Int J Legal Med (2003) 117 : 143–148
DOI 10.1007/s00414-003-0362-y

ORIGINAL ARTICLE

G. N. Rutty · A. Woolley · C. Brookfield · F. Shepherd
S. Kitchen

The PIVKA II test

The first reliable coagulation test for autopsy investigations

Received: 14 August 2002 / Accepted: 10 January 2003 / Published online: 28 February 2003
© Springer-Verlag 2003

Abstract To date there is no routinely used reliable diagnostic test that can be performed in the post-mortem period to investigate whether a deceased had a coagulation disorder. This paper describes a series of experiments to assess the use of an antigen-based method to investigate the vitamin K-dependent factor II function in the deceased. It illustrates that by using this approach the functional status of factor II can be investigated in the post-mortem period. The abnormal proteins that are investigated by this method appear to remain stable for at least 72 h and potentially up to at least 7 days. The method that is illustrated could thus be reliably used in the post-mortem period to identify a natural or drug-induced factor II abnormality. The potential for other protein components of the coagulation cascade to be investigated by similar antigen-based methodology is suggested.

Keywords Autopsy · Coagulation · PIVKA II · Warfarin

Introduction

To date, there is no routinely used reliable diagnostic test that can be performed in the post-mortem period to investigate whether the deceased had a coagulation disorder. In fact most pathologists would question the value of investigating haematological parameters let alone the coagulation profile of a deceased person. This is due to the fact that in the post-mortem period the blood will rapidly undergo a number of changes that will render the investigation of the function of the clotting cascade by traditional methods unreliable, if not impossible. Thus if a deceased person, be it a child or an adult, is suspected of having a natural or drug-induced clotting abnormality, reliance must be placed on tests performed on ante-mortem blood samples. However, in the majority of medico-legal cases samples may not be available for such tests due to the circumstances in which death occurred.

Certain natural diseases, for example haemorrhagic disease of the newborn (HDN), may present with a clinical picture identical to non-accidental injury. Thus there is the potential to misdiagnose a natural disease as a non-accidental event which could lead to the wrongful conviction of an innocent party. The identification of a reliable coagulation test that can be used in the post-mortem period would assist the investigation of such deaths. During life, factors II, VII, IX and X of the coagulation cascade require vitamin K for carboxylation of the glutamic residues of the precursors to produce a normal function. Failure of this carboxylation results in the production of non-functioning proteins which are then found in the blood. These proteins are known as "proteins induced by vitamin K absence" (PIVKA) [1] and are produced by natural diseases that interfere with the absorption and action of vitamin K, for example HDN, or by drugs that alter the function of vitamin K, for example warfarin. The ability to test the status of each of these proteins in post-mortem blood would allow the pathologist to investigate both natural and drug-induced abnormalities of the coagulation cascade.

A single case report illustrating the use of PIVKA analysis in the post-mortem period to assist in the differentiation between HDN and non-accidental injury was published by Rutty et al. in 1999 [2]. Since then no other studies on the use of PIVKA analysis on post-mortem samples have been published. We present a series of studies illustrating the use of PIVKA II analysis in the post-mortem period. It is shown that the protein appears to be stable in the post-mortem period and that quantification can be used to identify a factor II abnormality. This study thus identifies a reliable but limited coagulation test for

G. N. Rutty (✉)
Division of Forensic Pathology, University of Leicester,
Robert Kilpatrick Building Leicester Royal Infirmary,
PO Box 65, Leicester, LE2 7LX, UK
Tel.: +44-116-2523221, Fax: +44-116-2523174,
e-mail: gnr3@le.ac.uk

A. Woolley · C. Brookfield · F. Shepherd · S. Kitchen
Sheffield Haemophilia and Thrombosis Centre,
Royal Hallamshire Hospital,
Glossop Road, Sheffield, S10 2JF, UK

use during an autopsy. Consideration is given to other protein-based post-mortem coagulation tests which may also be stable in the post-mortem period.

Methods

Three sets of data were recorded during this study. The first set of data investigated the coagulation parameters of 22 deceased individuals where autopsies were undertaken under the legal authority of H.M. Coroner. The investigation of the clotting status was required as part of the autopsy examination. In each case a full blood count (FBC) was performed using a Coulter Gen S cell counter. Blood samples for clotting tests were collected into trisodium citrate (9 parts blood:1 part 0.105 M trisodium citrate, Vacuratainer, Becton-Dickinson, UK) and centrifuged at 2,000 *g* for 10 min prior to analysis. The clotting screen was performed using a Sysmex CA6000 autoanalyser and included the prothrombin time (PT) determined using Innovin thromboplastin (Dade-Behring, UK), activated partial prothrombin time (APPT) performed using the Synthasil reagent (Instrumentation Laboratory), thrombin time (TT) with thrombin from Instrumentation Laboratory, fibrinogen level by the Clauss assay (Dade-Behring, UK) and D-dimer analysis was by latex agglutination (Sigma Diagnostics, UK). The PIVKA II analysis was performed using an ELISA method (Stago, UK). In each case the age and sex of the deceased was known as well as the clinical history including, where appropriate, whether the deceased had been prescribed warfarin or not. If the patient was known to have been prescribed warfarin, then a toxicological analysis was undertaken.

The second data set investigated whether or not PIVKA II was detectable in the post-mortem period in corpses where the protein was expected to be present. To do this 14 deceased individuals, 7 of whom were known to have been prescribed warfarin in life and 7 who had no warfarin drug history were investigated. In each case written informed consent was granted by the immediate relatives of the deceased to undertake the laboratory investigations. In each case blood was taken from the femoral vein and placed into the same tubes as previously described and the same investigations including toxicology were performed in each case.

The third data set used a laboratory model to assess the stability of PIVKA II in a sample after collection. As it was not possible to store a cadaver at room temperature over a period of days and withdraw blood at predetermined time intervals, we had to resort to a stored blood test-tube model. This model was also used as it was unknown whether the protein was stable after death and if it was not, then the delay between death and the taking of the sample could result in errors in interpretation of the results. It was also unknown whether there was a difference between plasma and serum samples. Thus this part of the project analysed the presence of PIVKA II in 10 living patients, 5 of whom were prescribed warfarin and 5 who were not. Ethical permission was granted for this part of the project and written informed consent was obtained from each of the volunteers. For testing, 10 ml of blood was collected into citrated samples as described and also into tubes without anticoagulant which was then allowed to clot. The blood was separated into serum and plasma and stored for 7 days in the tubes within the laboratory at room temperature. On each of the 7 days an aliquot of the blood sample was removed, centrifuged as described and a PIVKA II analysis was performed.

Results

A total of 46 individuals were investigated in the 3 parts of the project. In each case appropriate legal authority, be

Table 1 The coagulation test results for 22 deceased individuals (part 1)

Case	Time since death (h)	Platelets ($\times 10^9$/l) (140–370)	PT (9.5–11.5 s)	APPT (24.0–35.0 s)	TT (17.0–23.0 s)	Fibrinogen (1.7–3.3 g/l)	D-Dimer (<0.25µg/ml)	PIVKA II (<8 ng/ml)	Coagulation status
1	12			36.2		7.3		925	HDN
2	Unknown		>180	>300		<0.1		2,926	Warfarin
3	72	255	>180	>300		<0.2		2,000	Warfarin
4	72	237	>300	>300		<0.1		8.1	Haemophilia
5	18		>180	>300		<0.2		16.8	NCA
6	6							4	NCA
7	24	327	>300	>300		<0.2		10	NCA
8	6							10.2	NCA
9	8							8.6	NCA
10	11		>240	>240				10.4	NCA
11	48		>300	>300		<0.2		6.9	NCA
12	19		>180	>300	>180	<0.2	4,000	31.1	NCA
13	48	735						<0.2	NCA
14	9	13	>300	>300		<0.1		11	NCA
15	31	207	>300	>300		<0.2	>64	8.6	NCA
16	12		>300	>300	33	<0.2	1,000	7.7	NCA
17	5	99	>300	>300	<0.2			30.3	NCA
18	6	162						<0.2	NCA
19	29		>180	>300		<0.2	>400	7.7	NCA
20	48						>40	5.7	NCA
21	19		>180	>300	>180	<0.2	4,000	31.1	NCA
22	4	272	>300	>300		<0.2	>64	<2.0	NCA

Reference laboratory ranges for normal healthy adults are given in brackets.
PT prothrombin time.
APPT activated partial prothrombin time.
TT thrombin time.
NCA no coagulation abnormality.

Table 2 The coagulation test results for 14 deceased individuals 7 of whom were known to have been prescribed warfarin in life and 7 who had no warfarin drug history (part 2)

Case Normal range	Platelets ($\times 10^9$/l) (140–370)	PT (9.5–11.5 s)	APPT (24.0–35.0 s)	TT (17.0–23.0 s)	Fibrinogen (1.7–3.3 g/l)	D-Dimer (<0.25 µg/ml)	PIVKA II (<8 ng/ml)	Warfarin (1–3 mg/l)	Warfarin status
1	168	>300	>300	>100	<0.2	250	7.5	<0.2	Prescribed
2	138	>300	>300	>100	<0.2	8,000	780	<0.2	Prescribed
3	138	>300	>300	>100	<0.2	2,000	1,504	1	Prescribed
4	162	>300	>300	27.8	<0.2	750	3,240	0.8	Prescribed
5		>240	>240	>100	<0.2	750	6,184		Prescribed
6	119	>300	>300	>100	<0.2	>1,000	3,900	<0.2	Prescribed
7	204	>300	>300	>180	<0.2	>10,000	5,560	1.7	Prescribed
8	233	>300	>300	26.4	<0.2	300	11.1	<0.2	Not prescribed
9	89	>300	>300	>240	<0.2	250	8.6	Not detected	Not prescribed
10	132	>300	>300	53.6	<0.2	200	52.6	<0.2	Not prescribed
11	393	>300	>300	38.4	<0.2	>1,000	14.4		Not prescribed
12	493	>300	>300	>150	<0.2	375	7.9	Not detected	Not prescribed
13	212	>300	>300	77.6	<0.2	375	7	Not detected	Not prescribed
14	406	>300	>300	>100	<0.2	750	9.3	Not detected	Not prescribed

PT prothrombin time.
APPT activated partial prothrombin time.
TT thrombin time.
The reference ranges are for healthy normal adults.

Table 3 The serum and plasma PIVKA II results for non-warfarinised patients on each day for 7 days

Case sample	PIVKA II (<8 ng/ml)						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Normal 1 plasma	5.20	4.80	5.00	5.70	4.70	4.80	5.80
Normal 1 serum	4.20	4.60	4.90	4.60	4.70	5.90	5.90
Normal 2 plasma	1.00	1.00	1.00	1.00	4.60	1.00	3.90
Normal 2 serum	1.00	1.00	1.00	1.00	1.00	5.30	1.00
Normal 3 plasma	7.80	8.70	9.20	9.80	9.90	6.00	4.92
Normal 3 serum	6.70	8.10	8.80	8.30	9.00	4.23	6.15
Normal 4 plasma	5.13	4.84	5.58	10.80	5.40	9.10	6.36
Normal 4 serum	5.70	5.87	6.31	11.30	5.70	9.50	5.76
Normal 5 plasma	5.00	5.87	6.41	5.93	4.38	6.15	6.31
Normal 5 serum	5.40	5.20	5.13	4.99	5.13	5.58	5.93

Table 4 The serum and plasma PIVKA II results for warfarinised patients on each day for 7 days

Case sample	PIVKA II (<8 ng/ml)						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Warfarin 6 plasma	3,619	4,203	4,247	3,878	4,007	3,570	4,274
Warfarin 6 serum	3,119	5,502	3,986	3,878	4,088	4,196	3,802
Warfarin 7 plasma	2,188	2,236	2,374	2,264	2,806	2,006	2,704
Warfarin 7 serum	2,022	2,148	2,164	2,180	1,846	2,170	2,368
Warfarin 8 plasma	1,598	1,706	1,846	1,714	1,568	1,672	1,626
Warfarin 8 serum	1,558	1,496	1,548	1,574	1,654	1,566	1,362
Warfarin 9 plasma	172.1	141.4	202.6	181.4	174	177.2	169.2
Warfarin 9 serum	173.1	183.1	177.3	173.4	179.6	178	135.6
Warfarin 10 plasma	2,248	2,262	2,496	2,172	2,204	2,084	1,902
Warfarin 10 serum	2,142	2,214	2,280	2,216	2,230	2,200	2,140

it from H.M. Coroner or by written informed consent from the living volunteers or deceased relatives, was granted to undertake the investigations.

In part 1 a total of 22 deceased individuals were investigated, 12 females and 10 males with 16 cases aged 3 years or younger. In all cases the blood samples were taken no greater than 72 h after death. Two cases, both adults, were known to have been prescribed warfarin in life, one case had haemophilia and another HDN. All other cases were found to have no evidence of a coagulation abnormality during life. The results of the platelet count (from the FBC, the other red cell and white cell parameters are not illustrated as this study concerns coagulation only and not all haematological parameters) and coagulation tests

Table 5 Mean post-mortem PIVKA II plasma levels in normal and warfarinised subjects

Mean values (cases)	PIVKA II (<8 ng/ml)						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Normal plasma (1–5)	4.83	5.04	5.44	6.45	5.80	5.41	5.46
Normal serum (1–5)	4.60	4.95	5.23	6.04	5.11	6.10	4.95
Warfarin plasma (6–10)	1,965	2,109	2,233	2,041	2,151	1,901	2,135
Warfarin serum (6–10)	1,802	2,308	2,031	2,004	1,999	2,062	1,961

Table 6 INR plasma levels in normal and warfarinised patients at collection

Plasma	INR at collection	Plasma	INR at collection
Normal 1	1.0	Warfarin 6	2.5
Normal 2	1.0	Warfarin 7	2.0
Normal 3	1.0	Warfarin 8	1.9
Normal 4	1.0	Warfarin 9	1.1
Normal 5	1.1	Warfarin 10	1.9

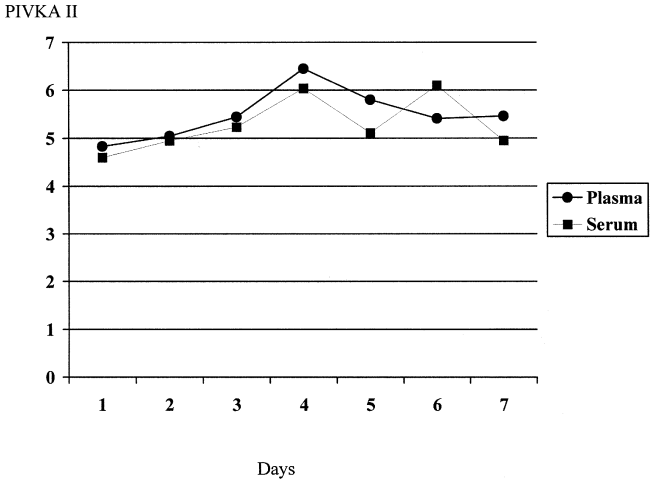

Fig. 1 A graph of the PIVKA II values (ng/ml) plotted against time (days) of the mean values of the control cases

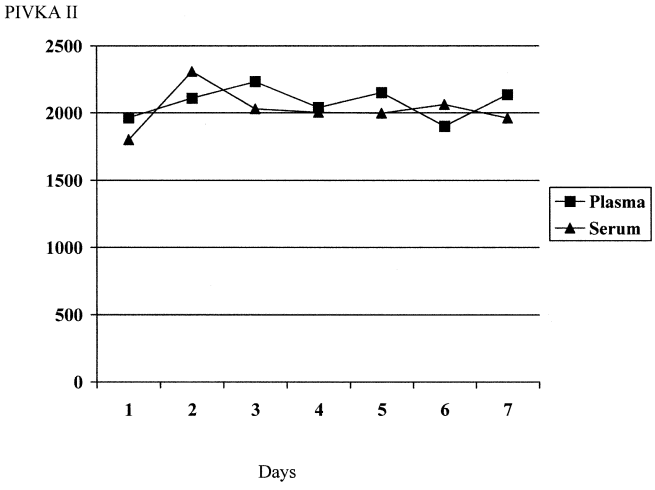

Fig. 2 A graph of the PIVKA II values (ng/ml) plotted against time (days) of the mean values of the warfarin cases

on each of the post-mortem blood samples are shown in Table 1. It was found that there was no significant correlation between the time since death (hours) and the PIVKA II results in the 19 deceased who had not received warfarin and did not have HDN (Pearson correlation coefficient r=0.18). This would suggest that there is no time-dependent leakage of PIVKA II from cellular storage after death.

In part 2 a total of 14 deceased were investigated, 9 males and 6 females with an age range from 64 to 84 years old at death. Of these 7 were known to have been prescribed warfarin during life but only 2 had levels within the therapeutic range at the time of death. The results of the coagulation tests on post-mortem blood are shown in Table 2.

In part 3 a total of 10 living patients volunteered for the project, 5 were known to have been prescribed warfarin and 5 were not. The results of the PIVKA II tests on each of the 7 days for each patient are shown in Tables 3, 4, 5 and 6. The mean values for each of the two types of samples for each of the two groups for each day are shown in Figs. 1 and 2. There were no significant differences (analysis of variance) between results in plasma and serum in normal subjects or in warfarinised subjects. Furthermore the differences between results on different days were not significant in plasma or serum in either of the two groups.

Discussion

During an autopsy examination the pathologist may have to undertake additional laboratory investigations using cadaver blood. To do this one must apply the same laboratory methods that are performed on blood samples taken from the living. As in the living, the correct sample must be taken from the correct site and placed into the correct container so that the correct analytical test can be performed. However, after death, due to the nature of the sample, for example if the blood is clotted or the body is in a state of advanced decomposition and assuming that a sample can be obtained in the first place, the results may be uninterpretable due to the rapid onset of post-mortem changes. Due to this, many would question the need to undertake such investigations if the results that are produced cannot be interpreted. An excellent example of this problem is the autopsy investigation of haematological param-

eters including the deceased's coagulation status. Under these circumstances one may become reliant upon ante-mortem tests or the existence of an ante-mortem blood sample which can be retrieved and tested.

In the post-mortem period the red and white cell morphology and parameters will alter with time but appear to remain stable in the early post-mortem period [3, 4]. Red cells may remain intact for up to 10 days whereas white cells undergo rapid change within the first 24-h period. The stability of individual types of leukocytes within the first 10 days is variable. Thus the analysis of the haematological state of the deceased can still be investigated for the purpose of diagnosis of disease states during the autopsy examination. One of the components of the ability to clot, the platelet count, has been shown to rise in the first 2 h after death followed by a steady decline over the next 10 h when it may reach 66% of the ante-mortem value [5]. After this initial rise and fall other authors have reported that the count will remain constant at the lower end of the standard range for up to 10 days after death [4]. Thus as illustrated in Tables 1 and 2, a platelet count can be performed after death, although the interpretation of the results becomes difficult due to the effect of post-mortem hypostasis and thrombolysis.

If the deceased is thought to have a natural or drug-induced abnormality of the coagulation cascade then, to date, the pathologist must rely upon the results of traditional clotting tests, i.e. the PT, APPT, TT and fibrinogen levels. However, as is illustrated in Tables 1 and 2, when post-mortem blood is used no interpretable results can be gained from these standard tests.

Rutty et al. [2] highlighted the importance of being able to test individual components of the coagulation cascade when trying to differentiate between natural disease and non-accidental injury in children. In this report they drew attention to the use of an antigen assay to analyse specific factors of the coagulation cascade in post-mortem blood. Under normal circumstances in life, the majority of circulating factors II, VII, IX and X have carboxylation of the appropriate glutamic residues in a vitamin K-dependent pathway [6]. Disruption of this system causes increases in the circulating levels of proteins induced by vitamin K absence (PIVKA). These are produced by natural diseases that interfere with the absorption and action of vitamin K, for example HDN, or by drugs that alter the function of vitamin K, for example warfarin. The ability to test the status of each of these proteins in post-mortem blood would allow the pathologist to investigate both natural and drug-induced abnormalities of this part of the coagulation cascade.

To our knowledge the only commercially available assay for PIVKA proteins is for PIVKA II and this project therefore concentrated on factor II. In living patients it has been shown previously that there is a significant correlation between the INR value and the PIVKA II value [7]. The first part of the project investigated whether PIVKA II was quantifiable in post-mortem blood. During life, all individuals will have a small quantifiable amount of PIVKA II which, from our laboratory reference range, should be less than 8 ng/ml. Table 1 illustrates the PIVKA II levels in 18 individuals with no evidence of a vitamin K abnormality. Values above the reference range were observed in these cases but no single value was found above 31.1 ng/ml. Thus this part illustrates that quantifiable results can be obtained in post-mortem blood but that the values may be above the upper reference range in normal individuals. However, in those with a vitamin K functional abnormality (case 1 with HDN, cases 2 and 3 were prescribed warfarin) the values are markedly raised compared to the normal cases and thus these individuals can be identified as having such an abnormal factor II function.

The ability to identify those with and without a vitamin K factor II abnormality based on the PIVKA II value was explored in part 2 of the project. In this section the samples were submitted to the laboratory blind of the clinical history that the patient was or was not prescribed warfarin. From Table 2 it can be seen again that in the normal subjects a PIVKA II value of up to 52.6 ng/ml was observed although again there was a significant difference between this value and 6 out of 7 of those prescribed warfarin. There were no false positive predictions made as to whether the patient was prescribed warfarin. Thus a raised value above that of the upper reference range can be seen in the post-mortem period in normal subjects but no value in the entire project was found to be above 52.6 ng/ml. Of the seven cases on warfarin six were predicted correctly from the PIVKA II value. Thus there was a single false negative case which was found to have a value within the normal reference range. As the warfarin value was sub-therapeutic it is possible that the deceased had been non-compliant with the therapy prior to death. Having said this, four of the warfarin subjects also had sub-therapeutic levels of warfarin present at death and yet had raised PIVKA II values. PIVKA II has been reported to have a half-life of approximately 16 h [1] by a different (less sensitive) method and thus although the patients warfarin levels have fallen to sub-therapeutic levels, exposure to warfarin (in these cases, therapeutic) can be confirmed in the post-mortem period by the analysis of PIVKA II.

The final part of the study used a model to consider the stability of the abnormal proteins in the post-mortem period and to investigate whether or not PIVKA II can arise after death. Ideally one would have taken a series of cadavers and left them at room temperature over the 7-day period, taking blood samples on each day to analyse for the presence of absence of PIVKA II. As this option was not possible to the investigators, blood was taken into test tubes with and without preservative and left for the 7-day period. It was hypothesised that if PIVKA II was already present at the time that the blood was drawn from the patient then this model could be used to assess its stability over this time period and equally if it was not present then this model would allow for the observation of whether the proteins would form under these conditions. The results are shown in Tables 3, 4, 5, 6, Figs. 1 and 2. The warfarin cases are again seen to have high values of 172 ng/ml or above compared with the control values when first drawn

from the body within the reference range. The differences between plasma and serum were not significant, indicating that either sample type could be used for the measurement of PIVKA II. In both normal and warfarinised subjects the measurement of PIVKA II was stable for at least 7 days. The differences between results on different days of sample storage were minor and not statistically significant. This suggests that the small variations observed reflect measurement variation rather than genuine changes in the levels of this protein. These results also support the assumption that once blood is drawn from the body, a delay in the time that the sample takes to get to the laboratory should not alter the interpretation provided that the sample arrives within 7 days. This is important if samples have to be sent by post to a distant reference laboratory.

This study highlights the ability to investigate the function of specific components of the clotting cascade by using antigen-based methods for the coagulation proteins rather than attempting to use more traditional clotting tests which become unusable in the post-mortem period. The proteins, in this case PIVKA II, have been shown to be quantifiable in the dead for at least 72 h after death and if one considers the test tube model results this could be extended to up to 7 days after death. The protein values do vary after death with an apparent initial rise and then fluctuation of values above the ante-mortem value. This observation is similar to that observed with other components of blood, for example the platelets and the haematocrit. However, as there is a marked difference between the control values and those with PIVKA II, the value can be used to identify those with either a natural or drug-induced factor abnormality. They can also be used to investigate drug compliance prior to death. Although this study has been confined to the investigation of PIVKA II, the other vitamin K-dependent factors could equally be investigated using the same methodology. The investigators have also undertaken the analysis of other coagulation proteins including Protein S and C and have observed that these too can be investigated in the post-mortem period. Further cadaver-based investigations are required to confirm the stability of these proteins in the post-mortem period but these results support that the use of protein-based antigenic coagulation tests can assist the pathologists in the post-mortem investigation of coagulation abnormalities.

Acknowledgements This project was funded by the Mason Medical Research Foundation. Ethical permission was granted for the live patient work by the South Sheffield Research Ethics Committee.

References

1. Presnell SR, Stafford DW (2002) The vitamin K-dependent carboxylase. Thromb Haemost 87:937–946
2. Rutty GN, Smith CM, Malia RG (1999) Late-form hemorrhagic disease of the newborn: a fatal case report with illustration of investigations that may assist in avoiding the mistaken diagnosis of child abuse. Am J Forensic Med Pathol 20:48–51
3. Laiho K, Penttila A (1981) Autolytic changes in blood cells and other tissues of human cadavers—viability and ions studies. Forensic Sci Int 17:109–120
4. Penttilia A, Laiho K (1981) Autolytic changes in blood cells of human cadavers. II. Morphological studies. Forensic Sci Int 17:121–132
5. Thomsen H, Kaatsch H-J, Krisch B (1999) How and why does the platelet count in postmortem blood change during the early postmortem interval? Forensic Sci Int 101:185–194
6. Boos J, Pollman H, Dominick HC (1989) Vitamin K-dependent coagulation parameters during the first 6 days of life. Incidence of PIVKA II in newborns. Paediatr Haematol Oncol 6:113–119
7. Malia RG, Ames PRJ, Brancaccio V, Iannaccone L, Brookfield C, Hampton KK (1999) Anti-phospholipid antibodies do not affect the action of warfarin in anti-phospholipid syndromes, but may affect the INR. Thromb Haemost [Suppl] 1:276