}}$ (95% confidence interval -76.61, -21.53) in D-dimer and $1.97\,\mathrm{mg\,L^{-1}}$ (95% CI -3.83, -0.12) in CRP levels, $P < 0.05$ in both cases. Mean reduction in IL-6 was also shown but not reaching significance ($P = 0.053$). It should be noted that IL-6 levels would have reached significance if those patients failing to reach target INR were excluded. By day 29 the fall in CRP from baseline failed to reach significance.

We confirmed reduction in D-dimer after warfarinization of atrial fibrillation patients to a target INR of 2–3. We also showed for the first time reductions in CRP and IL-6 after 15 days of warfarin, consistent with our hypothesis that coagulation activation products (e.g. D-dimer) are proinflammatory. At day 29 INR was less well controlled and reductions in D-dimer, CRP and IL-6 were no longer statistically significant. This suggests that an adequate level of warfarinization may be required for its anti-inflammatory effect. Although this study is small it does illustrate parallel trends in the activity within the systems of coagulation and inflammation which warrant further investigation in larger studies.

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Asymptomatic excessive coumarin anticoagulation is a risk factor for thrombotic and bleeding complications of oral anticoagulant therapy

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Dear Sir

The major adverse event of oral anticoagulant therapy (OAT) is bleeding and its risk increases with International Normalized Ratio (INR) levels. The measurement of time in therapeutic range is used to evaluate the clinical quality of OAT. The time spent within the therapeutic range represents a global index of the clinical quality of the treatment, but does not provide information about episodic overanticoagulation. Temporary coumarin stopping and the use of low-dose vitamin K1 are commonly recommended in the management of outpatients with asymptomatic elevation of INR [1,2]. Previous studies outlined that the risk of bleeding events rises to 200 per 100 patient-years if INR is higher than 7 [3]. In this study we have evaluated the rate of asymptomatic overanticoagulation in the routine practice of an anticoagulation clinic, the clinical characteristics of patients in whom this alteration is registered, and if the occurrence of episodic asymptomatic overanticoagulation is a predictor of adverse events of OAT.

From June 1995 to December 2001 we prospectively followed up 1068 patients with a total follow-up period of 2329 patient-years, 1021 on warfarin (2254 patient-years) and 47 on acenocumarol (75 patient-years), in Florence Anticoagulation Clinic. We defined asymptomatic overanticoagulation an INR >7 in any patient or >6 in patients over 75 years or considered at high risk of bleeding, according to criteria of vitamin K administration stated by Italian Federazione Centri Sorveglianza Anticoagulati (FCSA) [2]. For practical purpose we focused on those patients who received vitamin K administration. This condition is recorded in Anticoagulation Clinic files and may be easily retrieved to characterize patients as being at risk of instability. In patients with asymptomatic overanticoagulation therapy was stopped for 1 day and 2 mg of vitamin K1 (Konakion Roche®, Basel, Switzerland) was also orally administered, according to FCSA recommendations [2]. Vitamin K1 was also administered in patients at high risk of bleeding or in patients over 75 years, if INR was >6.

The occurrence of all types of bleeding and thromboembolic complications was recorded. During the study period, 141 patients (70 males and 71 females) presented asymptomatic overanticoagulation on 185 occasions. Thirty-one patients needed repeated vitamin K1 administrations during follow-up.

Asymptomatic overanticoagulation requiring vitamin K1 administration was significantly more frequent in women (rate 7.6×100 patient-years) than in men (rate 5.0×100 patient-years), with an incidence rate ratio (RR) of 1.5 [95% confidence interval (CI) 1.0, 2.1, $P = 0.01$]. Patients older than 75 years had asymptomatic overanticoagulation more frequently than younger patients [rate 21.1 vs. 4.4×100 patient-years; relative risk (RR) 4.8 (95% CI 3.3, 6.8) $P = 0.000$]. Patients treated with acenocoumarol had a significantly higher rate of vitamin K1 administration in comparison with patients treated with warfarin [rate 18.6 vs. 5.6×100 patient-years, respectively; RR 3.3 (95% CI 1.7, 5.7), $P = 0.0003$]. No difference was found in relation to weekly mean dosage for both drugs in comparison with the whole population followed by our Anticoagulation Clinic (data not shown). The quality of anticoagulation achieved was measured using Rosendaal's method [4]. The time spent below, within, and above the intended therapeutic range was 22%, 60% and 18%, respectively, in the other patients followed by our clinic and 24.5%, 57.5% and 18% in the patients with at least one episode of asymptomatic overanticoagulation. In patients with episodic asymptomatic overanticoagulation we observed a higher number of visits for dose adjustment than in patients who never required vitamin K administration (25 ± 6 vs. 17 ± 3, for patients on warfarin, $P = 0.000$, and 29 ± 7 vs. 22 ± 5 for patients on acenocoumarol, $P = 0.00$).

No difference in the rate of asymptomatic overanticoagulation was found in relation to the indication for OAT and target INR.

During the 3 months before and the 3 months after vitamin K1 administration neither bleeding nor thrombotic complica-

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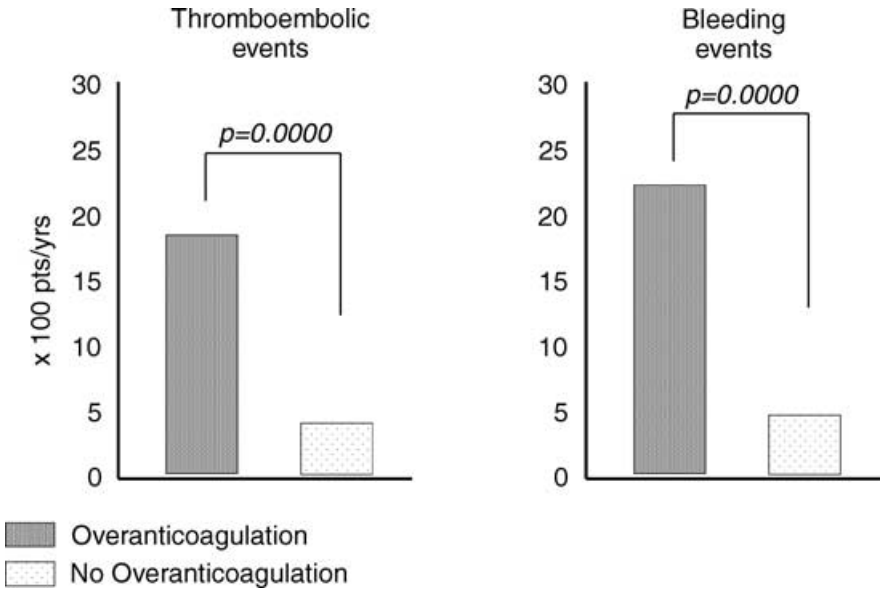

Fig. 1. Rates of all thrombotic and bleeding complications in patients with and without asymptomatic overanticoagulation.

tions were registered. One patient died 2 weeks after vitamin K administration because of renal failure. During follow-up we observed five minor bleeding events temporarily related to elevated INR levels that required vitamin K1 administration. However, considering both bleeding and thrombotic complications occurred in these patients during the total follow-up period, we found a higher rate of adverse events compared with the other patients referring to our Anticoagulation Clinic during the same period (Fig. 1). In particular, we recorded 22 thrombotic events in patients who had asymptomatic overanticoagulation and 71 in the other patients followed by our Anticoagulation Clinic [rate 18.5 per 100 patient-years vs. 3.2 per 100 patient-years; RR 5.7 (95% CI 3.3, 9.3), $P = 0.0000$]. Twenty-six and 79 bleeding events were recorded, respectively, in the two groups [rate 21.8 per 100 patient-years vs. 3.5 per 100 patient-years; RR 6.1 (95% CI 3.7, 9.6), $P = 0.0000$]. Major bleeding events were 2 and 23 (rate 1.7 per 100 patient-years vs. 1 per 100 patient-years), respectively. However, this difference was not statistically significant. None of the adverse events, except the five mentioned above, was temporarily related to the vitamin K1 administration.

The relationship between the time spent in therapeutic range and the occurrence of adverse events is well known [1,3,5]. In this study we focused our attention on the increase of INR without symptoms in patients referred to our Anticoagulation Clinic and we found that this phenomenon was related to patient characteristics. In particular, the rate of vitamin K1 administration was significantly higher in elderly, in women and in patients treated with acenocoumarol.

Considering both bleeding and thrombotic complications, we found higher rates of events in patients with at least one episode of asymptomatic overanticoagulation with respect to the other patients followed by our Anticoagulation Clinic. The risk of developing adverse events was almost 6-fold higher in patients who also required vitamin K1 administration. The adverse events registered were either thrombotic or hemorrhagic and were not time-related to the episode of overanticoagulation. These results are surprising, because one would expect overanticoagulated patients to be at increased risk of hemorrhagic and not of thrombotic events. This phenomenon probably reflects the instability of the anticoagulation which is not easily detectable by the measurement of time spent within therapeutic range, as confirmed by the higher number of visits for dose adjustment needed in these patients than in others. Episodic asymptomatic excessive INR possibly represents *per se* an index to easily identify unstable OAT patients at high risk of complications. We suggest that episodic excessive coumarin anticoagulation should be considered in the evaluation of the personal risk related to OAT.

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Low physician compliance of prescribing anticoagulant prophylaxis in patients with solid tumor or hematological malignancies and central vein catheters

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Dear Sir,

Thrombosis is a well-recognized complication of a central venous catheter (CVC) in oncology and hematology patients. In the American College of Chest Physicians (ACCP) consensus on anticoagulant prophylaxis, it is suggested that warfarin (1 mg daily) or a low molecular weight heparin (LMWH) should be given to cancer patients with a CVC [1]. The Dutch consensus is in concordance with this. Data on the implementation of these consensus guidelines are limited to a report from a single hospital in the USA and suggested that physicians are reluctant to prescribe anticoagulant prophylaxis in cancer patients with CVCs [2].

We conducted a nationwide survey to assess physician compliance with consensus guidelines at medical departments in the Netherlands where oncology and hematology patients are treated with chemotherapy. The questionnaire contained three topics: (i) the use of CVCs for chemotherapy; (ii) systematic use or non-use of anticoagulant prophylaxis in patients with CVCs; and (iii) rationale for complying with the anticoagulant guidelines.

In addition, questions were asked about the type and the dose of anticoagulant prophylaxis; about the influence of platelet counts on this treatment, and of the expected duration of CVC placement (short-term vs. long-term CVCs) in relation to the initiation of anticoagulation.

Of 157 questionnaires sent to medical, oncology and hematology departments involved in the treatment of cancer patients, 116 were returned (response 74%). The response rate was similar for departments where oncology (79 out of 106; 75%) and hematology patients (37 out of 51; 73%) were treated. In most departments CVCs were used to administer chemotherapy and supporting treatment for oncology (85%) and hematology (84%) patients. The results of the questionnaire are summarized in Fig. 1.

Overall, in 14 of 67 (21%) of the oncology and in three of 31 (10%) of hematology departments, prescription of anticoagulant prophylaxis for CVC-related thrombosis was routine policy. The most frequently used type of anticoagulant prophylaxis was a LMWH (88%), at a low dose of 2850 anti-Xa units, although in some centres a therapeutic dose (up to 7600 anti-Xa units) was given. Low-dose coumarin derivatives (1 mg acenocoumarol once daily) were used at two departments where anticoagulant prophylaxis was prescribed as a standard policy.

The platelet count was an important parameter in the decision to continue prophylaxis. In nine of 17 clinics (53%) where anticoagulant prophylaxis was used, this was not given if the platelet count dropped below $50 \times 10^9\,L^{-1}$. The expected duration of stay of the catheter was not a major factor; in only two of 17 (12%) of the departments was anticoagulant prophylaxis not started when short-term CVCs were used (expected duration $<$14 days).

The two most important reasons for not having a routine policy of anticoagulant prophylaxis were a low expected incidence of clinically manifest thrombosis and the fear of hemorrhage (62%). This was also reflected in the preference for heparin locks or flushes (38%), which some respondents thought would give less systemic anticoagulant effects. In addition, several physicians mentioned spontaneously that they did not feel the consensus guidelines were sufficiently convincing to prescribe anticoagulant prophylaxis (29%). Only a minority of physicians reported being not familiar with the national or international ACCP consensus (18%).

This survey reveals that in a minority of Dutch clinics, where chemotherapy is given, anticoagulant prophylaxis for CVC-related thrombosis is prescribed according to national and international guidelines, in spite of general awareness of consensus guidelines. The low compliance among physicians to prescribe anticoagulant prophylaxis in this large survey is similar to that reported in the study by Carr and Rabinowitz,

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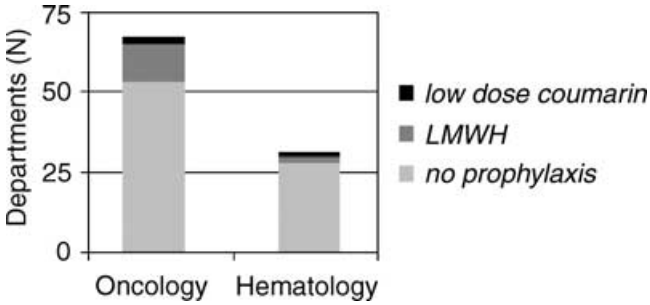

Fig. 1. The policy of prescribing anticoagulant prophylaxis (no prophylaxis, low molecular weight heparin (LMWH) or low-dose coumarin) for central vein catheter-related thrombosis amongst centres involved in treatment of oncology and hematology patients.

i.e. an initial 10% compliance, which increased to 20% after notification of the physicians about the policy and benefits of anticoagulant prophylaxis [2]. However, reasons for a poor compliance amongst physicians remain unclear from this study. In our survey, the most important reasons given for not prescribing anticoagulant prophylaxis were the fear of bleeding under anticoagulant prophylaxis and a low expected incidence of clinically manifest thrombosis. Thus, in general, the presumed risk of anticoagulant prophylaxis (bleeding) outweighs the benefit from it (less thrombosis) in clinical practice.

The incidence of clinically manifest thrombosis in the clinics that participated in our survey is unknown. The reported incidence of clinically manifest CVC-related thrombosis varies between studies, with recent figures ranging from 6 to 12% [2,3] Available data from randomized trials concerning the safety of anticoagulant prophylaxis are limited, and restricted to patients with normal or only slightly decreased platelet counts ($>100 \times 10^9\,L^{-1}$) [4,5]. Moreover, in patients undergoing bone marrow transplantation or intensive chemotherapy for hematological disease, usually developing severe thrombocytopenia, such data from randomized trails are not available.

The presumed increased risk of bleeding in patients treated with chemotherapy seems to be a matter of great concern and depended largely on the occurrence of thrombocytopenia, which is commonly observed in patients undergoing chemotherapy. Alternatively, selecting patients with a high risk profile for developing manifest thrombosis in the presence of (severe) thrombocytopenia might help clinicians to decide in whom anticoagulant prophylaxis is warranted or not, i.e. to individualize instead of routine anticoagulant prophylaxis [3]. Unfortunately, data on the contribution of commonly found (independent) risk factors to CVC-related thrombosis are limited and not always consistent. For example, in carriers of factor (F)V Leiden who need to undergo bone marrow transplantation the cumulative incidence of clinically manifest thrombosis may be as high as 54% [3]. However, in two other studies there was no evidence of a significant contribution of FV Leiden to CVC-related thrombosis [6,7]. Clearly, before implementation of anticoagulant prophylaxis according to risk factors in venous thromboembolism such as FV Leiden, there is more need for studies in which the outcome of such stratification is evaluated.

The use of acenocoumarol by two clinics in our survey warrants comment. Data concerning the use of acenocoumarol as anticoagulant prophylaxis for CVC-related thrombosis are not available. Low-dose warfarin, which is mentioned in the ACCP consensus guidelines as one of the drugs of choice, is, however, not registered in the Netherlands. In general, the use of coumarin drugs in these vulnerable patients, who sometimes have impaired liver function or low vitamin K intake, might induce a prolonged International Normalized Ratio (INR) in some patients and therefore INR monitoring seems warranted [4].

In conclusion, despite an evidence-based recommendation of anticoagulant prophylaxis for CVC-related thrombosis, this survey shows that routine use is not generally implemented and more evidence is needed to establish firmly the risk–benefit ratio for broader implementation of consensus guidelines. In a patient group at high risk of thrombosis with common occurring (severe) thrombocytopenia, individualizing anticoagulant prophylaxis might be a useful strategy, but needs to be established.

Acknowledgements

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Does negative heparin-platelet factor 4 enzyme-linked immunosorbent assay effectively exclude heparin-induced thrombocytopenia?

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Dear Sir,

Heparin-induced thrombocytopenia (HIT) is a potentially fatal side effect of heparin therapy that is difficult to diagnose on clinical criteria alone [1]. A reliable sensitive test would therefore be very valuable to exclude HIT. It would make it possible to continue heparin safely and effectively in patients with negative result and to restrict the diagnostic work-up, including a time-consuming activation assay, to the fraction of patients with positive result. It is usually mentioned that 95% of HIT patients have antibodies that recognize heparin-platelet factor 4 complexes (H-PF4) [2].

We undertook a systematic review of the published validation studies for Asserachrom HPIA® (Diagnostica Stago, Asnières, France), an enzyme-linked immunosorbent assay (ELISA) for H-PF4 antibodies which interlaboratory reproducibility was formally assessed and proved to be excellent [3]. We followed the methodological standards for systematic reviews of diagnostic and screening tests [4]. The MEDLINE database was used to find appropriate studies during the 10-year period from 1992 (first description of the H-PF4 ELISA by Amiral) through 2002 using 'platelet factor 4' and 'heparin-induced thrombocytopenia' as keywords. One hundred and sixteen articles were retrieved and manual searching revealed five more. We ex-

Table 1 Classification performance and estimated sensitivity established in the clinically defined HIT patients and in those with heparin-dependent platelet-activating antibodies

		Percentage of positive H-PF4 ELISA (95% CI)	
	Platelet activation test	Among clinically defined HIT patients	Among HIT patients with platelet activating antibodies
Classification performance			
Amiral *et al. Thromb Haemost* 1992 [5]	PAT	96.4% (81.7-99.9) 27/28	95.8% (78.9-99.9) 23/24
Greinacher *et al.** *Thromb Haemost* 1994 [6]	SRA	–	97.0% (84.7-99.9) 33/34
Amiral *et al. Thromb Haemost* 1995 [7]	PAT	97.7% (88.0-99.9) 43/44	97.4% (86.5-99.9) 38/39
Arepally *et al.** *Am J Clin Pathol* 1995 [8]	SRA	–	90.6% (75.0-98.0) 29/32
Amiral *et al. Thromb Haemost* 1997 [9]	PAT	87.8% (83.3-92.7) 164/187	85.3% (80.0-90.8) 134/157
Pouplard *et al. Am J Clin Pathol* 1999 [11]	SRA	97.7% (88.0-99.9) 43/44	97.6% (87.4-99.9) 41/42
Walenga *et al.*† *Semin Hematol* 1999 [12]	PAT or SRA	64.3% (57.6-71.0) 128/199	81.8 % (74.9-88.7) 99/121
Total		80.7 (77.2-84.1) 405/502	88.4% (85.4-91.4) 397/449
Estimated sensitivity			
Samama *et al. Bull Acad Natle Med* 1998 [10]	PAT	72.4% (61.8-81.5) 63/87	74.7% (63.3-84.0) 56/75
Rugeri *et al.*† *Hematology* 1999 [13]	PAT	92.6% (75.7-99.1) 25/27	88.9% (65.3-98.6) 16/18
Gruel *et al.**† *Blood* 2000 [14]	SRA	–	96.6% (88.3-99.6) 57/59
Lindhoff-Last *et al. Br J Haematol* 2001 [15]	HIPA	91.1% (78.8-97.5) 41/45	89.7% (75.8%-97.1%) 35/39
Total		81.1 (75.0-87.2) 129/159	85.9 (81.0-90.8) 164/191

*Positive SRA is one of the criteria for the diagnosis of HIT. †Prospective study. PAT, platelet agregation test; SRA, ^{14}C-serotonin release assay; HIPA, heparin- induced platelet activation assay.

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cluded reviews and studies without explicit HIT diagnosis. Eleven articles were finally retained [5–15]. Every effort was made to inform the authors of published studies and to obtain their agreement on the analysis we made of their data. Four of us independently reviewed each article.

The Asserachrom HPIA® was used according to the package insert in seven studies [8,10–15], and the so-called home-made tests of the other four studies [5–7,9] were performed by J. Amiral, the designer of the commercial Asserachrom HPIA® kit. We checked that authors' diagnostic criteria fitted with the standardized criteria proposed by George *et al.* [16] based on the time course of the platelet count (PC) in relation to heparin therapy and the possibility of other causes of thrombocytopenia. The occurrence of thrombotic complications, the most common sequelae of HIT, was also taken into account [1]. The presence of platelet activating antibodies as assessed by serotonin release assay was included in the diagnostic criteria in three studies [6,8,14]. As a crucial point, the results of ELISA were interpreted blind to diagnosis of HIT, and conversely the ELISA results were never taken into account to establish the diagnosis of HIT. Four studies included non-selected, i.e. consecutive patients [10,13–15]. As previously proposed, classification performance and sensitivity measured the capacity of the Asserachrom HPIA® to detect HIT among selected or non-selected HIT patients, respectively [17]. Classification performance was estimated in seven studies to be 80.7% (95% CI 77.2–84.1) for clinically defined HIT patients and 88.4% (95% CI 85.4–91.4) for those who also had detectable platelet activating antibodies. Sensitivity was estimated in four studies to be 81.1% (95% CI 75.0–87.2) in the clinically defined group of HIT patients and 85.9% (95% CI 81.0–90.8) for the subgroup with platelet activating antibodies that was used to characterize the HIT population study as well as possible (Table 1).

According to the results of this systematic review, Asserachrom HPIA® would fail to diagnose HIT in about 20% of HIT patients. Taking into account the severity of the syndrome, there is no sufficient evidence to support the exclusion of HIT in cases of negative results on Asserachrom HPIA®. These results suggest that there are indeed other targets for HIT antibodies such as interleukin-8 and neutrophil activating peptide 2 (NAP-2) [18,19]. Another explanation was suggested by Harenberg *et al.* who showed that HIT patients without antibodies to H-PF4 might seroconvert after the cessation of heparin therapy [20].

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Increased plasma levels of soluble platelet glycoprotein V in patients with acute myocardial infarction

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Dear Sir,

Soluble glycoprotein V (GPV) is a 69-kDa fragment of platelet GPV which is selectively released into plasma during activation of platelets by thrombin [1]. Soluble GPV can now be quantified by ELISA (Asserachrom® Soluble GPV; Stago, Asnières, France) [2] and has already been shown to be a specific indicator of thrombin-induced platelet activation (i) *in vitro* in platelet concentrates for transfusion [2], (ii) *in vivo* in experimental thrombosis in the rat [3], and (iii) in patients with atherosclerosis [4] or atrial fibrillation [5]. In acute coronary syndromes, thrombin and platelets play key roles in the development of thrombosis. The aim of this study was to measure the release of soluble GPV during intracoronary thrombosis in patients with an acute coronary syndrome.

Among all patients admitted between December 1998 and August 1999 to the Intensive Care Unit of the Cardiology Department at the University Hospital of Strasbourg for suspicion of an acute coronary syndrome, 47 consecutive patients were recruited for this study on the criterion of the presence of chest pain at rest within the preceding 24 h. At admission and before administration of any antithrombotic treatment, a 4.5-mL venous blood sample was taken into a DiatubeTM H CTAD (Becton Dickinson, Plymouth, UK) for measurement of soluble GPV, von Willebrand factor (VWF), platelet factor 4 (PF4), thrombin–antithrombin complexes (TAT) and prothrombin fragment 1+2 (F1+2). On the basis of electrocardiograms and biochemical markers of myocardial injury [creatinine phosphokinase (CPK) and cardiac troponin I], these patients were assigned to one of the following categories: unstable angina or non-ST-elevated myocardial infarction ($n = 19$), acute myocardial infarction with ST segment elevation and Q-wave formation ($n = 8$) or no acute coronary syndrome ($n = 20$). The group of acute myocardial infarction with ST segment elevation was composed of inaugural infarctions and among these patients the median delay to admission after the onset of chest pain was 2.5 h (interquartile range 2–4 h).

Soluble GPV was significantly increased in the group of acute myocardial infarction and ST segment elevation with respect to the group without acute coronary syndrome at admission (Table 1). At this time, the median level of CPK in patients with acute myocardial infarction and ST segment elevation was lower than twice the upper reference limit of the normal value for our laboratory [272 (123–530) vs. 440 IU L^{-1}], while cardiac troponin I was hardly detectable [0.46 (0.41–2.04) vs. 0.35 ng mL^{-1} as the limit of detection and 3.5 ng mL^{-1} for a diagnosis of myocardial infarction according to our laboratory values]. However, the large increases in CPK [peak 2635 (1352–3372) IU L^{-1}] and cardiac troponin I [peak 47.16 (38.20–136.71) ng mL^{-1}] observed during hospitalization support the diagnosis of myocardial infarction. No increase in soluble GPV was found in the group of unstable angina or non-ST-elevated myocardial infarction, whereas cardiac troponin I was detectable during hospitalization in the blood of 17 of the 19 patients of this group. These 17 patients displayed a median level of soluble GPV of 20.0 ng mL^{-1} (17.4–40.1) ($P = 0.011$ vs. group of acute myocardial infarction with ST segment elevation). Analysis of VWF levels showed a rise in VWF as a function of the degree of severity of the coronary syndrome (no acute coronary syndrome <unstable angina or non-ST-elevated myocardial infarction <acute myocardial infarction with ST segment elevation; Table 1). The other markers of thrombosis (PF4, TAT and F1+2) were also increased in the group of acute myocardial infarction with ST segment elevation, but statistical significance was not attained (Table 1).

The principal result of this study was the detection of a significant increase in plasma levels of soluble GPV in patients presenting myocardial infarction with ST segment elevation. Contrary to other markers, significance was reached despite the limited number of subjects in this group, which testifies to the reliability of the soluble GPV test. In this work, blood samples were taken during the first hours of coronary occlusion, before the rise in markers of myocardial injury. Elevated levels of soluble GPV were therefore observed at an early stage during the development of an acute coronary thrombus, indicating that

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Table 1 Characteristics of the patients and levels of thrombosis markers

	Patients without acute coronary syndrome	Patients with unstable angina or non-ST-elevated myocardial infarction	Patients with acute myocardial infarction and ST segment elevation	*P*-value
n	20	19	8	
Age (years)	62.5 (55.4–67.1)	70.1 (59.8–77.9)	51.0 (43.1–65.9)	0.054
Male gender, *n* (%)	11 (55.0)	15 (78.9)	8 (100.0)	0.039
Prior CAD, *n* (%)	4 (20.0)	10 (52.6)	0 (0.0)	0.011
Current smokers, *n* (%)	5 (25.0)	8 (42.1)	3 (37.5)	0.517
Diabetes mellitus, *n* (%)	5 (25.0)	4 (21.1)	2 (25.0)	0.952
Soluble GPV ($ng\,mL^{-1}$)	22.7 (20.0–31.5)	23.4 (17.8–46.8)	55.3* (35.5–69.1)	0.041
VWF ($IU\,L^{-1}$)	1.49 (1.20–1.82)	2.07 (1.18–2.79)	2.71** (2.49–3.02)	0.002
PF4 ($ng\,mL^{-1}$)	3.18 (3.00–8.47)	10.28 (3.03–19.30)	12.55 (5.55–36.63)	0.065
TAT ($ng\,mL^{-1}$)	2.48 (1.62–3.06)	2.02 (1.29–4.08)	3.74 (3.39–58.03)	0.103
F1+2 ($nmol\,L^{-1}$)	1.43 (1.07–1.92)	1.55 (1.31–2.34)	1.84 (1.16–4.78)	0.497

Soluble GPV, Soluble glycoprotein V; VWF, von Willebrand factor; PF4, platelet factor 4; TAT, thrombin–antithrombin complexes; F1+2, prothrombin fragment 1+2. Results are expressed as the number (percentage) or median (interquartile range: 25th to 75th percentile). Values of *P* were calculated using Fisher's exact test for qualitative data and the test of Kruskal–Wallis for quantitative data. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. group of patients without acute coronary syndrome.

the liberation of soluble GPV is a very early event with respect to the increase in biochemical markers of myocardial injury (CPK and cardiac troponin I). This precocity and the lack of correlation between soluble GPV and cardiac troponin I or CPK (data not shown) suggest that soluble GPV is a marker of intracoronary thrombus formation rather than myocardial damage. PF4, TAT and F1+2 were also increased in the group of acute myocardial infarction with ST segment elevation, although statistical significance was not reached owing to the small number of cases. On the basis of our calculations, 40 patients would have been required to achieve significance with these markers while eight proved sufficient using soluble GPV.

The absence of a detectable rise in soluble GPV in the group of unstable angina or non-ST-elevated myocardial infarction could be due to the transitory nature of the thrombosis occurring in unstable angina or the variable delays between thrombotic/ischemic events and blood withdrawal. These chronological factors could explain the lack of detection of soluble GPV at a later time, and the window of 24 h in the inclusion criterion was probably too large. Other markers of thrombin generation have likewise been reported as failing to identify patients with unstable angina [6]. Plasma VWF increased significantly in the group of acute myocardial infarction with ST segment elevation, in agreement with previous studies [7]. Circulating VWF is most probably essentially of endothelial origin and therefore reflects vascular damage. Hence VWF and soluble GPV could prove to be complementary tools to elucidate the mechanisms of coronary thrombosis and the physiopathological differences between unstable angina and myocardial infarction with or without ST elevation.

In conclusion, this work has revealed a significant increase in plasma soluble GPV levels during acute coronary thrombosis leading to myocardial infarction. Larger scale studies will be necessary to assess the informative, diagnostic and prognostic value of this new marker in coronary, cerebral and peripheral arterial thrombosis.

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Cerebral vein thrombosis and right foot ischemia in a 21-year-old man

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Dear Sir,

A 21-year-old previously healthy Guyanese student with no significant past medical history was referred to our emergency department in July 2001 complaining of generalized headaches for 4 months and a 24-h history of severe headache, nausea, vomiting, L facial droop and left arm weakness.

On history taking, he recounted that 10 months previously, he began experiencing neck pains and increasingly frequent headaches. He sought help from a chiropractor in March 2001 and following his first neck manipulation developed a generalized seizure. He was immediately admitted to a community hospital where a brain computed tomography (CT) scan demonstrated a small right frontal intracerebral hemorrhage. He had been transferred to Neurosurgery at our hospital and a magnetic resonance imaging (MRI) of the brain showed slow flow through the right transverse sinus. In June 2001, a transfemoral venogram demonstrated complete occlusion of the right transverse sinus. No specific therapy was prescribed at this time with the exception of Dilantin. He had been discharged with outpatient follow-up with the thrombophilia and neurosurgical services. On examination during this particular presentation (July 2001) he had new left hemiparesis, left facial nerve palsy and splenomegaly. MRI and cerebral angiogram confirmed superior sagittal and right transverse sinus thromboses and small subacute and chronic intracerebral bleeds consistent with cerebral vein infarcts.

CT abdomen demonstrated only isolated homogeneous mild/moderate splenomegaly (15.4 cm). Blood work, including complete blood count (CBC), lytes, blood urea nitrogen (BUN), creatinine, liver and calcium profile were all within normal limits. On four occasions, his mean corpuscular volume (MCV) was noted to be borderline low at 76–77 (normal 78–96 FL), but a ferritin and Hg electrophoresis were normal and alpha thallesemia trait was entertained, although genotyping was not performed. An extensive thrombophilia screen was normal (ATIII, proteins C, S, factor V Leiden, prothrombin variant, homocysteine and lupus anticoagulant) with the exception of a slightly increased IgM anticardiolipin antibody (ACA) of 17 MPL (normal 0–11). There was no family history of thrombosis.

He was anticoagulated with heparin followed by warfarin. The patient slowly recovered and was discharged home 3 weeks later with follow-up. The patient remained well for 3 months but re-presented to the emergency department complaining of a painful right foot and bluish discoloration of his right 3–5 toes. Doppler and digital plethysmography showed no evidence of large vessel occlusion and he was treated for vasospasm with prostaglandin infusion, calcium channel blockers and nitrates with modest effect. A vasculitis work-up was found to be negative and no vegetations were detected on 2-dimensional echo of his heart. During this admission, his ACA IgG had risen to 43 GPL units (0–20) and his IgM ACA remained elevated at 18 MPL units (0–11). Antiphospholipid antibody syndrome (APLA) was considered, ASA was added and the patient's anticoagulation was increased (target INR 2.5–3.5). On this occasion, his MCV was consistently low at 72–76, and cells were noted to be hypochromic and microcytic with occasional tear drops on blood film, but ferritin remained in the normal range, albeit lower than previously. Hg and WBC were consistently normal, although his RBC count was noted to be elevated on several occasions ranging from 6.35 to $6.7 \times 10^{12}\,L^{-1}$ (normal $4–6 \times 10^{12}\,L^{-1}$). A repeat ultrasound was performed upon discharge and hepatomegaly (15.2 cm) and persistent splenomegaly (19.3 cm) were documented. Peripheral blood was sent for CFU-GEMM colony growth by plating mononuclear cells with and without erythropoietin at $2\,U\,mL^{-1}$ (methods previously published) [1,2]. These results are presented in Table 1 and supported spontaneous erythroid colony growth. A bone marrow aspirate and biopsy were then done and demonstrated again spontaneous erythroid colonies and findings consistent with a myeloproliferative disorder (MPD) such as polycythemia vera (erythroid and megakaryocyte hyperplasia, increased reticulin, absent iron stores) evolving into the spent phase (Fig. 1).

The patient is currently asymptomatic and thrombosis-free for 10 months on warfarin and ASA. His blood counts remain in

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Table 1 Serial spontaneous erythroid colony results on peripheral blood and marrow

Date	Sample type	Cells plated (cm^{-3})	BFU-E without erythropoietin	BFU-E with erythropoietin
13 March 2002	Peripheral blood	1×10^5	45	125
9 July 2002	Bone marrow	5×10^4	32	96
9 July 2002	Peripheral blood	1×10^5	80	166

BFU-E, Blast forming units erythroid.

the normal range. ACA IgG and IgM levels have also normalized, 8 months following the ischemic foot episode. He has just started alpha-interferon and will be evaluated for an allogeneic marrow transplant.

Both antiphospholipid syndromes and MPD are included in the small list of prothrombotic states in which thrombosis occurs within both the venous and arterial beds [3]. Polycythemia rubra vera (PV) is a clonal MPD classically associated with increased red cell mass, leukocytosis, thrombocytosis, splenomegaly, thromboses and bleeding. Idiopathic arterial and venous thromboses have been reported to be the presenting feature of previously undiagnosed MPD with common sites including the portal, hepatic or splanchnic veins [4–6]. Recent stroke registries (HART) have shown that 0–7% of cerebral infarcts may be attributed to hematological causes. Indeed, two patients described by Haan *et al*. [7] presented with cerebral vein thrombosis and were diagnosed with MPD based on histological findings. Spontaneous erythroid colonies from peripheral blood or marrow samples in erythropoietin-poor media are considered a usual feature of PV, and can be detected before any overt manifestations of the disease occur [4–6,8]. This method has been reported to be particularly helpful in diagnosing cases of PV involving iron deficiency, where the usually elevated blood counts are not found [9], as in this case.

The delay in diagnosing this patient's MPD is not surprising given his young age, consistently normal blood counts, and normal ferritins. Furthermore, we suspected alternative causes for his microcytosis prior to the bone marrow (alpha thal trait) and thrombosis (APLA syndrome). APLA syndrome is associated with arterial and venous thrombosis, including deep vein thrombosis and pulmonary embolism, premature coronary artery disease, premature cerebrovascular disease (including stroke, transient ischemic attack, cerebrovascular thrombotic stroke), and retinal arterial and venous occlusive disease. Thrombotic episodes associated with APLA syndrome may occur in vascular beds that are infrequently affected by other prothrombotic states with cutaneous manifestations that include distal cutaneous ischemia, infarcts of the skin, and acrocyanosis [3]. IgG, IgM and IgA anticardiolipin antibodies are all associated with thrombosis. Of patients with thrombosis and anticardiolipin antibodies 17% have isolated IgM idiotype [10]. There is no apparent association between the type of thrombotic event and type or titer of anticardiolipin antibody present [11,12]. The relationship of his anticardiolipin antibodies to his cerebral venous thromboses and lower leg arterial insufficiency is not entirely clear. Although one study found elevated levels of ACAs in 45.6% of young stroke patients, correlation is not necessarily causality. Furthermore, low positive values may be transient or reactive phenomena [13]. The literature reports the temporal sequence of antibody levels following a thrombotic event as showing peaks in IgM at 5–7 days and in IgG in up to 2 weeks. The IgG peak occurred during his foot ischemia episode, but not following the cerebral vein thrombosis. Finally, the mere presence of antiphospholipid antibodies may be insufficient to generate thrombosis. A second 'hit' in combination may be required for thrombosis to occur. In this instance, the second hit may have been his MPD. Nevertheless, given the strong association of MPD with thrombosis, one cannot

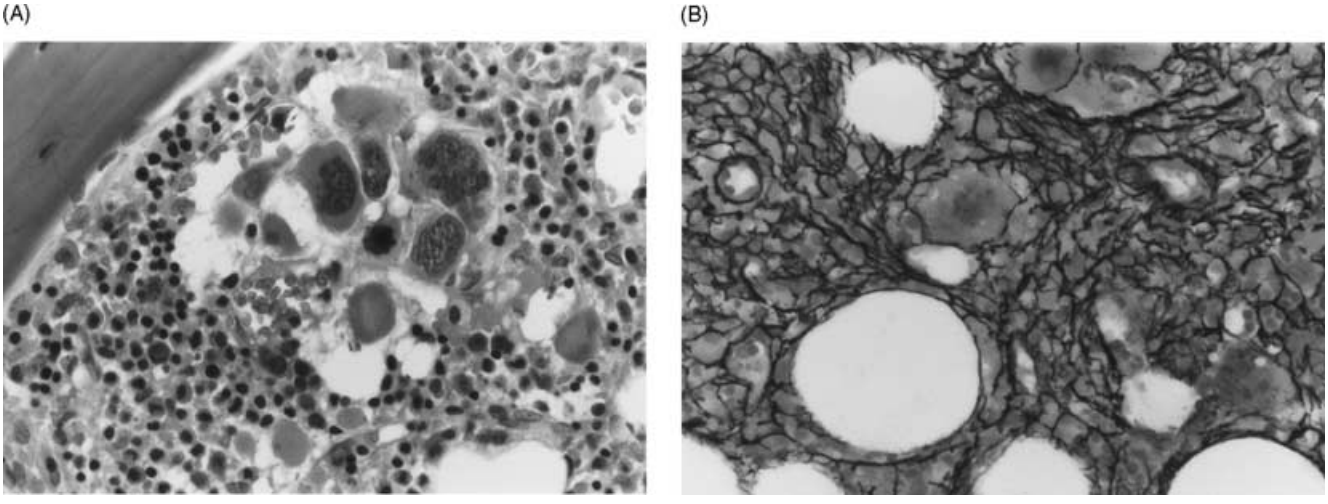

Fig. 1. Photomicrograph of bone marrow biopsy, (A) H&E. Erythroid and megakaryocytic hyperplasia. Clusters of megakaryocytes with hyperchromatic, dysmorphic nuclei, and atypically located near bone trabeculum (× 1000). (B) Reticulin fibrosis of the bone marrow (× 1000).

rule out the possibility that these anticardiolipin antibodies were unrelated, merely reactive phenomena.

We believe this case is interesting to physicians for the following reasons: it reinforces the need to consider occult MPD in cases of unexplained venous and arterial thromboses, even in very young patients; it demonstrates that anticardiolipin antibodies and lupus anticoagulants may be reactive and transient phenomena and not necessarily the cause of thromboses, even when elevated; and finally, it illustrates an interesting and unusual case of a very young man with moderately advanced MPD being unmasked by idiopathic cerebral vein thromboses.

Acknowledgements

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Identification and characterization of a natural R96C EPCR variant

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Dear Sir,

The anticoagulant protein C (PC) pathway is an important regulatory system in coagulation because genetic defects affecting the components of this system are important risk factors for venous thromboembolism (VTE). More recently reduced levels of activated protein C (APC) have been suggested to be an important and prevalent risk factor for VTE [1]. The endothelial protein C receptor (EPCR) is the last characterized component of the PC anticoagulant pathway, which functions by presenting PC to the thrombin–thrombomodulin complex, increasing five to nine times the rate of activation of PC on the endothelial cell surface [2–4]. Therefore, it might be anticipated that genetic or acquired defects of this receptor would lead to increased risk of thrombosis. Recently a 23-bp insertion within the EPCR gene which creates a non-functional truncated EPCR has been reported in patients who had VTE or myocardial infarction, although the epidemiological studies conducted up to now have failed to show a significant association with thrombosis [5,6]. We have undertaken screening for mutations in the EPCR gene

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of patients with VTE trying to identify new mutations related with risk for VTE.

We analyzed 96 patients randomly selected from 217 patients diagnosed with VTE. When a sequence variation was found we extended the study to the whole group of 217 patients and also 239 controls without personal or familial history of VTE. The population studied is described elsewhere [7]. All exons and intron–exon boundaries of the EPCR gene as well as 373 bp of the 5′ flanking sequence were studied by single-stranded conformational polymorphism analysis (SSCP) following polymerase chain reaction. Specific primers and experimental conditions of SSCP are available upon request. All potential band shifts were characterized by direct sequencing (ABI 373; Applied Biosystems, Norwalk, CT, USA). A previously unreported heterozygous C to T variation at position 4031 was identified, which predicts a substitution of Arg to Cys at position 96 in the mature EPCR protein. The prevalence of this variation was 0.44% in patients (1/217) and 0.83% in controls (2/230) [odds ratio =0.88 (95% confidence interval 0.05, 23.38)]. None of the patients or controls carried the 23-bp insertion. Three previously reported polymorphisms were also detected, which are not expected to be associated with risk for VTE: A4600 G that predicts Ser219 Gly, T2532A (16 bp upstream of the start of exon II), and T3997A (20 bp upstream of the start of exon III) [8,9].

Soluble EPCR (sEPCR) (residues 1–193, mature protein numbering) [8] was expressed in *Pichia pastoris* system (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. In brief, we amplified the cDNA of wild-type sEPCR sequence and cloned it into pPIC2αC following the *Saccharomyces cerevisiae* α-factor secretion signal that allows for efficient secretion of many proteins from *P. pastoris*. An alanine residue was added at the beginning of the sequence of sEPCR by the cloning process. Mutagenesis to produce sEPCR R96C was performed using the Quickchange™ (Stratagene, La Jolla, CA, USA) method followed by sequencing/subcloning to eliminate the possibility of unwanted mutations in the vector during the mutagenesis process. Yeast was transformed with the linearized vector, resulting in the integration of coding sequence for sEPCR into the endogenous methanol-responsive promoter by homologous recombination. Samples of yeast culture supernatants were electrophoresed under reducing conditions in 12% NuPAGE Bis-Tris gels (Invitrogen) and electroblotted. sEPCR and sEPCR R96C were detected with a rabbit anti-sEPCR polyclonal antibody followed by murine horseradish peroxidase (HRP)-conjugated antirabbit IgG (Alpha Diagnostica International, San Antonio, CA, USA). N-Glycosylation of the wild-type and R96C mutant was studied by digestion with endoglycosidase F/Peptide-N-glycosidase (New England Biolabs, Beverly, MA, USA). In order to study the PC binding to wild-type and R96C sEPCR, microplate wells were coated with the anti-EPCR monoclonal antibody RCR-2 (kind gift of Dr K. Fukudome) to capture sEPCR or sEPCR R96C. Increasing concentrations of human PC (ERL, South Bend, IN, USA) (12.5–1200 nmol L^{-1}) in Hank's balanced salt solution supplemented with 3 mmol L^{-1} $CaCl_2$ and 0.6 mmol L^{-1} $MgCl_2$ were added to the wells. After washing, bound PC was detected with a peroxidase-conjugated anti-PC polyclonal antibody (Dako, Glostrup, Denmark). Calculations of the apparent Kd [Kd(app)] were performed with Enzfitter software (Biosoft, Cambridge, UK) using the one-site ligand-binding model. To compare Kd of wild-type and variant sEPCR, Student's *t*-test was used. The Kd(app) results are expressed as mean ± SD. Wild-type sEPCR and sEPCR R96C expressed in *P. pastoris* showed the same migration pattern on denaturing SDS–PAGE with a band about 45 kDa. Incubation of both wild-type and R96C with N-glycosidase F resulted in a unique and uniform lower band (25 kDa), indicating that glycosylation was similar in both wild-type and R96C (Fig. 1). The Kd(app) of R96C sEPCR–PC binding [61.9 ± 8.5 nM for ($n = 3$)] was similar to that exhibited by the wild-type form [67.9 ± 17.6 nM ($n = 6$), $P = 0.5$].

The epidemiological study performed does not support a role for Arg96 Cys in VTE because it is not more frequent in patients than in controls. However, the size of the study was such that it could not exclude a small risk of VTE and functional characterization was required to eliminate the possibility of a dysfunctional variant. The *in vitro* expression and characterization of EPCR R96C reveals that the binding affinity of R96C EPCR for PC was indistinguishable from that of wild-type EPCR. Since the EPCR effect on PC activation is mediated through the high-affinity binding of PC to EPCR, our results suggest that R96C will behave similarly to wild type in PC activation on the cell surface. R96C variant does not seem to alter molecular stability as the variant is expressed with similar efficiency to wild-type EPCR. Therefore, based on data from

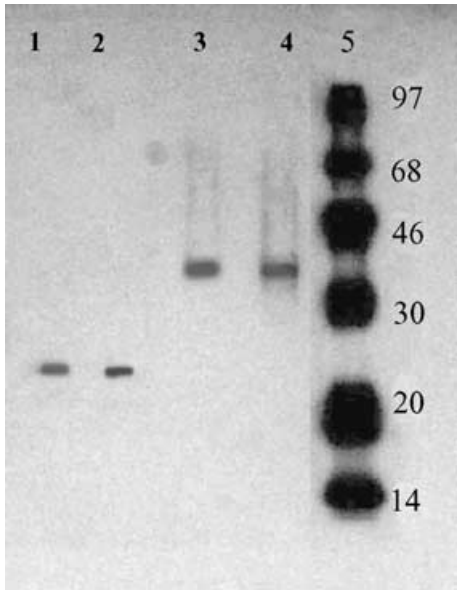

Fig. 1. Expression of recombinant soluble endothelial protein C receptor (sEPCR) and sEPCR R96C in *Pichia pastoris*. Recombinant sEPCR wild-type and R96C preparations from the supernatants of stably transformed *P. pastoris* were subjected to SDS–PAGE under reducing conditions and Western blot with a polyclonal anti sEPCR antibody. Lanes 1 and 2 show wild-type and R96C sEPCR after deglycosylation. Lanes 3 and 4 show wild-type and R96C sEPCR. Lane 5 shows molecular weight markers (kDa).

the epidemiological and biological studies, we conclude that R96C variant is highly unlikely to present any increased risk for VTE.

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Pattern of symptoms in 93 Iranian patients with severe factor XIII deficiency

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Dear Sir,

The deficiency of factor (F)XIII causes a rare but severe bleeding disorder and is associated in women with the frequent inability to carry pregnancy to term [1]. Since the disorder has an average frequency of one case in 2–3 million in the general population, only single cases or small series have been described [2]. We report the clinical symptoms in the largest series ever studied, which consists of 93 Iranian patients who had less than 5% FXIII in plasma measured by a method based upon the solubility of clots in 5 M urea and immunoassays of the A and B subunits of FXIII [3]. The evaluation of such a large group of patients with the rarest coagulation disorder was possible only in a country like Iran, where the high frequency of consanguineous marriages increases by six to eight times the frequency of all recessive disorders [4].

The 93 patients with severe FXIII deficiency from 77 unrelated families (56 males and 37 females, age 3–60 years) were frequently born from consanguineous marriages (82%) and were diagnosed and regularly followed at the Imam Khomeini Hospital Hemophilia Center in Tehran, where detailed records of clinical events occurring in hospital and at home were kept. Data were collected by the same physicians (M.L. and R.S.) who examined the patients, drew family pedigrees and collected clinical histories, with a questionnaire tailored to evaluate the significance of the bleeding symptoms reported. For hematomas and hemarthrosis questions and physical examinations were

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Table 1 Prevalence of each bleeding symptom in 93 patients with factor XIII deficiency

Symptoms	Patients	%
Mucosal tract bleeding		
Mouth bleeding	45/93	48
Menorrhagia	7/20*	35
Epistaxis	30/93	32
Hematuria	10/93	10
GI bleeding	10/93	10
Soft tissue bleeding		
Umbilical cord bleeding	68/93	73
Hematoma	54/93	58
Hemarthrosis	51/93	55
CNS bleeding	23/93	25
Other symptoms		
Surgical bleeding	27/32	84
Miscarriages	3/6**	50

*Twenty of 37 women are of reproductive age. **Only 6/20 women had experience of pregnancy.

focused on the presence or not of a traumatic cause, on localization and frequency; for epistaxis, on frequency (at least three episodes from both nostrils without a history of trauma) and need for compression of the nostrils for treatment; for menorrhagia, on duration of the menstrual period ($\geq$6 days), number of absorbent pads used ($\geq$10) or occurrence of iron deficiency with no other source of bleeding; for gastrointestinal (GI) bleeding, hematuria and central nervous system (CNS) bleeding, on need for hospital admission and treatment. Miscarriages were recorded when no cause other than FXIII deficiency was found. Mouth bleeding after dental extraction or from lips or tongue cuts and major/minor surgery (including circumcision) was also recorded, except for those procedures carried out under replacement therapy. Umbilical cord bleeding was recorded when it led to blood transfusion. Phenotype analysis showed that all patients had $<$5% of subunit A activity by clot solubility in urea and from 25% to 170% of subunit B activity by enzyme immunoassays, so that they were classified as severe FXIIIA-deficient patients.

The most frequent symptom of mucosal tract bleeding (Table 1) was bleeding in the oral cavity (lips, tongue, gum), followed by menorrhagia and epistaxis. GI bleeding was not unusual. On gastroscopic examination, none of the patients with this complication had lesions that could explain bleeding. There was also no obvious cause for macroscopic hematuria.

Umbilical cord bleeding was the most frequent symptom of soft tissue bleeding (Table 1). Spontaneous hematoma and hemarthrosis occurred in a large proportion of patients. Nine cases of retroperitoneal hematoma (10%) were observed (six women and three men). CNS bleeding occurred in 23 patients, 11 intracerebral and 12 subdural. Twenty-seven of 32 patients (84%) who underwent minor and major surgery without replacement therapy had post-surgical bleeding and required blood transfusion. Twenty of 37 women were of reproductive age: in four of these women intraperitoneal bleeding occurred at the time of ovulation and in one patient this complication led to hysterectomy. Only six women became pregnant and half of them had at least one miscarriage. One patient had 13 consecutive miscarriages, all before the 20th week of gestation. She is now on prophylactic treatment with two bags of cryoprecipitate per month, which allowed her to bring to term two pregnancies.

Of our multitransfused patients, 50% were positive for hepatitis C virus (anti-HCV), two patients were positive for HBsAg and none was anti-HIV positive.

The results of this large study confirm that FXIII deficiency is associated with a severe bleeding tendency characterized by a high rate of life-endangering episodes such as CNS and umbilical cord bleeding. Mutation finding and prenatal diagnosis in families at risk will be the best weapon to reduce the incidence of this rare bleeding disorder in a country such as Iran with a high rate of consanguineous marriages.

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Common single nucleotide polymorphisms in the promoter region of the human factor XI gene

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Dear Sir,

Nucleotide polymorphisms that may impact thrombotic risk have been identified in genes for plasma, platelet and blood vessel proteins [1], and epidemiological studies indicate that plasma concentrations of coagulation proteases influence risk for venous thromboembolism (VTE) [2–4]. Factor (F)XI is the zymogen of a plasma protease that contributes to hemostasis by activating FIX. Using the registry of the Leiden Thrombophilia Study, Meijers *et al*. demonstrated that the 10% of the population with the highest plasma FXI levels have a 2.2-fold increased risk for VTE when compared to the remaining 90% [4]. FXI level was an independent risk factor, even after other common risk factors such as FV Leiden and the prothrombin G20210A polymorphism were taken into account. Plasma FXI appears to be synthesized largely, if not exclusively, in the liver [5]. The protein is not known to be stored in secretory granules, nor have mobilizable pools been identified. This suggests that plasma FXI concentration is regulated primarily at the level of transcription and translation within hepatocytes. We wished to determine if the recently characterized promoter region of the human FXI gene [6] contains common single nucleotide polymorphisms (SNPs) that could influence FXI gene expression.

The promoter region of the FXI genes (base pairs −412 to +16) for 65 West African (Ghanaian), 34 Caucasian, and 34 East Asian volunteers were amplified by PCR, using DNA from peripheral blood. PCR products were screened for SNPs by dideoxyfingerprinting as previously described [6], and polymorphisms were verified by direct sequencing of PCR products. Two common SNPs were identified at positions −403 (G/T) and −273 (C/G) in all three populations tested. The predominant allele contained G at −403 (allele frequency 0.71) and C at −273 (allele frequency 0.72). Allele frequencies for −403G in West Africans, Caucasians and East Asians were 0.58, 0.87, and 0.81, respectively; while frequencies for −273C were 0.61, 0.87 and 0.78, respectively. Interestingly, the public data base for the human genome displays the minor allele (− 403T and −273G) for both SNPs (http://www.ensemble.org). We noted that within a population the allele frequencies for the −403 SNP were similar to those for the −273 SNP, and carried out an analysis of haplotype frequencies to look for evidence of linkage disequilibrium between the two loci. The analysis was done using the EHPlus program [7]. Results are shown in Fig. 1(a), and demonstrate in all populations studied a significant deviation from the haplotype distribution expected if the two loci were associating randomly ($P < 10^{-4}$). A subsequent analysis of the degree of linkage disequilibrium using 2LD software [8] confirmed that the two loci are in marked linkage disequilibrium, indicating little recombination has occurred between the two sites since the origination of the SNPs. The significance of this finding is as yet unclear.

We performed a preliminary analysis of the effect of the SNPs on transcription factor binding with gel mobility shift assays, using nuclear extracts from a human hepatoma cell line (Hep G2) and normal rat liver. A significant difference in transcription factor binding was noted between the two versions of the −273 SNP. A major shifted band is noted when a ^{32}P-labeled oligonucleotide representing promoter base pairs −289 to −267, and containing cytosine at position −273 (− 273C), was mixed with Hep G2 nuclear extract (Fig. 1b). This band is effectively competed away by an excess of cold −273C oligo, but not with cold oligo containing G at position −273 (− 273G). Similar results were obtained with normal rat liver nuclear extract (Fig. 1c). Labeled −273G oligonucleotide failed to produce a shifted band with rat liver (Fig. 1c) or Hep G2 (data not shown) extract. Analysis of the sequence surrounding the −273 locus using Transfac – the Transcription Factor Data Base (http://transfac.gbf.de/TRANSFAC/) [9] identified possible binding sites for several transcription factors. Oligonucleotides containing consensus binding sites were prepared for each candidate transcription factor, and were used as competitors in gel mobility shift assays with rat liver nuclear extract. The oligonucleotide containing the binding site for one candidate, the ubiquitously expressed transcription factor CP2, was an effective competitive inhibitor for binding to rat nuclear extract (Fig. 1c).

Plasma FXI levels were measured by ELISA for 57 of the 65 West African volunteers. For this analysis, G at −403 and C at

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(a)

SNP		Ghanaian (n = 65)*		Caucasian (n = 34)*		East Asian (n = 34)*		Total (n = 133)*	
403	273	Expect	Observe	Expect	Observe	Expect	Observe	Expect	Observe
1	1	0.35	0.57	0.75	0.86	0.63	0.78	0.51	0.70
1	2	0.23	0.00	0.12	0.00	0.17	0.02	0.20	0.01
2	1	0.26	0.03	0.12	0.00	0.15	0.00	0.21	0.02
2	2	0.16	0.39	0.02	0.14	0.04	0.19	0.08	0.27

* $p < 10^{-4}$ between expected and estimated haplotype frequencies.

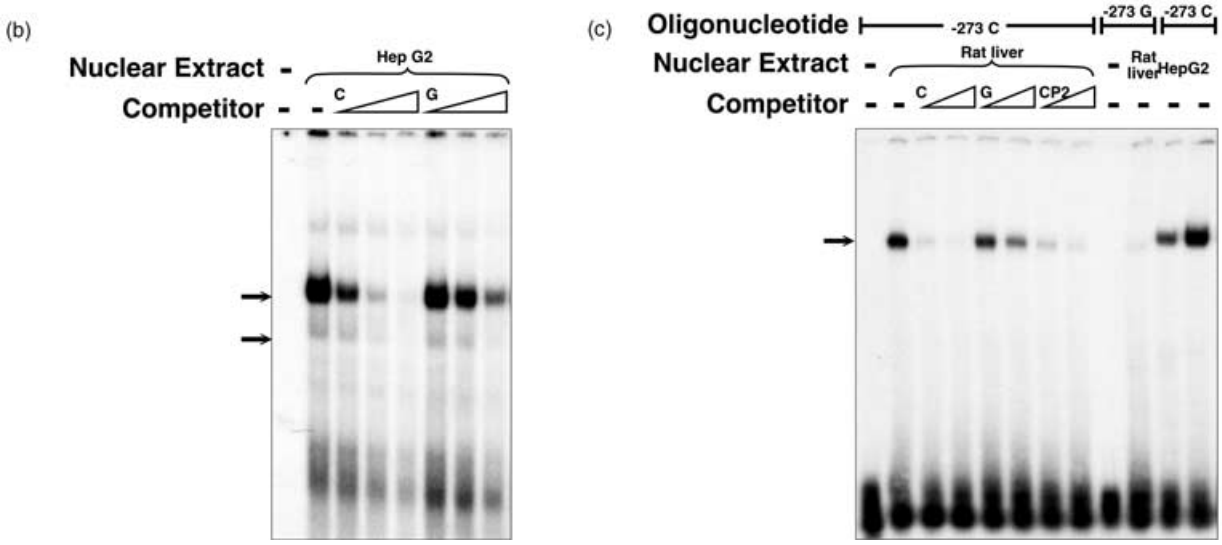

Fig. 1. (a) Estimated haplotypes frequencies for human FXI gene promoter SNPs. Expected haplotype frequencies are compared with observed frequencies for the −403 and −273 SNPs. Expected frequencies are calculated by multiplying allele frequencies at each of the two sites (EHPlus software program). In the SNP column, 1 represents G at −403 or C at −273, and 2 represents T at −403 and G at −273. (b) Gel mobility shift assay for the −273 SNP using Hep G2 nuclear extract. Binding reactions for nuclear extract (10 μg) and ^{32}P-labeled double-stranded oligonucleotide [6] were size-fractionated on 4% non-denaturing polyacrylamide gels, followed by autoradiography. Triangles in the competitor row represent 50-, 100-, and 200-fold excess (left to right) of cold FXI oligonucleotide containing either C or G at position −273. Sequences for the complementary −273C oligonucleotides are 5′-GGCACACAGGCAAAATCAAGTTC and 5′-GAACTTGATTTTGCCTGTGTGCC. The −272 G oligonucleotides are identical except for substitutions at the underlined base pair (G and C, respectively). (c) Gel mobility shift assay with rat liver nuclear extract. ^{32}P-labeled FXI oligonucleotides containing C or G at position −273 were incubated with rat hepatocyte or Hep G2 nuclear extract (10 μg) and analyzed as in (b). Triangles in the competitor row represent 100- or 200-fold molar excesses of cold FXI oligonucleotides containing C or G at position −273, or an oligonucleotide containing a consensus binding site for transcription factor CP2. Sequences for the complementary CP2 oligonucleotides are 5′-CAAGTTTTACTGGGTAGAGCAAGCACAAACCAG and 5′-CTGGTTTGTGCTTGCTCTACCCAGTAAAACTTG.

−273 were considered wild type, and T at −403 and G at −273 variant alleles. A pool of plasma from all participants was prepared as a control. The mean for the individually determined FXI levels for the entire group was 109 ± 35.5% of the pooled control. The mean FXI level for individuals with only wild-type alleles was slightly higher (115.5 ± 39.1% of control) than the mean for those with one or two variant (105.6 ± 37.5%) or three or four variant (105.7 ± 33.3%) alleles. However, ranges were wide in all populations and no statistically significant difference based on haplotype could be determined in this small population.

In summary, we have identified common polymorphisms at nucleotides −403 and −273 in the promoter of the human FXI gene in members of diverse populations. This wide distribution is consistent with an origin for both SNPs predating the exit of modern *Homo sapiens* from Africa to Asia and Europe. The −273 SNP influences transcription factor binding *in vitro* and may therefore affect gene transcription *in vivo*. The FXI −403 and −273 SNPs should be examined in larger study populations for correlation with plasma FXI levels and association with thrombotic disorders.

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No Val34Leu polymorphism of the gene for factor XIIIA subunit was detected by ARMS-RACE method in three Asian populations

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Dear Sir,

During the course of studies to find genetic defects of XIIIA or XIIIB deficiency, a G→T substitution was identified at codon 34 in exon II of the XIIIA gene (Val34Leu), and turned out to belong to common polymorphisms of the XIIIA gene. Recently, the Val34Leu polymorphism was found to be inversely associated with thrombotic phenotypes, primarily in Caucasians [1–3]. In contrast to the high prevalence of this polymorphism in Caucasians, a low prevalence has been reported in investigation of a limited number of Japanese subjects [4,5]. Moreover, to our knowledge the prevalence of this polymorphism has not been determined in Asian populations besides Japanese. To allow the simple and reliable determination of the Val34Leu polymorphism in larger studies, we developed a new genetic diagnostic method by combining an amplification refractory mutation system (ARMS) with rapid automated capillary electrophoresis (RACE) [6], and applied it to about 500 Asian subjects including healthy Japanese, Korean, Chinese and Italian individuals, and Japanese patients with ischemic heart disease, cerebrovascular dementia, or Alzheimer's disease.

To determine genotypes by ARMS, two differently fluorescence-labeled sense primers, 20 mer with FAM for the Val34 allele (5′-CACAGTGGAGCTTCAGAGCG-3′) and 22 mer with TET for the Leu34 allele (5′-CCCACAGTGGAGCTTCAGAGCT-3′), and a common antisense primer (5′-CTGGACCCAGAGTGGTGG-3′) were used for polymerase chain reaction. Amplified products were analyzed by RACE using an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer, Foster City, CA, USA), as described previously [6].

The application of the ARMS-RACE method to 103 healthy Japanese revealed that none had the Val34Leu polymorphism, though its gene frequency was high in Italians (Table 1). This was also true when 201 Japanese patients with ischemic heart disease, cerebrovascular dementia, or Alzheimer's disease were examined. Among the 304 Japanese subjects in this study, the Val34Leu polymorphism was not detected at all, which is inconsistent with the two previous reports that it was found in one out of 46 and one out of 40 Japanese individuals [4,5]. The reason for this discrepancy is currently unclear; however, the presence of the polymorphism in Suzuki's study could be an error, since it was judged by a mobility shift in single-strand

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Table 1 Prevalence of XIIIA Val34Leu genotypes

Ethnic group	Total	Val/Val	Val/Leu	Leu/Leu	Frequency*
Control					
Japanese	103	103	0	0	0.00**
Chinese	95	95	0	0	0.00**
Korean	100	100	0	0	0.00**
Italian	75	42	30	3	0.24
Patients (Japanese)					
Ischemic heart disease	87	87	0	0	0.00**
Cerebrovascular dementia	20	20	0	0	0.00**
Alzheimer's disease	94	94	0	0	0.00**

*Gene frequency of Leu allele. **$P < 0.005$ against Italian control subjects.

conformation polymorphism analysis. It is also possible that the subjects of the latter study [5] may not be pure Japanese, since they were 'Japanese descendents' in Brazil and may have been admixed with other ethnic groups having high gene frequencies of the polymorphism.

It was of note that the polymorphism was detected neither in 100 Korean nor in 95 Chinese individuals. Although a total of 499 subjects were examined, the Val34Leu polymorphism was not detected in three Asian populations. In contrast, much higher distribution of the Val34Leu allele has been reported in 160 and 191 Asian subjects in the UK [7,8]. However, these were Asian Indian in the former study, and originated from India, Pakistan, or Bangladesh in the latter study; therefore, they must be Caucasoid. The Val34Leu polymorphism may have appeared after the Caucasoid–Mongoloid diversion. This is less likely, however, because the Val34Leu allele has also been found to occur frequently in American Indians and Africans [5]. It is more likely therefore that individuals having only the Val34 allele were ancestors of the three Asian populations. Alternatively, codon 34 of the XIIIA gene may be a hotspot for mutation, and the G→T transversion may have occurred more than once in the other ethnic groups.

Since the Val34Leu polymorphism is inversely associated with myocardial infarction, cerebral infarction, and venous thrombosis in Caucasians [1–3], it is important to study the genuine prevalence of the Val34Leu polymorphism among different ethnic groups. The polymorphism was absent in Japanese individuals both with and without thrombosis. Thus, this polymorphism is not a discriminative risk factor for thrombosis, at least among Japanese. Furthermore, the incidence of thrombosis among Japanese is lower than in Americans (Caucasian) [9]. Since thrombosis is a multifactorial disease, the absence of other genetic risk factors, such as the factor V Leiden and prothrombin G20210A polymorphisms, may compensate for the prothrombotic phenotype of the Val34 allele in Japanese (and also in Korean and Chinese).

The prevalence or frequency of each gene polymorphism is quite different between ethnic groups, as is the case with the Ala601Thr variant, a common single nucleotide polymorphism (SNP) of the plasminogen gene in Asian populations alone, including Japanese, Korean, and Chinese [6]. Thus, the Val34-Leu polymorphism is yet another example of the ethnic specificity of gene polymorphisms. It is important to accumulate data on SNPs of thrombosis-related genes in each ethnic group in order to understand fully these groups' genetic risk factors for thrombosis.

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Preserving eye function in prematurely born children with severe protein C deficiency

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Dear Sir

Homozygous or double heterozygous protein C deficiency is very rare and usually presents life-threatening thrombosis such as cerebral venous sinus thrombosis and purpura fulminans. Another presentation is blindness, which is believed to occur *in utero* during the third trimester period and soon after birth. The occurrence of vitreous hemorrhage and retinal detachment results in permanent blindness [1–6]. Its pathophysiology is poorly understood.

The treatment options include giving low molecular weight heparin during infancy, oral anticoagulant, intravenous or subcutaneous replacement of protein C concentrate, which still has some limitations in many countries, and liver transplantation [4,7,8].

We report on two 6-year-old twin children born as 33-week monozygotic preterm infants who had delayed thrombotic clinical presentations. The first twin, birth weight 2030 g, presented with purpura fulminans at 1 year of age. He received supportive and symptomatic treatment and was diagnosed with severe protein C deficiency (protein C activity 2%) at 4 years of age. His father and mother have protein C activity of 19% and 62% (normal 64–140), respectively. He was doing well, although the brain magnetic resonance imaging showed evidence of cerebral infarction and perfusion-ventilation of lungs revealed evidence of pulmonary embolisms.

The second twin, birth weight 1680 g, developed meningitis in the neonate period complicated by cerebral palsy. He also presented with purpura fulminans at 1 year of age and received supportive and symptomatic treatment. At 4 years of age, he exhibited deep vein thrombosis of lower extremity combined with pulmonary embolisms inducing cyanosis and dyspnea. Consequently, he was diagnosed with severe protein C deficiency (protein C activity 2%).

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Both twins have been anticoagulated with warfarin to maintain the International Normalized Ratio (INR) levels at 3–4. However, both twins exhibited thrombotic complication while their INR levels were at 4 and 2, respectively. Fresh frozen plasma at a dose of 10 mL kg^{-1} was additionally given once a week. The plasma is called quarantine plasma prepared from three regular donors by the National Blood Center, Thai Red Cross Society. The plasma is used after the donors have two negative anti-HIV testings with an interval of 3 months. From 4 to 6 years of age, both twins have not shown any thrombotic event. They are still negative for the hepatitis B, C and HIV infection.

At 6 years of age, the complete eye examination was performed. In the first twin, the corrected visual activity was $20/30^{-2}$ in the right eye and $20/40^{-2}$ in the left eye. The corneas, anterior chambers, pupils and lenses were normal. Dilated fundus examination revealed clear vitreous and normal posterior segment. Refraction showed high myopia with a spherical equivalent of −7.50 diopters right eye and −13.00 diopters left eye. In the second twin, the visual acuity in both eyes measured by the preferential looking test revealed $20/300^{-2}$. The patient could fix on and follow the testing target. Anterior segment and dilated fundus examinations were within normal limits. His poor vision is most likely due to the underlying cerebral palsy.

The illustrated preserved eye function in prematurely born children with severe protein C deficiency supports the hypothesis that eye complications from severe protein C deficiency commonly occur *in utero* during the third trimester. Although the management of females at risk is prenatal diagnosis [2,8–10], early delivery during the third trimester with immediate protein C replacement after birth will be another favorable option.

Prophylactic replacement therapy with fresh frozen plasma benefits the patients in decreasing thrombotic events. The patients are less likely to suffer from deep vein thrombosis and pulmonary embolisms. Awareness of transfusion-transmitted diseases is warranted. Quarantine plasma from regular donors is another option where protein C concentrate is not available or too expensive.

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