Journal of Thrombosis and Haemostasis, **6**: 797–803 DOI: 10.1111/j.1538-7836.2008.02934.x

ORIGINAL ARTICLE

Vitamin K-induced modification of coagulation phenotype in VKORC1 homozygous deficiency

G. MARCHETTI,* P. CARUSO,* B. LUNGHI,* M. PINOTTI,* M. LAPECORELLA,† M. NAPOLITANO,† A. CANELLA,* G. MARIANI‡ and F. BERNARDI*

**Department of Biochemistry and Molecular Biology, University of Ferrara, Ferrara; †Hemophilia and Thrombosis Centre, O.U of Internal Medicine 2, San Salvatore Hospital, L'Aquila; and ‡Department of Internal Medicine and Public Health, L' Aquila University, L'Aquila, Italy*

To cite this article: Marchetti G, Caruso P, Lunghi B, Pinotti M, Lapecorella M, Napolitano M, Canella A, Mariani G, Bernardi F. Vitamin K-induced modification of coagulation phenotype in VKORC1 homozygous deficiency. *J Thromb Haemost* 2008; **6**: 797–803.

Summary. *Background:* Combined vitamin K-dependent clotting factor (VKCF) deficiency type 2 (VKCFD2) is a rare bleeding disorder caused by mutated vitamin K 2,3-epoxide reductase complex subunit 1 (VKORC1) gene. *Methods and results:* An Italian patient with moderate to severe bleeding tendency was genotyped, and found to be homozygous for the unique VKORC1 mutation (Arg98Trp) so far detected in VKCFD2. The activity levels of VKCFs were differentially reduced, and inversely related to the previously estimated affinity of procoagulant factor propeptides for the γ-carboxylase. The normal (factor IX) or reduced antigen levels (other VKCFs) produced a gradient in specific activities. Vitamin K supplementations resulted in reproducible, fast and sustained normalization of PT and APTT. At 24 h the activity/antigen ratios of VKCFs were close to normal, and activity levels were completely (factor VII and IX), virtually (prothrombin, factor X and protein C) or partially (protein S) restored. Thrombin generation assays showed a markedly shortened lag time. The time to peak observed at low tissue factor concentration, potentially mimicking the physiological trigger and able to highlight the effect of reduced protein S levels, was shorter than that in pooled normal plasma. At 72 h the thrombin generation times were normal, and the decrease in activity of procoagulant VKCFs was inversely related to their half-life in plasma. The improved coagulation phenotype permitted the uneventful clinical course after invasive diagnostic procedures. *Conclusions:* Modification of coagulation phenotypes in VKCFD2 after vitamin K supplementation was clinically beneficial, and provided valuable patterns of factor specific biosynthesis, half-life and decay.

Correspondence: Francesco Bernardi, Department of Biochemistry and Molecular Biology, via Fossato di Mortara 74, I-44100 Ferrara, Italy. Tel.: +39 0532 974425; fax +39 0532 974484.
E-mail: ber@unife.it

Received 31 August 2007, accepted 8 February 2008

Keywords: VKCFD2, VKORC1 mutation, coagulation factor levels, thrombin generation, vitamin K supplementation.

Introduction

Impaired γ-carboxylation [1,2] produces multiple deficiency of vitamin K-dependent coagulation factors (VKCFD), a rare bleeding disorder inherited as an autosomal recessive trait [3]. The VKCFD type 2 (VKCFD2) is caused by defective regeneration of vitamin K hydroquinone by the vitamin K 2,3-epoxide reductase complex subunit 1 (VKORC1) [4]. Only one causative missense change (VKORC1 Arg98Trp) has been found in the homozygous condition in families of Lebanese and German origin [5,6].

In VKCFD2, the activity of the γ-glutamyl carboxylase enzyme, impaired by the limiting concentration of the vitamin K hydroquinone, can be transiently restored by vitamin K supplementation. These features make this condition an ideal model to investigate *in vivo* the temporal variations in levels of vitamin K-dependent factors, driven by changes in γ-glutamyl carboxylation activity. Comparison of this natural condition with the pharmacological inhibition of the vitamin K cycle could provide valuable information to interpret observations obtained in plasma of patients on anticoagulant therapy [7–11].

The study of plasma phenotype variation in VKCFD2 would also contribute to validate, through *in vivo* observations, the cellular and molecular investigations [4,12–16] aimed at dissecting the mechanisms through which components of the vitamin K cycle [17] participate in the regulation of circulating factor level activity. Moreover, an extended analysis of levels after vitamin K supplementation in VKCFD2 would also provide information about the natural decay of vitamin K dependent clotting factors, of great interest for replacement therapy in inherited coagulation disorders [18].

As a thorough characterization of this deficiency by single factor parameters and by assays integrating the contribution of procoagulant and anticoagulant components has not been reported, we have investigated an VKCFD2 Italian patient over time after vitamin K supplementation.

Patients, materials and methods

Patient and family

The proposita, a 34-year-old woman, had experienced since childhood repeated nose and gum bleeds, menorrhagia, a severe post-partum hemorrhage at the age of 23 requiring blood transfusions, hemoperitoneum following ovarian cyst rupture, and rectal bleeding. In addition she presented with osteoporosis.

Laboratory testing of the patient's plasma revealed reduced activity levels of several procoagulant factors. Family members showed coagulation parameters in the normal range. Informed consent was obtained from the family members entering this study.

DNA analysis

The screening of the molecular defect responsible for the altered coagulation profile in the proposita was performed in both the γ-glutamyl-carboxylase (GGCX) and VKORC1 genes.

Direct scanning of exons and intron-exon boundaries of GGCX and VKORC1 genes were performed by PCR amplification followed by automated sequencing with the ABI Prism 377 DNA Sequencer (PE Applied Biosystems, Foster City, CA, USA). Specific primers for GGCX and VKORCI genes were derived from the Gene Bank database (references U65896 and AY587020, respectively). In the GGCX gene three single nucleotide changes, the g.1156G > C (intron 1), the g.9167T > C (exon 9), that predicts the Arg406Arg synonymous change, and the g.11665G > C (intron 14) were detected.

Coagulation laboratory assay

Plasma samples were withdrawn before (0 h) and after (at 4, 24, 28 and 72 h) intravenous administration of 10 mg vitamin K (Konakion–Roche, Basel, Switzerland). Plasma samples before and after 24 h were also collected during a second course of vitamin K application conducted 5 months later, before an esophagogastroduodenoscopy and colonoscopy with biopsy. Venous blood was drawn in sodium citrate (12.9 mmol L^{-1}) and immediately centrifuged at 2000 *g* for 20 min at 4 °C. Plasma was separated, snap-frozen, and stored in aliquots at −80 °C.

Factor clotting activities were assessed by conventional PT (factor (F) II, FVII, FX and PS) or APTT -based assays (FIX and PC) with the corresponding commercial factor-depleted plasma (HemosIL^{TM}; Instrumentation Laboratory, Milan, Italy). PT was also assayed in a FII, FVII, FIX and FX-depleted bovine plasma triggered with bovine thromboplastin (Pro-IL-Complex kit, HemosIL^{TM}). PC amidolytic activity was measured chromogenically (HemosIL^{TM} Protein C).

The total activity of the protein C pathway was evaluated by the APTT-based method Pro-C® Global (Dade Behring Diagnostics, Marburg, Germany). The normalized ratio (PCAT-NR) of the APTT determined in the presence and in the absence of activator Protac (PCAT:PCAT/0 ratio) was reported.

Antigen assays

Antigen levels were measured by ELISA using a mouse monoclonal anti-human FVII antibody, a sheep polyclonal anti human-FVIII antibody, a goat polyclonal anti-human FIX antibody (Affinity Biologicals Inc, Ancaster, Canada), a rabbit polyclonal anti-human FX antibody (DakoCytomation, Glostrup, Denmark) and a mouse monoclonal anti-human free PS antibody (Instrumentation Laboratory) directed to the C4bBP domain. Prothrombin levels were evaluated by Western blot analysis as previously reported for FX [19]. Upon electrophoresis of diluted patient's plasma (1:70) on a Novex Tris-Glycine 4–20% PAA Gel (Invitrogen, Carlsbad, CA, USA), and electroblotting onto nitrocellulose membranes (Schleicher & Schuell Microscience, Keene, NH, USA), prothrombin was probed by a sheep polyclonal anti-human prothrombin-HRP (Affinity, Carlsbad, CA, USA). Densitometric analysis of bands was conducted by the GS-700 instrument (BIORAD, Hercules, CA, USA).

FXa generation

Plasma FVII activity towards FX was assayed essentially as previously described [20]. Generation of activated FX (FXa) was monitored continuously by exploiting a specific fluorogenic substrate ($MeSO_2$-D-CHA-Gly-Arg-AMCAcOH, American Diagnostica, Greenwich, CT, USA). Fluorescence (360 nm excitation, 465 emission) was measured on Spectra-FluorPlus microplate reader (TECAN, Salzburg, Austria).

Thrombin generation

Platelet-poor plasma (PPP) samples were centrifuged at 23 000 *g* at 4 °C for 1 h before testing. Calibrated automated thrombin activity measurement was conducted according to Hemker *et al.* [21] in an automated microtiter plate fluorometer (Fluoroskan Ascent; Thermo Labsystems, Helsinki, Finland) using the Thrombinoscope software (Synapse BV, Maastricht, The Netherlands). The assays were carried out at 37 °C essentially as previously reported [22]. Coagulation was triggered in recalcified PPP under the following experimental conditions: 4 μM phospholipids (PLP, 20% phosphatidyl serine-80% phosphatidyl choline); 1 pM recombinant human tissue factor (TF)/4 μM PLP; 5 pM TF/4 μM PLP; 10 pM TF/8 μM PLP. The snake venom Protac (HemosIL^{TM}; Instrumentation Laboratory) was used in the presence of 3.5 pM TF/10 μM PLP. Thrombin generation was evaluated overtime by exploiting a specific fluorogenic substrate (Z-Gly-Gly-Arg-AMC) and was conducted in parallel in each plasma sample supplemented with a thrombin calibrator (Synapse BV). Experiments were carried out in duplicate or triplicate.

Results

DNA studies

Sequencing of the VKORC1 gene showed that the proposita was homozygous for the c.292C > T transition resulting in the amino acid change Arg98Trp. The proposita's mother, two brothers and one sister were found to be carriers, as indicated by the AciI restriction analysis.

Frequent polymorphisms predicting variation in coagulation factor levels were also investigated. The proposita was found to be homozygous for the −323 ins10 in F7 gene [23], a condition associated with a remarkable reduction in FVII levels [24].

Coagulation studies

To get a comprehensive evaluation of the hemostatic profile and of its variations following vitamin K supplementation, APTT and PT were measured in the patient's plasma before and after intravenous supplementation of 10 mg vitamin K (Table 1). Before treatment the APTT was mildly prolonged and the PT-INR was remarkably increased. At 4 h a normal APTT and a remarkable decrease of the PT-INR were observed. The APTT was shorter than that of PNP (30 s) both at 24 and 28 h, and at the same time points PT was normalized. At 72 h the beneficial effects of vitamin K supplementation were noticeable, particularly for APTT.

Variations in PT and APTT were evaluated in the light of level variations of the specific vitamin K-dependent factors.

Table 1 Coagulation times and activity levels of vitamin K-dependent coagulation factors measured before (0 h) and after vitamin K treatment

	Plasma sampling						
	0 h		4 h	24 h		28 h	72 h
APTT	33	39*	26	23	24*	23	26
PT-INR	3.18	3.46*	1.66	1.15	1.14*	1.11	1.79
PT†	107			37			
PCAT-NR	0.64			1.29			
FVII	14	10*	30	113	115*	117	19
FIX	26	21*	58	142	93*	108	67
FX	8	8*	30	68	79*	66	39
	31‡			77‡			64‡
FII	26	13*	42	67	84*	67	43
PC	36	34*	55	92	105*	89	48
PS	27	21*	36	54	46*	50	45

*Values obtained before and after the second course of vitamin K administration. The APTT and PT† are expressed in seconds (normal range 25–35 s and 18–27 s, respectively). The PT† was measured in a FII, FVII, FIX and FX-depleted bovine plasma triggered with bovine thromboplastin. PCAT-NR, normalized ratio of the APTT determined in the presence and in the absence of the protein C activator Protac. Clotting activity (FVII, FIX, FX, FII) and amydolitic activity (PC) levels are reported as percentage of PNP (70–120, normal range). Free protein S antigen is reported as percentage of PNP (53–109, normal range). ‡FXa levels (% of PNP) measured at the peak of the FXa generation curves.

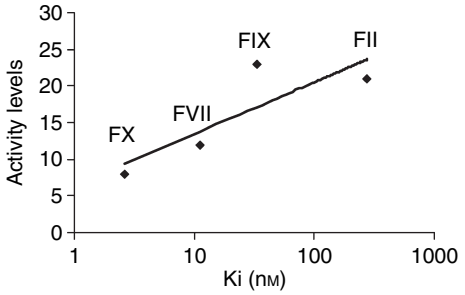

Fig. 1. Baseline activity level of procoagulant factors and inhibition constants (Ki) of coagulation factor propeptides for γ-carboxylase [25]. The mean activity levels measured in two independent plasma samples are reported. Logarithmic relation between variables (R^2 = 0.70).

Procoagulant factors The baseline activity levels, measured on different occasions (Table 1), were constant for FX and showed modest (FVII and FIX) or pronounced (prothrombin) variations.

As the differentially reduced activity values might reflect the residual carboxylase activity, we investigated their relationship with the binding affinities of carboxylase for the propeptide of the vitamin K-dependent procoagulant factors, previously estimated through peptide inhibition constants [25]. In two plasma samples obtained 5 months apart before vitamin K supplementation, mean activity levels showed a logarithmic relation (R^2 = 0.70) with the inhibition constants (Fig. 1). The best relation was found for FVII, FX and prothrombin levels (R^2 = 0.99), which indicated that their activity was inversely related with the affinity of the vitamin K-dependent carboxylase for coagulation factor propeptides.

For all factors an appreciable and reproducible (Table 1) increase in activity levels was measured after vitamin K supplementation. Plasma activity levels reached normal values for FVII and FIX, and borderline values for FX and prothrombin.

Levels higher than those at baseline were still detectable at 72 h, with the exception of FVII, which decreased at pretreatment levels (Table 1). The decrease in levels from the peak to 72 h was inversely related to (Fig. 2) the known half-life values of coagulation factors [26]. Particularly, the decrease showed

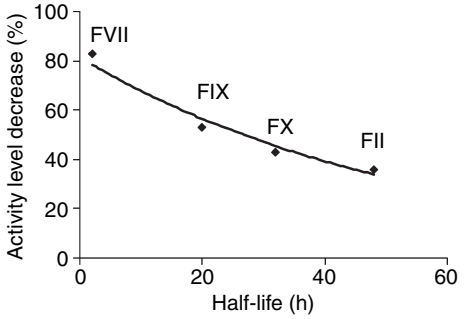

Fig. 2. Decrease in activity levels (expressed as percentage) from peak to 72 h and half-life of procoagulant factors. The lowest values in the half-life range [26] were used (R^2 = 0.97, exponential curve).

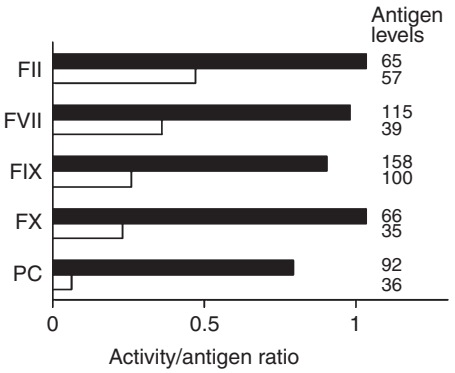

Fig. 3. Activity/antigen ratios measured before (white column) and after vitamin K treatment (black column). For PC the anticoagulant/amydolitic activity ratio is reported. The antigen levels are indicated. Prothrombin antigen levels were evaluated by densitometric analysis of Western blot.

the best fitting (exponential curve, $R^2 = 0.97$) with the lowest values of the half-life range. The R^2 using the highest half-life values was 0.75.

Defective VKOR activity appeared to affect activity and secretion of vitamin K-dependent factors to a different extent, and was responsible for the presence of dysfunctional molecules in plasma, as indicated by an excess of antigen levels (Fig. 3). FIX antigen levels were normal at baseline whereas those of FVII and FX were similarly reduced. At 24 h antigen levels were restored (FVII) or remarkably increased (FX).

The activity/antigen ratios at baseline and their variations induced by vitamin K supplementation after 24 h are summarized in Fig. 3. Before vitamin K administration, the unbalance between activity and antigen levels was remarkable with ratios of 0.2 (FX), 0.3 (FIX) and 0.5 (FVII and FII), which would strongly indicate the presence of partially carboxylated forms of vitamin K dependent clotting factors (PIVKA).

The overall dysfunctional effect of poorly carboxylated forms was indicated by PT assays conducted in a FII, FVII, FIX and FX-depleted bovine plasma triggered with bovine thromboplastin (Table 1). The PT observed in the patient (107 s, 5% of PNP) was more prolonged than those measured in plasma from patients on anticoagulant therapy (range 44–89 s, 7–28% of PNP).

Vitamin K administration substantially increased activity/antigen ratios of all procoagulant factors. The increase in amount of functional molecules was also indicated by the amelioration of parameters of PT and APTT assays (Table 1). However, the PT obtained in the bovine plasma (clotting time 37 s and 42% activity) was still far from normal (18–27 s).

As expected, the antigen level of FVIII (1.31 IU mL^{-1}), not requiring vitamin K for biosynthesis, did not change after vitamin K administration (1.33 IU mL^{-1}).

Anticoagulant factors Repeated measurements indicated that the constant but defective amydolitic activity of PC at baseline was substantially improved by vitamin K supplementation (Table 1). Anticoagulant assays indicated a very ample variation in PC activity (from 2% to 73% of PNP). Assuming that amidolytic activity parallels or is lower than antigen levels, the increase in PC specific activity (ratio from 0.06 to 0.8; Fig. 3) appears the most pronounced among the evaluated serine proteases.

Protein S free antigen levels at baseline (Table 1) were similar to those found in type I PS deficiency. Although levels were doubled at 24 h, they were only partially restored. This pattern was confirmed in the second course of vitamin K supplementation (Table 1).

At 24 h the PS anticoagulant function, evaluated in a PT-based assay, resulted in 58% of PNP, which roughly normalizes the activity/antigen ratio, as observed for vitamin K-dependent serine proteases.

The protein C pathway was further investigated through activation of endogenous protein C by the venom Protac in the APTT-based assay ProC global [27] (Table 1). The normalized ratio at baseline (NR = 0.64) was comparable with that of a heterozygous FV Leiden plasma (NR = 0.72), and was substantially increased by vitamin K supplementation (NR = 1.29).

Thrombin and FXa generation assays

The vitamin K-induced modification of thrombin generation parameters was evaluated under different conditions (Fig. 4). At all TF concentrations the lag-time and time to peak were both prolonged before treatment. In two plasma samples, obtained several months apart and assayed at 1 pM TF, the lag-time ranged from 7.7 ± 0.01 to 9.8 ± 0.16 min and the time to peak from 10.3 ± 0.01 to 12.8 ± 0.16 min. By comparison, the PNP lag-time (4.5 ± 0.5 and 4.6 ± 0.16 min) and time to peak (8.2 ± 1.17 and 8.6 ± 0.16 min) were constant. In the absence of TF thrombin generation was undetectable at 40 min (not shown).

At 24 h after vitamin K supplementation, the lag-time was similar to normal (Fig. 4). When measured at 1 pM TF (Fig. 4)

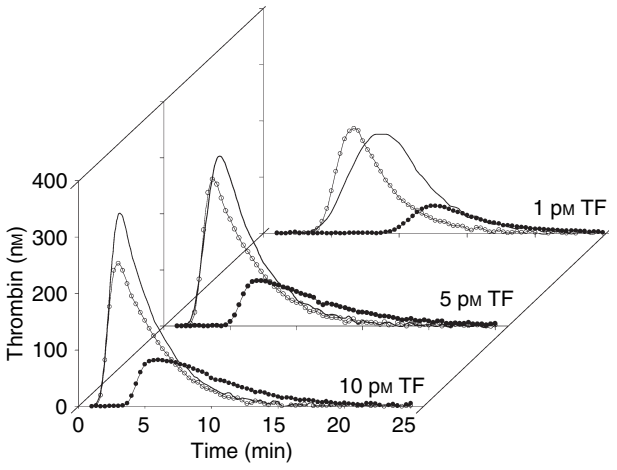

Fig. 4. Thrombin generation triggered with the indicated TF concentrations in patient plasma, before (●) and 24 h after (○) vitamin K administration, and in PNP (–).

the time to peak was reproducibly shorter (5.7 ± 0.33 and 6.6 ± 0.16 min) than in PNP.

Before treatment ETP values were clearly reduced at all TF concentrations (Fig. 4), ranging from 26% (1 pM TF) to 48% of PNP (10 pM TF). After treatment, the ETP was close to normal, ranging from 70% (1 pM TF) to 78% (10 pM TF) of PNP.

Normal time parameters (lag-time 4.7 ± 0.01 min, time to peak 7.0 ± 0.01 min) and sustained ETP (61% of PNP) were still present in the patient's plasma at 72 h.

In order to enhance the contribution of the APC pathway, thrombin generation was also evaluated in the presence of the venom Protac (not shown). This protein C activator substantially inhibited thrombin generation in PNP (61% reduction in ETP), but was poorly effective in the patient's plasma, both before (21% reduction) and after (25% reduction) vitamin K administration. The ETP measured in plasma from a protein S-deficient subject (PS:C and PS:Ag, 40%) was poorly modified (11% decrease) after the addition of Protac.

FXa generation was measured with a specific FXa fluorogenic substrate. The FXa generation after extrinsic activation (Table 1) substantially increased following vitamin K administration (from 31% to 77% of PNP), and was maintained at 72 h (64% of PNP). The time to maximal FXa generation was consistently shortened at 24 h (from 3.3 to 1.0 min) and similar to that of PNP (1.1 min). The FXa generation levels at baseline were comparable with FX antigen levels, and higher than FX activity in the coagulation assay, further confirming the presence of dysfunctional FX molecules (Fig. 3).

Discussion

The genotyping and characterization of the coagulation phenotype in the VKCFD2 patient provided us with information that significantly extends the knowledge of this rare condition, and has general implications for vitamin K-dependent regulation of plasma factor levels.

It is intriguing that among the several CpG sites (n = 30), potential hot spots for missense changes in VKORC1, only that contained in codon 98 and responsible for very low VKORC1 activity has been found to be mutated in VKCFD2 patients from different countries [5]. Differently, several VKORC1 mutations have been associated with warfarin resistance [5,28,29], a major clinical phenotype in the anticoagulant therapy. However, the mutational pattern of VKCFD2 is based on very few cases, which prevents an informative comparison.

The homozygous VKORC1 Arg98Trp change has been found associated with heterogeneous clinical pictures, ranging from fatal intracerebral hemorrhage after birth [6] to moderate/severe bleeding in adult life in the proposita. Noticeably, this Italian patient is also homozygous for FVII polymorphisms predicting further reduction in FVII levels, which could exacerbate bleeding tendency. From these observations we infer that VKCFD2, caused by a single VKORC1 mutation, interacts with other genetic/environmental factors with a major role in the clinical phenotype. The variations of coagulation parameters observed in the proposita before vitamin K supplementations further support the role of environmental components.

The homozygous condition for the VKORC1 Arg98Trp substitution provided us with a valuable model to investigate *in vivo* the effect of the limited availability of vitamin K hydroquinone on coagulation factor levels, and to compare biosynthetic efficiency of hepatocytes subjected to genetically induced depletion of vitamin K hydroquinone with those poisoned by coumarin derivatives. FIX, as strongly indicated by its normal antigen levels, showed the lowest sensitivity to the defective vitamin K cycle, either induced by the genetic deficiency of VKORC1 or warfarin therapy [8,11].

Interestingly, we found that the functional levels of procoagulant factors before vitamin K supplementation, which integrate several biosynthetic and secretion steps *in vivo*, inversely correlated with the affinity of the vitamin K-dependent carboxylase for coagulation factor propeptides. Our observation supports the hypothesis that binding affinities, found *in vitro* to vary over a considerable range [25] and to influence the extent of γ-carboxylation [30], contribute to predict activity level differences *in vivo*, particularly for FVII, FX and prothrombin levels and to a lesser extent for FIX.

The similarly reduced protein levels of FVII, FX and PC in the presence of a wide range of activity levels, produced a gradient in specific activity among factors (Fig. 3), with PC displaying the lowest value. The major effect of VKORC1 deficiency on this inhibitor does not appear to be mediated by a preferential impairment in biosynthesis/secretion [14]. The low affinity of PC for membranes [31], further decreased by reduced γ-carboxylation of the Gla domain, should substantially contribute to the extremely reduced PC functional levels in clotting assays, in the lower range of those observed in patients on stabilized warfarin treatment [32].

Vitamin K supplementation enabled us to investigate temporal variations in levels of the vitamin K-dependent factors, their effects on overall function tests and clinical coagulation phenotype. An intravenous single dose of vitamin K resulted in a fast, efficient and sustained normalization of coagulation times, as indicated by the integrated assays we used.

The fast amelioration of clotting times and of coagulation factor activity levels at 4 h after vitamin K supplementation could reflect, in addition to a '*de novo*' biosynthesis, a short time release of proteins accumulated at the intracellular level because of impaired γ-carboxylation. This complexity limits our ability to measure in plasma the steady state conditions of the vitamin K-dependent factors. The restoration of the carboxylase activity by vitamin K supplementation could be also favoured by DT-diaphorase, a NAD(P)H dehydrogenase, that might play a role in the generation of the hydroquinone form of vitamin K in the presence of a high concentration of vitamin K quinone [33], as probably occurs after vitamin K intravenous administration.

The evaluation of the thrombin generation offered several quantitative parameters clearly demonstrating the beneficial

effects of vitamin K supplementation, particularly the markedly shortened lag-time and time to peak. In the presence of normalized or borderline activity levels of all procoagulant factors and PC, the partial restoration of protein S levels would contribute to the short time parameters of thrombin generation, and to the low response to Protac. Comparison of thrombograms obtained with different TF concentrations provided clear evidence for the gain of information obtained at 1 pM TF concentration, potentially mimicking the physiological trigger and able to highlight the effect of reduced protein S levels [34].

The follow up of the coagulation factor levels after transient vitamin K-induced increase permits a parallel evaluation of their natural decay in plasma, of great interest for replacement regimens in inherited deficiencies. Strikingly, the differential decrease in functional levels of endogenous procoagulant vitamin K-dependent factors from the peak to 72 h supports the lowest half-life values previously estimated in plasma for infused single factors.

The functional rescue and the uneventful clinical course after invasive diagnostic procedures suggest that intravenous vitamin K administrations should be considered the first-line treatment in this condition. However, intravenous and oral long-term administrations should be tested in a formal study to evaluate the effectiveness of vitamin K administration in prophylactic terms.

Taken together our findings in VKCFD2 validate *in vivo* the cellular and molecular investigations focused on regulation of circulating factor level activity by vitamin K cycle components, and provide evidence for the interplay between coagulation factor specific biosynthesis and decay after vitamin K supplementation.

Addendum

F. Bernardi and G. Marchetti conceived and designed the study and wrote the paper. P. Caruso and M. Pinotti designed the functional study and performed coagulation functional assays. B. Lunghi performed the DNA study. M. Lapecorella and M. Napolitano performed the clinical coagulation laboratory assays. A. Canella performed antigen level determinations. G. Mariani did the clinical study design. All authors critically contributed to the interpretation of results, review and editing. F. Bernardi supervized the whole work and was responsible for the final approval of the version to be submitted.

Acknowledgements

The authors thank D. Gemmati, M. Baroni and S. Calzavarini for helpful suggestions and discussion. The study was supported by 'Programmi Ricerca Cofinanziati' MIUR and by Telethon grant GGP05214.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

References

1 Furie B, Bouchard BA, Furie BC. Vitamin K-dependent biosynthesis of γ-carboxyglutamic acid. *Blood* 1999; **93**: 1798–808.
2 Hansson K, Stenflo J. Post-translational modifications in proteins involved in blood coagulation. *J Thromb Haemost* 2005; **3**: 2633–48.
3 Zhang B, Ginsburg D. Familial multiple coagulation factor deficiencies: new biologic insight from rare genetic bleeding disorders. *J Thromb Haemost* 2004; **2**: 1564–72.
4 Oldenburg J, Watzka M, Rost S, Muller CR. VKORC1: molecular target of coumarins. *J Thromb Haemost* 2007; **5**(Suppl. 1): 1–6.
5 Rost S, Fregin A, Ivaskevicius V, Conzelmann E, Hortnagel K, Pelz HJ, Lappegard K, Seifried E, Scharrer I, Tuddenham EG, Muller CR, Strom TM, Oldenburg J. Mutations in VKORC1 cause warfarin resistance and multiple coagulation factor deficiency type 2. *Nature* 2004; **427**: 537–41.
6 Oldenburg J, von Brederlow B, Fregin A, Rost S, Wolz W, Eberl W, Eber S, Lenz E, Schwaab R, Brackmann HH, Effenberger W, Harbrecht U, Schurgers LJ, Vermeer C, Müller CR. Congenital deficiency of vitamin K dependent coagulation factors in two families presents as a genetic defect of the vitamin K-epoxide-reductase-complex. *Thromb Haemost* 2000; **84**: 937–41.
7 Viganò S, Mannucci PM, Solinas S, Bottasso B, Mariani G. Decrease in protein C antigen and formation of an abnormal protein soon after starting oral anticoagulant therapy. *Br J Haematol* 1984; **57**: 213–20.
8 Kumar S, Haigh JR, Tate G, Boothby M, Joanes DN, Davies JA, Roberts BE, Feely MP. Effect of warfarin on plasma concentrations of vitamin K dependent coagulation factors in patients with stable control and monitored compliance. *Br J Haematol* 1990; **74**: 82–5.
9 Hirsh J, Dalen JE, Anderson DR, Poller L, Bussey H, Ansell J, Deykin D, Brandt JT. Oral anticoagulants: mechanism of action, clinical effectiveness, and optimal therapeutic range. *Chest* 1998; **114**: 445S–69S.
10 Watson HG, Baglin T, Laidlaw SL, Makris M, Preston FE. A comparison of the efficacy and rate of response to oral and intravenous Vitamin K in reversal of over-anticoagulation with warfarin. *Br J Haematol* 2001; **115**: 145–9.
11 Penning-van Beest FJ, Gomez Garcia EB, van der Meer FJ, van Meegen E, Rosendaal FR, Stricker BH. Levels of vitamin K-dependent procoagulant and anticoagulant proteins in over-anticoagulated patients. *Blood Coagul Fibrinolysis* 2002; **13**: 733–9.
12 Stanton C, Wallin R. Processing and trafficking of clotting factor X in the secretory pathway. Effects of warfarin. *Biochem J* 1992; **284**: 25–31.
13 McClure DB, Walls JD, Grinnell BW. Post-translational processing events in the secretion pathway of human protein C, a complex vitamin K-dependent antithrombotic factor. *J Biol Chem* 1992; **267**: 19710–7.
14 Tokunaga F, Wakabayashi S, Koide T. Warfarin causes the degradation of protein C precursor in the endoplasmic reticulum. *Biochemistry* 1995; **34**: 1163–70.
15 Wu W, Bancroft JD, Suttie JW. Differential effects of warfarin on the intracellular processing of vitamin K-dependent proteins. *Thromb Haemost* 1996; **76**: 46–52.
16 Wallin R, Hutson SM. Warfarin and the vitamin K-dependent gamma-carboxylation system. *Trends Mol Med* 2004; **10**: 299–302.
17 Stafford DW. The vitamin K cycle. *J Thromb Haemost* 2005; **3**: 1873–8.
18 Mannucci PM, Duga S, Peyvandi F. Recessively inherited coagulation disorders. *Blood* 2004; **104**: 1243–52.
19 Pinotti M, Marchetti G, Baroni M, Cinotti F, Morfini M, Bernardi F. Reduced activation of the Gla19Ala FX variant via the extrinsic coagulation pathway results in symptomatic CRMred FX deficiency. *Thromb Haemost* 2002; **88**: 236–41.
20 Pinotti M, Etro D, Bindini D, Papa ML, Rodorigo G, Rocino A, Mariani G, Ciavarella N, Bernardi F, Pinotti M, Etro D, Mariani G.

Residual factor VII activity and different hemorrhagic phenotypes in CRM(+) factor VII deficiencies (Gly331Ser and Gly283Ser). *Blood* 2002; **99**: 1495–7.
21 Hemker HC, Giesen PLA, Ramjee M, Wagenvoord R, Beguin S. The thrombogram: monitoring thrombin generation in platelet rich plasma. *Thromb Haemost* 2000; **83**: 589–91.
22 Regnault V, Be′guin S, Lecompte T. Calibrated automated thrombin generation in frozen-thawed platelet-rich plasma to detect hypercoagulability. *Pathophysiol Haemost Thromb* 2003; **33**: 23–9.
23 Marchetti G, Patracchini P, Papacchini M, Ferrati M, Bernardi F. A polymorphism in the 5′ region of coagulation factor VII gene (F7) caused by an inserted decanucleotide. *Hum Genet* 1993; **90**: 575–6.
24 Bernardi F, Marchetti G, Pinotti M, Arcieri P, Baroncini C, Papacchini M, Zepponi E, Ursicino N, Chiarotti F, Mariani G. Factor VII gene polymorphisms contribute about one third of the factor VII level variation in plasma. *Arterioscler Thromb Vasc Biol* 1996; **16**: 72–6.
25 Stanley TB, Jin DY, Lin PJ, Stafford DW. The Propeptides of the vitamin K-dependent proteins possess different affinities for the vitamin K-dependent carboxylase. *J Biol Chem* 1999; **274**: 16940–4.
26 Hedner U, Davie EW. Introduction to hemostasis and the vitamin K-dependent coagulation factors. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The Metabolic Basis of Inherited Disease*, 6th edn. New York: McGraw-Hill, 1989: 2107–27.
27 Dati F, Hafner G, Erbes H, Prellwitz W, Kraus M, Niemann F, Noah M, Wagner C. ProC Global: the first functional screening assay for the complete protein C pathway. *Clin Chem* 1997; **43**: 1719–23.
28 Bodin L, Horellou MH, Flaujac C, Loriot MA, Samama MM. A vitamin K epoxide reductase complex subunit-1 (VKORC1) mutation in a patient with vitamin K antagonist resistance. *J Thromb Haemost* 2005; **3**: 1533–5.
29 Rieder MJ, Reiner AP, Gage BF, Nickerson DA, Eby CS, McLeod HL, Blough DK, Thummel KE, Veenstra DL, Rettie AE. Effect of VKORC1 haplotypes on transcriptional regulation and warfarin dose. *N Engl J Med* 2005; **352**: 2285–93.
30 Camire RM, Larson PJ, Stafford DW, High KA. Enhanced gamma-carboxylation of recombinant factor X using a chimeric construct containing the prothrombin propeptide. *Biochemistry* 2000; **39**: 14322–9.
31 Shen L, Shah AM, Dahlback B, Nelsestuen GL. Enhancing the activity of protein C by mutagenesis to improve the membrane-binding site: studies related to proline-10. *Biochemistry* 1997; **36**: 16025–31.
32 D'Angelo A, Vigano-D'Angelo S, Esmon CT, Comp PC. Acquired deficiencies of protein S. Protein S activity during oral anticoagulation, in liver disease, and in disseminated intravascular coagulation.. *J Clin Invest* 1988; **81**: 1445–54.
33 Wallin R, Martin LF. Warfarin poisoning and vitamin K antagonism in rat and human liver. Design of a system in vitro that mimics the situation in vivo. *Biochem J* 1987; **241**: 389–96.
34 Seré KM, Rosing J, Hackeng TM. Inhibition of thrombin generation by protein S at low procoagulant stimuli: implications for maintenance of the hemostatic balance. *Blood* 2004; **104**: 3624–30.