

Problems relating to the laboratory diagnosis of factor XIII deficiency: a UK NEQAS study

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Summary. Familial (F)XIII deficiency is an extremely rare bleeding disorder. In most laboratories the diagnosis is initially established through a clot-solubility screening test. We report here results from a series of UK NEQAS (Blood Coagulation) Proficiency Testing investigations, in which laboratories were provided with samples from normal individuals and from various subjects with FXIII deficiency with a request to perform their usual test for this disorder and to provide an interpretation of their results. Over 95% of centers were able to diagnose severe familial FXIII deficiency in previously untreated patients and to identify samples from normal subjects. However, both quantitative and qualitative methods produced widely variable results on samples obtained from previously treated individuals with FXIII deficiency but having measurable levels of FXIII. Data generated by UK NEQAS investigations suggested that solubility tests employing thrombin show greater sensitivity to FXIII deficiency, and this was confirmed in a subsequent single-center study. Our results lead us to recommend the use of thrombin and acetic acid in the clot-solubility screening test. Use of sensitive screening tests, and improvement in the accuracy and precision of quantitative FXIII assays will aid study of the clinical importance of moderate FXIII deficiency.

Keywords: external quality assessment, factor XIII, screening tests.

Introduction

Familial deficiency of factor (F)XIII is an extremely rare autosomal recessive disorder, with an estimated prevalence in the UK of 1 in 3 million [1]. Neonatal umbilical stump bleeding and delayed onset bleeding following trauma are

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characteristic features of this disorder [2]; other bleeding manifestations include soft tissue, subcutaneous and intramuscular hematomas. A major problem is the susceptibility of severely affected individuals to spontaneous intracranial hemorrhage. Fetal loss is common in women with FXIII deficiency [3]. Acquired deficiency has also been described in a number of disorders including liver disease and ulcerative colitis [1,4].

The test most widely used in routine hemostasis laboratories for the detection of FXIII deficiency is the clot-solubility test, in which plasma is clotted with calcium and/or thrombin, and resistance to lysis by urea or acetic acid is determined. These screening tests have been used in routine laboratories to diagnose severe FXIII deficiency. Methods employing urea as a lysing agent have been reported to be sensitive to $<1 \text{ U dL}^{-1}$ FXIII, with those employing acetic acid reported to be sensitive to $<2 \text{ U dL}^{-1}$ FXIII in plasma [5].

In this report, we present the results of a series of external quality assessment investigations undertaken by the UK National External Quality Assessment Scheme for Blood Coagulation (UK NEQAS) in respect of screening tests and quantitative assays for FXIII deficiency. Results obtained in these investigations prompted us to carry out a single-center study to investigate solubility test sensitivity. Our results have allowed us to determine the relative sensitivity of different procedures for the detection of FXIII deficiency.

Materials and methods

UK NEQAS proficiency testing investigation

Lyophilized plasma samples were prepared from the following groups of patients:

- 1 A pool of plasma prepared from three related subjects with severe familial FXIII deficiency, prior to receiving their first treatment with FXIII concentrate (untreated FXIII deficiency).
- 2 Two unrelated subjects with familial FXIII deficiency, who were receiving FXIII prophylaxis. Samples were taken immediately prior to treatment, at least 3 weeks after previous treatment, when their plasma FXIII level would have been at the lowest concentration (treated FXIII deficiency).

3 A subject with acquired FXIII deficiency. This patient had a short history of intramuscular bleeding and spontaneous bruising. The FXIII level in this patient was 8 U dL^{-1} . A low-grade FXIII inhibitor was identified by measurement of residual FXIII using a Berichrom kit (Dade-Behring, Liederbach, Germany) following a 2-h incubation of patient and normal plasma. An inhibitor was detected at a level of 1.1 U mL^{-1} , where 1 U mL^{-1} is defined as inhibiting 50% of added FXIII after 2 h at 37°C .

4 Two donors with normal levels of FXIII.

5 Two pools of normal donor plasma, one collected into trisodium citrate, the other into EDTA.

With the exception of the EDTA pool, all venous blood was collected into 0.105 mol L^{-1} trisodium citrate. All samples were centrifuged (minimum $2000 \times g$ for 10 min), and plasma frozen below -40°C until use. Plasma was then thawed, buffered with 0.8% HEPES, and lyophilized in 0.5 mL aliquots, as previously described [6]. Samples were numbered in chronological sequence, in order of distribution over a period of 5 years:

- Sample 1: normal level of FXIII.
- Sample 2: acquired FXIII deficiency.
- Sample 3: untreated FXIII deficiency.
- Sample 4: normal level of FXIII.
- Samples 5 & 6: treated FXIII deficiency.
- Sample 7: normal donor pool (citrate)
- Sample 8: normal donor pool (EDTA).

Lyophilized samples were distributed in four investigations between 1996 and 2002; results were returned from an average of 143 centers in 17 countries. Participants were asked to perform their routine screening test for FXIII deficiency, and report their method details together with an interpretation for each sample. In the investigation distributed in May 2002, details were requested for the source and concentration of thrombin used by centers using thrombin-based clot-solubility tests.

Plasma collected into EDTA anticoagulant and clotted with thrombin is expected to give a positive solubility screen with either urea or acetic acid. For the plasma sample from a pool of normal donors taken into EDTA anticoagulant, participants were asked to employ the following test protocol:

- 1** Reconstitute the sample in 0.5 mL distilled H_2O . Mix by swirling and leave to stand for 5 mins.
- 2** Transfer 0.2 mL of the sample to a glass test tube. Add 0.2 mL of 10 U mL^{-1} thrombin. Leave to clot for 30 min at 37°C .
- 3** Add 3 mL 5 mol L^{-1} urea or 3 mL 2% acetic acid. Incubate at room temperature, and check at 30-min intervals for clot lysis.

Single-center studies

Samples In order to confirm on fresh plasma samples results obtained by the NEQAS participants on lyophilized samples in the proficiency testing investigation, plasma samples were collected from eight untreated subjects with severe FXIII deficiency, and 12 normal subjects (laboratory staff with no bleeding history). Samples were also obtained 3 weeks after treatment with FXIII concentrate from two of the FXIII-

deficient patients. Samples were centrifuged (minimum $2000 \times g$ for 10 min), and plasma frozen below -40°C until use. Spiked plasma samples were prepared by addition of 1, 5 and 10% normal pooled plasma to FXIII-deficient plasma (Sigma Diagnostics, Poole, Dorset, UK).

Screening tests The following screening tests were used for all FXIII-deficient and normal samples. In a glass test-tube, 0.2 mL plasma was mixed with either 0.2 mL 0.025 mol L^{-1} CaCl_2 or 0.2 mL saline + 0.1 mL 30 U mL^{-1} thrombin. The tube was left at 37°C for 30 min to clot. 3 mL of 5 mol L^{-1} urea, 2% acetic acid or 1% monochloroacetic acid was added, and the tube was capped, mixed by inversion, and incubated at either room temperature or 37°C . The tube was inspected for presence or absence of a clot at regular intervals.

Factor XIII assays FXIII was measured quantitatively in the FXIII-deficient samples using a photometric FXIII assay kit (Dade-Behring). A pool of frozen plasma obtained from 20 normal subjects was used to construct a reference curve.

FXIII A and B subunits were measured by sandwich ELISA using capture (American Diagnostics Inc., Greenwich, CT, USA), and detection antibodies (Affinity Biologicals, Ancaster, Ontario, Canada).

Results

Reagents and kits used by UK NEQAS participants

Results with corresponding interpretations were reported by 133–147 centers for the four investigations described here.

Screening tests Between 121 and 127 centers used clot-solubility screening tests in the UK NEQAS investigations. In the first investigation (samples 1 and 2, May 1997), 60 centers used calcium, 46 thrombin, and 20 a combination of both to clot the plasma samples. Fifty-eight centers used urea, 25 acetic acid, 30 a combination of urea and acetic acid, and seven monochloroacetic acid (MCA), to lyse the clot. Eleven different combinations of clotting agent and lysing agent were used, and normal ranges of >30 min to >24 h were reported. In addition, differences were noted in concentrations, volumes and sources of reagents, and reference ranges; ranges varied from <1 h to 24 h for each of the method combinations.

Quantitative assays The number of centers employing quantitative FXIII assays increased from eight in May 1997 to 30 in May 2002. Eight of these centers also performed clot-solubility screens. An average 84% of centers used the Behring Berichrom FXIII assay; other methods included the Pentapharm Pefakit, Stago FXIII kit, Binding site RID, and an in-house ELISA.

A summary of quantitative assay results and overall interpretations for all samples is shown in Table 1. Wide variation was seen between results obtained with each sample, for example $20\text{--}136 \text{ U dL}^{-1}$ for sample 7 (normal plasma pool) and $<1\text{--}50 \text{ U dL}^{-1}$ for sample 3 (untreated FXIII deficiency).

Table 1 Interpretations and quantitative assay results reported by UK NEQAS participants for samples distributed for FXIII testing. See text for detailed descriptions of sample types

Survey date	Sample	Description	n	Interpretations (%)			FXIII assay results		
				Normal	Border-line	Abnormal	n	Median (U dL ⁻¹)	Range (U dL ⁻¹)
May 97	1	Normal	137	100.0	0.0	0.0	8	125.0	84–158
May 97	2	Acquired FXIII deficiency	137	30.2	11.9	57.9	7	7.8	3–67
September 98	3	XIII-deficient pool	137	2.9	1.5	95.6	18	7.6	<1–50
September 98	4	Normal	143	97.8	0.0	2.2	18	104.5	62–155
September 99	5	Treated XIII deficiency	133	69.9	5.3	24.8	19	8.0	3–130
May 02	6	Treated XIII deficiency	147	32.7	12.9	54.4	30	10.1	0–55
May 02	7	Normal pool	145	95.9	1.4	2.7	30	100.6	20–136
May 02	8	EDTA plasma	140	32.9	3.6	63.5	30	126.4	1–200

Table 2 Interpretations by UK NEQAS participants, treated FXIII deficiency (sample 6), by thrombin reagent source

Source of thrombin	n	Final concentration range (U mL ⁻¹)	Interpretations (median FXIII 10.1 U dL ⁻¹)		
			Normal	Borderline	Abnormal
Biomerieux	1	NS	0	1	0
Dade-Behring	11	6–15	3	1	7
Diagnostic Reagents	17	2–17	0	0	17
Diamed	1	5	0	0	1
Instrumentation Laboratory	10	0.6–15	4	2	4
Organon	2	7–15	0	0	2
Sigma	2	6	0	0	2
Technoclone	2	5–7.5	0	1	1
Thrombosis reference center	3	3.1–6.2	0	0	3

NS, not stated.

Data on the thrombin reagents used in the investigation distributed in May 2002 are shown in Table 2, together with the corresponding interpretations provided for the FXIII-deficient sample distributed in this investigation.

Normal plasma

Screening tests and interpretations Over 95% of centers were able to correctly identify plasmas with normal levels of FXIII (samples 1, 4 and 7).

Assays Results reported by centers employing quantitative assays ranged from 84 to 158 U dL⁻¹ [coefficient of variation (CV) 24.5%], 62–155 U dL⁻¹ (CV 19.3%) and 20–136 U dL⁻¹ (CV 17.5%), in samples 1, 4 and 7, respectively.

Severe, untreated FXIII deficiency

Screening tests and interpretations An abnormal result was reported by 95.6% of centers for a sample (no. 3) prepared from a pool of plasma from untreated donors with severe familial FXIII deficiency.

Assays The expected FXIII level in this sample was <1%; however, the median level reported by 18 centers was

7.6 U dL⁻¹ (range <1–50 U dL⁻¹, CV 110.3%). A histogram of these results is shown in Fig. 1.

Treated FXIII deficiency

Screening tests and interpretations A lack of consensus between centers was observed for samples from donors receiving prophylaxis for FXIII deficiency, obtained at least 3 weeks after treatment (samples 5 and 6). Interpretations were heavily influenced by choice of method; on average, 66% of centers employing thrombin as a clotting agent reported an abnormal screen for these samples, compared with 17% of centers who used calcium. Figures 2 and 3 show the pattern of interpretations by clotting agent and lysing agent, respectively, for the FXIII-deficient sample distributed in May 2002 (sample 6).

Assays The median FXIII level reported in this sample by 30 centers using quantitative assays was 10.1 U dL⁻¹, range 0–55 U dL⁻¹.

Acquired FXIII deficiency

Screening tests and interpretations This sample was identified as abnormal by 57.9% of centers.

Assays The median FXIII level was 7.8 U dL⁻¹, range 3–67 U dL⁻¹.

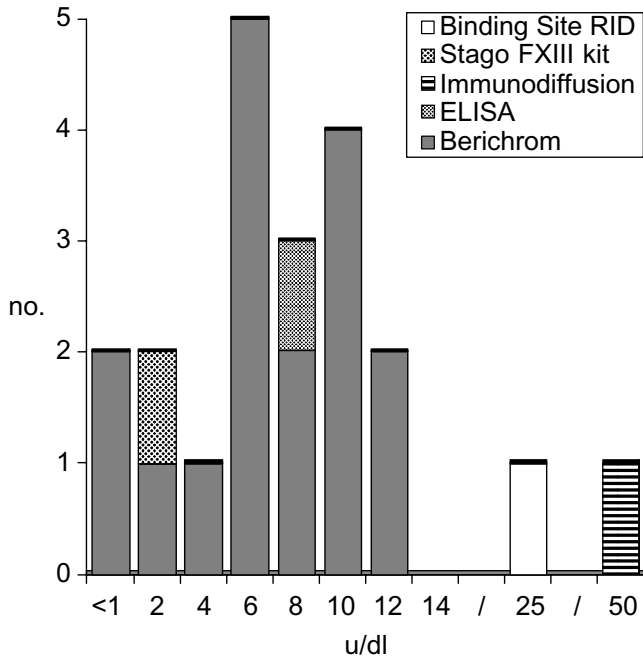


Fig. 1. Quantitative FXIII assay results, untreated FXIII deficiency (sample 3).

EDTA plasma

Screening tests and interpretations Using the method provided to participants, this plasma was expected to give a positive FXIII screening test. However, 32.9% of centers reported a normal screen for this sample. Some thrombin reagents were associated with a higher proportion of negative screening tests (Table 2).

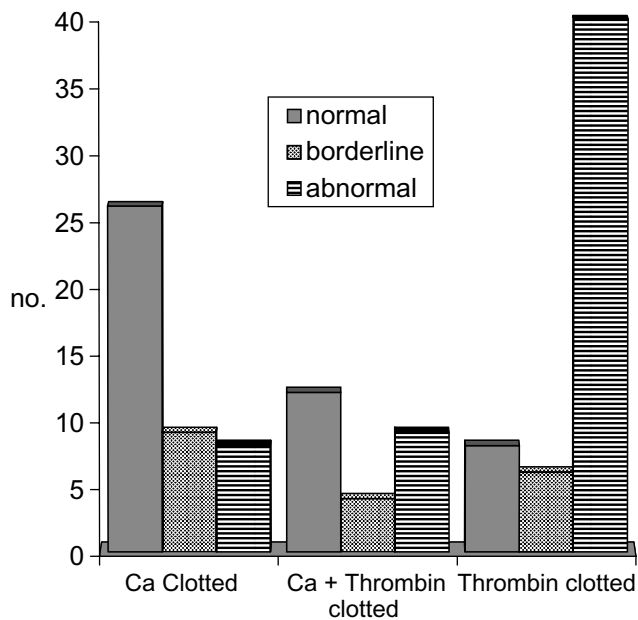


Fig. 2. Interpretations by clotting agent; treated FXIII deficiency (sample 6, median FXIII 10.1 U dL⁻¹).

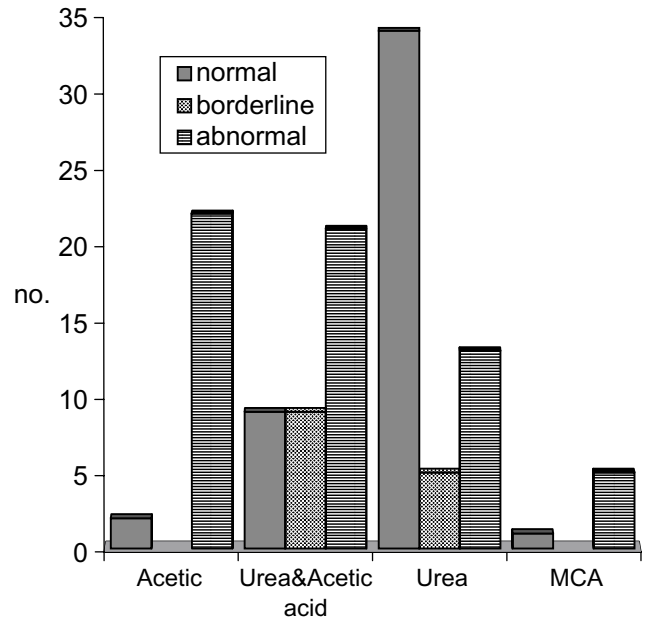


Fig. 3. Interpretations by lysing agent; treated FXIII deficiency (sample 6, median FXIII 10.1 U dL⁻¹).

Single-center studies

Screening tests and interpretations Table 3 shows results obtained in this laboratory by different methods for samples from subjects with FXIII deficiency, together with results from FXIII-deficient plasma spiked with normal plasma. Assay results from photometric and ELISA assays are also shown.

Normal subjects

Screening tests and interpretations A reference range was established for each combination of reagents, using the shortest clot-lysis time obtained with 20 normal plasmas as the limit of normal. No clot lysis was observed within 24 h when urea, acetic acid or MCA was added to calcium-clotted plasma, nor was it observed when urea was added to thrombin-clotted plasma. However, out of 12 normal samples clotted with thrombin to which acetic acid was then added, one lyzed at 12 h, and one sample with MCA lyzed at 6 h.

Untreated FXIII deficiency

Screening tests and interpretations For all eight untreated subjects with severe familial FXIII deficiency, clot-solubility tests were positive, irrespective of method. For calcium and urea-based tests, clots dissolved between 2 and 4 h. For calcium and thrombin-clotted methods with acetic acid, clots dissolved within 40 min.

Assays The FXIII levels in these plasmas measured by photometric assay ranged from <3 to 8 U dL⁻¹, and by ELISA ranged from 3.7 to 5 U dL⁻¹ (Table 3).

Table 3 FXIII screen lysis times (in hours), and assay results – single-center investigation

Sample	Calcium			Thrombin			Photo-metric assay (U dL ⁻¹)	ELISA (U dL ⁻¹)
	Urea	Acetic acid	MCA	Urea	Acetic acid	MCA		
Normal subjects (n = 20)	>24	>24	>24	>24	=12	=6	–	–
Congenital FXIII deficiency								
Untreated (n = 8)	2.0–4.0	0.5–0.6	0.5–2.0	0.5–2.5	0.5–0.6	0.5–2.0	<3.0–8.0	3.7–5.0
Treated (n = 2)	>24	12.0–24.0	ND	24.0	5.0	ND	8.0–9.0	7.1–7.7
Acquired FXIII deficiency (n = 1)	>24	2.5	ND	1.0	1.0	ND	8.0	8.5
FXIII 0% (spiked plasma)	1.8	0.5	0.5	1.2	0.5	ND	<3.0	3.6
FXIII 1% (spiked plasma)	7.0	0.6	ND	1.0	0.6	ND	ND	ND
FXIII 5% (spiked plasma)	>24	10.0	ND	10.0	0.6	ND	ND	ND
FXIII 10% (spiked plasma)	>24	>24	ND	>24	6.0	ND	ND	ND

ND, not done.

Treated FXIII deficiencies

Screening tests and interpretations For two samples from patients receiving prophylactic therapy, taken at least 3 weeks after treatment, and a sample from the subject with acquired FXIII deficiency, calcium and urea-based screens were negative (i.e. no lysis within 24 h). In contrast, screens employing thrombin and acetic acid were positive. **Assays** The FXIII levels in these plasmas measured by photometric assay ranged from 8 to 9 U dL⁻¹, and by ELISA ranged from 7.1 to 8.5 U dL⁻¹.

Spiked plasma

Screening tests and interpretations All screening tests performed on a commercial FXIII-deficient plasma sample were positive. When this sample was spiked with a pooled normal plasma, the calcium/urea screen was positive with 1% normal plasma, but negative at 5%, while the thrombin/acetic acid screen was positive even after addition of 10% normal plasma. Intermediate results were obtained with calcium/acetic acid and thrombin/urea combinations.

Discussion

FXIII deficiency is a rare but important familial bleeding disorder. The clinical complications associated with severe (< 1%) deficiency are well-documented. However, reports also indicate that mild FXIII deficiency (between 5 and 40% of normal) is also associated with severe bleeding complications [7]. It is therefore essential that the screening tests used are sufficiently sensitive to detect clinically important reductions in FXIII levels. Detection of reduced levels of FXIII in certain disease states is also useful as a prognostic indicator [8,9], and replacement of FXIII in relatively mild acquired deficiency seen in inflammatory bowel disease has been shown to be effective in reducing bleeding [10].

Because cross-linking of fibrin occurs after initial clot formation, coagulation tests employing clot formation as an endpoint cannot be used. Specific assays are available for measurement of FXIII in plasma. Immunological assays include

ELISA and radioisotope methods [11,12], and 'functional' photometric assays [13,14]. Over a 5-year period, the number of centers employing specific FXIII assays in UK NEQAS (blood coagulation) investigations increased from eight to 30. However, the most widely used method for screening for FXIII deficiency is the clot-solubility method.

A recent (May 2002) UK NEQAS survey revealed that 125/150 (83%) continue to use a clot-solubility screening test. Currently, a large range of methods remain in use; 15 combinations of calcium, thrombin or thromboplastin as clotting agents with urea, acetic acid or monochloroacetic acid as lysing agents. Nine different thrombin sources were reported, with concentrations ranging from 0.6 to 17 U mL⁻¹.

There is no consensus in the literature in respect of the relative sensitivity of clot-solubility methods employing calcium and urea. On the one hand, Francis [15] reported sensitivity to FXIII levels of less than 0.5%, whilst Jacobsen and Godal [16] reported sensitivity to between 3 and 5% FXIII. Francis also reported sensitivity of calcium and acetic acid methods to between 0 and 3% FXIII in plasma [15].

Our data show that over 95% of centers employing solubility tests are able to identify samples from normal subjects and a pool of plasma from patients with severe untreated FXIII deficiency.

For samples from subjects with mild/moderate reduction in FXIII levels (either through prophylaxis or acquired deficiency), there was a lack of consensus in interpretation amongst solubility test users. The data suggested that thrombin-based solubility tests have a greater sensitivity to mild or moderate FXIII deficiency.

This was investigated further in a single-center study, which confirmed that all clot-solubility methods may be sufficiently sensitive to detect severe FXIII deficiency. However, for samples from subjects receiving FXIII prophylaxis, and therefore with detectable levels of FXIII, there were marked differences in the sensitivity of the different reagent combinations. These samples were most reliably detected by reagent combinations that included thrombin as the clotting agent in the absence of calcium, with the thrombin/acetic acid combination being the most sensitive.

We have demonstrated that the calcium/urea method is sensitive to between 1 and 5 U dL⁻¹ of FXIII, and thrombin/

acetic acid is sensitive to at least 10 U dL^{-1} FXIII in plasma, with intermediate sensitivity for the calcium/acetic acid and thrombin/urea methods. The greater sensitivity of the thrombin-based clot-solubility test has been reported previously [16], and is probably due to a lack of exogenous calcium, required to complete activation of FXIII in plasma. This principle is used in the method described by Dacie and Lewis [17], in which plasma collected into EDTA anticoagulant is clotted with thrombin and lysed with urea or acetic acid. This method is recommended to give a positive screening test. It is noteworthy that 33% of centers using this method failed to report a positive screen. It is apparent from manufacturer's kit inserts that the thrombin reagents used by some centers also contain calcium, and it was evident that these reagents were associated with a greater proportion of negative screens for a sample with reduced FXIII levels.

Of those centers employing quantitative assays for FXIII, on average 93% were able to correctly identify normal and FXIII-deficient samples. Of note, however, was the marked imprecision of quantitative assays used by centers. For the untreated FXIII-deficient sample, a median XIII level of 7.6 U dL^{-1} was obtained, with results ranging from 0 to 55 U dL^{-1} . The poor reproducibility and precision of the quantitative FXIII assay results complicates interpretation of our data, particularly for samples with FXIII levels below 10 U dL^{-1} .

The relationship between severity of FXIII deficiency and clinical severity is unclear, and is complicated by difficulty in measurement of FXIII. Seitz [7] has reported findings from a multicenter questionnaire, which revealed that 7/14 subjects with FXIII levels between 5 and 40% suffered severe bleeding complications. The methods by which levels were measured in that study were not identified. However, our data suggest that at these levels quantitative FXIII assays may not be able to distinguish between mild and more severe deficiencies of FXIII, and that many centers using clot-solubility tests would have been unable to identify these subjects.

We conclude that use of solubility screens employing thrombin alone to clot plasma and acetic acid for lysis allows greater sensitivity to mild or moderate FXIII deficiency. The provision of sensitive screening tests and FXIII assays with greater accuracy and precision will allow further elucidation of the relationship between moderate and mild FXIII deficiency and clinically important bleeding.

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