Specific and global coagulation assays in the diagnosis of discrepant mild hemophilia A

Annette E. Bowyer,[1] Joost J. Van Veen,[1] Anne C. Goodeve,[2,3] Steve Kitchen,[1] and Michael Makris[1,2]

[1]Sheffield Haemophilia and Thrombosis Centre, Sheffield; [2]Department of Cardiovascular Science, University of Sheffield, Sheffield; and [3]Sheffield Diagnostic Genetics Service, Sheffield Children's NHS Foundation Trust, Sheffield, UK

ABSTRACT

The activity of the factor VIII coagulation protein can be measured by three methods: a one or two-stage clotting assay and a chromogenic assay. The factor VIII activity of most individuals with mild hemophilia A is the same regardless of which method is employed. However, approximately 30% of patients show marked discrepancies in factor VIII activity measured with the different methods. The objective of this study was to investigate the incidence of assay discrepancy in our center, assess the impact of alternative reagents on factor VIII activity assays and determine the usefulness of global assays of hemostasis in mild hemophilia A. Factor VIII activity was measured in 84 individuals with mild hemophilia A using different reagents. Assay discrepancy was defined as a two-fold or greater difference between the results of the one-stage and two-stage clotting assays. Rotational thromboelastometry and calibrated automated thrombography were performed. Assay discrepancy was observed in 31% of individuals; 12% with lower activity in the two-stage assay and 19% with lower activity in the one-stage assay. The phenotype could not always be predicted from the individual's genotype. Chromogenic assays were shown to be a suitable alternative to the two-stage clotting assay. Thromboelastometry was found to have poor sensitivity in hemophilia. Calibrated automated thrombography supported the results obtained by the two-stage and chromogenic assays. The current international guidelines do not define the type of assay to be used in the diagnosis of mild hemophilia A and some patients could be misclassified as normal. In our study, 4% of patients would not have been diagnosed on the basis of the one-stage factor VIII assay. Laboratories should use both one stage and chromogenic (or two-stage) assays in the diagnosis of patients with possible hemophilia A.

Introduction

Hemophilia A is an X-linked bleeding disorder caused by a reduction in the factor VIII coagulation protein (FVIII:C). The severity of bleeding is usually related to the level of FVIII:C. According to the International Society on Thrombosis and Haemostasis (ISTH) definition[1] of hemophilia, patients with mild hemophilia A (MHA) have FVIII:C levels of 5-40 IU/dL and have fewer bleeding episodes than those with the severe or moderate forms of the disease. In many patients, MHA is initially diagnosed by the findings of a prolonged activated partial thromboplastin time (APTT) and reduction in FVIII:C level.

FVIII:C can be measured using the one-stage clotting assay (FVIII:C1), two-stage clotting assay (FVIII:C2) or a chromogenic (amidolytic) assay (FVIII:CR). The FVIII:C of most MHA patients is the same regardless of the assay method employed and in these cases the assays are considered to be non-discrepant. However, approximately 30% of patients show marked differences in FVIII:C activity when assayed by the three methods and are regarded as assay discrepant.[2-4] The differences in levels may be 2-fold or greater and for some patients, one or more of the FVIII:C assays may be in the normal range, potentially leading to an incorrect diagnosis.

It has recently become clear that two distinct types of assay discrepancy exist. One type of discrepancy is the lower two-stage form, in which patients have reduced FVIII:C2 or FVIII:CR compared to FVIII:C1 activity and exhibit bleeding patterns consistent with the lower FVIII:C2 value.[2] The other type of discrepancy is the lower one-stage form in which the FVIII:C1 is reduced compared to that determined by the FVIII:C2 or FVIII:CR assay and in which little bleeding is reported.[4,5] We assessed FVIII:C assay discrepancy in 84 patients and female carriers from the Sheffield Haemophilia and Thrombosis Centre with FVIII:C levels between 5 and 50 IU/dL using a number of FVIII:C assays and also examined the value of rotational thromboelastometry (ROTEM) and calibrated automated thrombography (CAT) in the diagnosis of these patients.

Methods

Patients' samples

Citrated plasma samples (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA) from 77 males with MHA and seven female carriers of hemophilia A, with FVIII:C levels between 5 and 50 IU/dL by at least one method, were obtained following informed consent. All experiments were performed with the approval of Sheffield Teaching Hospitals NHS Foundation Trust ethics committee.

Standard coagulation assays

APTT evaluations and FVIII:C1 assays were performed with Actin

 doi:10.3324/haematol.2013.088088
Manuscript received on March 16, 2013. Manuscript accepted on June 25, 2013.
Correspondence: m.makris@sheffield.ac.uk

FS APTT reagents and FVIII-deficient plasma (both from Siemens, Marburg, Germany) according to standard methods.[6] FVIII:C2 assays were performed by an in-house method using bovine factor V and platelet substitute (Diagnostic Reagents, Oxon, UK).[7] FVIII:CR assays were performed with three commercial kits, Siemens, Coamatic (Chromogenix, IL, Bedford, USA) and Technochrom (Pathway Diagnostics, Dorking, UK), using the manufacturers' assay conditions.

Definition of assay discrepancy

Assays were denoted discrepant if a 2-fold or greater difference in FVIII:C1 was observed between the Actin FS and FVIII:C2 results. Lower two-stage discrepancy was defined as a FVIII:C1/FVIII:C2 ratio of ≥2.0 and lower one-stage discrepancy as a FVIII:C1/FVIII:C2 (or FVIII:CR) ratio of ≤0.5. Ratios of 0.6-1.9 were deemed non-discrepant.

We also assessed another FVIII:C1 assay using Synthasil APTT reagent (Instrumentation Laboratories, Bedford, USA) and three FVIII:CR assays (from Siemens, Chromogenix and Pathway Diagnostics) instead of the FVIII:C2.

Global assays of hemostasis

ROTEM assays using citrated whole blood were performed on a ROTEM Gamma analyzer (TEM International, Munich, Germany). Activation was initiated with the INTEM reagent (Pentapharm, Munich, Germany) according to the manufacturer's recommendations. A modified in-house low tissue factor method, following the method of Sorensen *et al.*,[8] was also performed. Assay conditions, reproducibility and normal ranges for the modified assay have been described previously.[9]

CAT assays were performed with platelet-poor plasma. The assay conditions, reproducibility and normal ranges have been described previously[10] and were based on the method by Hemker *et al.*[11]

Genetic analysis

F8 mutations were analyzed using either confirmation sensitive gel electrophoresis followed by DNA sequencing of amplicons displaying a migration shift[12] or by direct sequencing of the entire coding region and intron-exon boundaries of *F8*[13]. Nucleotide numbering of the *F8* cDNA was from the A of the ATG initiation codon and protein from the initiator methionine at the start of FVIII using reference sequences for cDNA NM_000132.2 and protein NP_000123.1.

Results

Incidence of assay discrepancy

Eighty-four individuals, with FVIII:C between 5 and 50 IU/dL by at least one method, were included in this study; 59 (70%) had less than a 2-fold difference between FVIII:C1 and FVIII:C2 assays and were classified as non-discrepant. Ten patients (12%) had a FVIII:C1/FVIII:C2 ratio of ≥2.0 and were classified as having a "lower two-stage FVIII discrepancy"; four of these patients had a FVIII:C1 concentration in the normal range.

Fifteen patients (18%) had a FVIII:C1/FVIII:C2 ratio of ≤0.5 and were classified as having the "lower one-stage FVIII discrepancy"; 13 of these patients had a FVIII:C2 concentration in the normal range. Figure 1 shows the patients' distribution by FVIII level.

For those patients with non-discrepant assay results the mean FVIII:C1/FVIII:C2 ratio was 1.0 (mean FVIII:C1 and FVIII:C2 both 21 IU/dL). In patients with lower two-stage FVIII discrepancy the mean ratio was 2.8 (mean FVIII:C1 48 IU/dL, FVIII:C2 17 IU/dL) and in those with lower one-stage FVIII discrepancy the mean ratio was 0.3 (mean FVIII:C1 23 IU/dL, FVIII:C2 66 IU/dL) (Table 1).

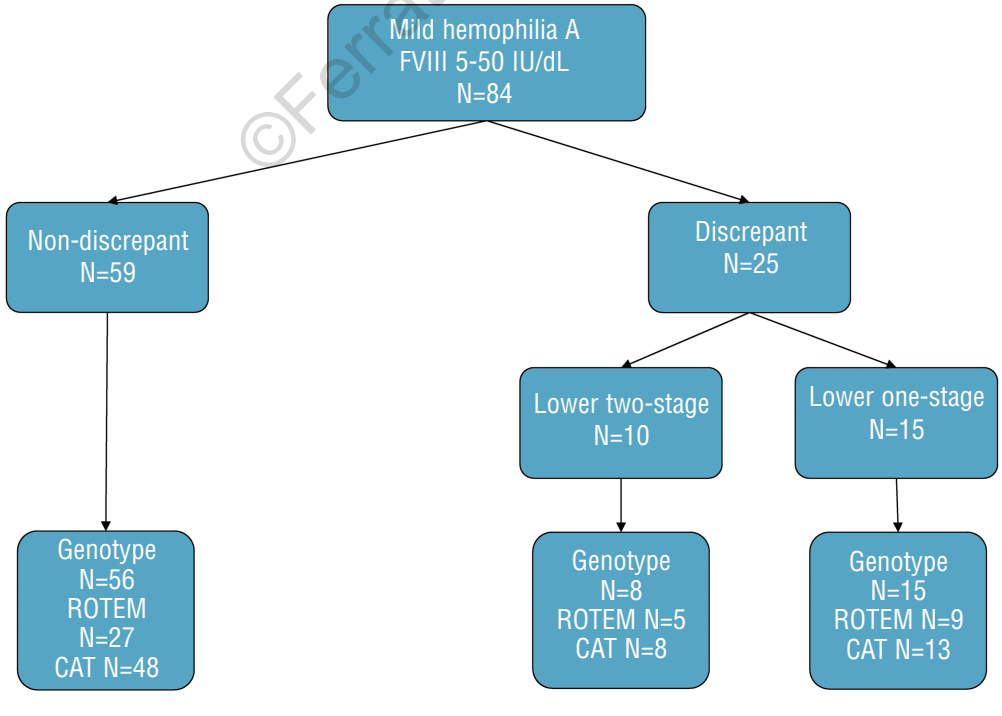

Figure 1. Flow chart representing the three groups of patients in this study; non-discrepant, lower two-stage discrepant and lower one-stage discrepant. The number of subjects with a mutation determined and the number of subjects who had global assays of hemostasis performed are also shown.

Genotype

The genotype was known for 79 patients (Table 2): the majority of mutations were missense mutations although cases of a frameshift, a splice site mutation and a female carrier of the intron 22 inversion, which is linked to severe hemophilia A, were also identified. The most frequent mutations found in the three groups were p.Arg1985Gln (15 non-discrepant patients, 2 with lower two-stage discrepancy), p.Tyr365Cys (13 lower one-stage discrepancy, 3 non-discrepant female carriers), and p.Arg602Gly (7 non-discrepant patients) and p.His1973Leu (5 lower two-stage discrepancy patients) (Table 2).

Prolongation of the activated partial thromboplastin time

The APTT was determined in 82 patients, and was prolonged above the top of the reference range in 73. Four individuals with non-discrepant FVIII:C assay results had a normal APTT ranging from 0.1 to 2.7 s below the upper limit of the normal range; their FVIII:C1 ranged from 32-40 IU/dL and FVIII:C2 from 23-68 IU/dL. Four subjects with the lower two-stage form of discrepancy had a normal APTT ranging from 0 to 4.3 s below the top of the normal range, with their FVIII:C1 ranging from 31-112 IU/dL and FVIII:C2 from 13-37 IU/dL. A female carrier of p.Tyr365Cys with the lower one-stage form of assay discrepancy had a normal APTT, 0.4 s below the top of the normal range, with an FVIII:C1 of 33 IU/dL and FVIII:C2 of 70 IU/dL (Table 1). Eight patients with a normal APTT had thrombin generation tested which was abnormal in six males with non-discrepant assays or lower 2 stage discrepancy but was normal in two female carriers of p.Tyr365Cys.

Alternative reagents

In 75 patients, FVIII:C1 assays were also performed with Synthasil APTT reagent and FVIII:CR with three kits from Siemens, Chromogenix and Pathway Diagnostics to compare these results against those with the Actin FVIII:C1 and FVIII:C2. The mean FVIII:C1 with Synthasil was 27 IU/dL while that with Actin FS was 23 IU/dL. The difference between the results obtained using the two reagents was statistically significant (paired t test, P=0.0009).

The mean FVIII:C2 was 27 IU/dL; the mean FVIII:CR results were 28 IU/dL with the Siemens kit, 29 IU/dL with the Coamatic kit and 28 IU/dL with the Technochrom kit. No statistically significant differences were observed between the results of the FVIII:C2 and the three FVIII:CR kits (ANOVA, P=0.2449) (Table 1).

Global assays of hemostasis

Rotational thromboelastometry

INTEM (a thromboelastometric test with mild activation of the contact phase of hemostasis) and modified thromboelastometry were performed on 27 patients with non-discrepant assays, five patients with lower two-stage discrepancy and nine patients with lower one-stage discrepancy. The results and normal ranges are shown in Table 3. For INTEM, the clotting time (CT) most reliably identified patients with MHA whilst in the modified method the CT, MAXV and MAXVT most reliably identified these patients. The mean FVIII:C1 of the non-discrepant patients with an abnormal INTEM result was 16 IU/dL (range, 4-39 IU/dL) and 27 IU/dL (range, 17-49 IU/dL) for those with a normal INTEM result (statistically significant: P<0.05). For the modified method, these values were 19 IU/dL (range 4-49 IU/dL) and 23 IU/dL (range, 9-37 IU/dL), respectively (not statistically significant: P>0.05). Overall, more than half of the patients had normal measurements by either method.

Calibrated automated thrombography

Forty-eight patients with non-discrepant assays, eight with lower two-stage discrepancy and 13 with lower one-stage discrepancy patients had thrombin generation measurements. As expected there was a large variability in endogenous thrombin potential (ETP) and peak thrombin generation at a given FVIII concentration irrespective of the FVIII assay used (Figure 2A-D). Overall, thrombin generation measurements were similar in the non-discrepant and lower two-stage groups (median ETP and peak thrombin generation 1336.5 nm/min and 173.8 nM and 1208 nm/min and 136.5 nM, respectively) (Table 4). Median thrombin generation measurements in the lower one-stage group (ETP 1852 nm/min and peak thrombin generation 308 nM) were similar to those in normal individuals (1860 nm/min and 408 nm, respectively) and both significantly

Table 1. APTT and FVIII assay results for the 84 patients and female carriers. APTT was performed in 82 patients and the number of patients in each group with a prolonged APTT is shown along with the median APTT (±1 SD from the mean). A negative number inicates a result within the adult reference range. The mean FVIII:C1 (performed with Actin FS APTT reagent) and FVIII:C2 in 59 non-discrepant, 10 lower two-stage discrepancy and 15 lower one-stage discrepancy patients is shown with the range in brackets. The adult reference range (ARR) is indicated for the standard assays. FVIII:C was tested in 75 patients using an alternative APTT reagent (Synthasil) for FVIII:C1 and three FVIII:CR kits. The mean FVIII:C is shown with the range in brackets.

	Non-discrepant	Lower two-stage	Lower one-stage
Overall patients consented (n.)	59	10	15
Number of APTT performed	58	10	14
Number of patients with prolonged APTT	54	6	13
Median APTT above the top of reference range (s)	5.9	0.75	5.5
	(-2.3 to 14.1)	(-3.3 to 4.8)	(-2.3 to 13.3)
FVIII:C2 IU/dL (ARR 52-184 IU/dL)	21 (4-68)	16 (4-37)	66 (10-111)
FVIII:C1 Actin IU/dL (ARR 58-184 IU/dL)	21 (4-76)	45 (11-112)	23 (4-37)
Alternative reagents (n.)	54	8	13
FVIII:C1 Synthasil IU/dL	23 (4-90)	42 (5-112)	30 (4-45)
FVIII:CR Siemens IU/dL	20 (3-78)	20 (5-84)	70 (4-116)
FVIII:CR Coamatic IU/dL	21 (3-80)	27 (6-96)	59 (9-106)
FVIII:CR Technochrom IU/dL	18 (2-61)	14 (5-24)	79 (7-134)

higher than those in the lower two-stage/non-discrepant patients (P<0.001 for ETP and P=0.0001 for peak thrombin). There were no differences between the groups in time to peak (P>0.05) but there was a significant difference in lag time (P=0.04). Among the patients with lower one-stage discrepancy, only one had a borderline abnormal ETP (1423 nm/min, lower limit 1475 nm/min) but seven had reduced peak thrombin. Of the latter, two (p.Arg602Gly and p.Pro2319Leu) had a peak thrombin generation less than 50% of the mean normal and were the only patients in the lower one-stage discrepancy group who had FVIII:C2 less than 40 IU/dL (FVIII:C2 10 and 26 IU/dL, FVIII:C1 4 and 13 IU/dL, respectively). The remainder (all p.Tyr365Cys) had mildly reduced or normal peak thrombin. Among the patients with lower two-stage discrepancy, three had FVIII:C1 levels greater than 40 IU/dL (FVIIIC1 42, 64 and 112 IU/dL, FVIII:C2 15, 14 and 37 IU/dL) but reduced ETP and peak thrombin generation. Eight patients with non-discrepant assay results had FVIII:C2 levels greater than 40 IU/dL. Of these, three had a normal ETP and peak thrombin (all female carriers of p.Tyr365Cys), one had a normal ETP and mildly reduced peak thrombin and four had reduced ETP and peak thrombin.

Significant correlations were found in lower one-stage discrepancy subjects with peak thrombin and Actin FS FVIII:C1 (r=0.77), Siemens FVIII:CR (r=0.83) and Technochrom FVIII:CR (r=0.75).

Discussion

Incidence of assay discrepancy

Assay discrepancy in patients with MHA was first highlighted in 1983[14] in four patients with markedly different

Table 2. The genotype and number of subjects with each mutation found in the non-discrepant, lower two-stage and lower one-stage groups. Five patients had no record of mutation analysis and are denoted unknown. * Indicates an amino acid change previously linked to assay discrepancy.

Nucleotide change	Mutation (in gene order)	N.	N. of females	Mean FVIII:C1 (IU/dL)	Mean FVIII:C2 (IU/dL)	FVIII:C1/C2 ratio
NON-DISCREPANT						
c.655G>A	p.Ala219Thr	5	1	10	11	0.91
c.1094A>G	*p.Tyr365Cys	3	3	39	59	0.66
c.1492G>A	p.Gly498Arg	1	-	6	5	1.20
c.1616G>A	p.Gly539Glu	1	-	23	12	1.92
c.1649G>A	*p.Arg550His	1	-	23	15	1.53
c.1804C>G	p.Arg602Gly	7	-	18	18	1.00
c.1834C>T	p.Arg612Cys	2	-	13	16	0.81
c.2149C>T	*p.Arg717Trp	2	-	24	15	1.60
c.3637delA	p.Glu1210fs	1	1	27	38	0.71
c.5101G>A	p.Glu1701Lys	2	-	17	18	0.94
c.5180A>T	p.Asp1727Val	1	-	40	47	0.85
c.5217C>T	p.Asn1739= (splice)	1	-	29	32	0.91
c.5320C>T	p.His1774Tyr	1	-	11	9	1.22
c.5348G>C	p.Arg1783Thr	1	-	37	56	0.66
c.5395T>C	p.Ser1799Pro	1	-	31	50	0.62
c.5398C>T	p.Arg1800Cys	1	-	4	5	0.80
c.5954G>A	p.Arg1985Gln	15	-	29	18	1.61
c.6082G>A	p.Gly2028Arg	3	-	22	19	1.16
c.6104T>C	p.Val2035Ala	1	-	17	12	1.42
c.6532C>T	p.Arg2178Cys	1	-	15	20	0.75
c.6956C>T	p.Pro2319Leu	4	-	8	8	1.00
ND	Inv intron 22	1	1	27	23	1.17
ND	unknown	3	-	25	26	0.96
LOWER TWO-STAGE FORM						
c.1649G>A	*p.Arg550His	1	-	31	13	2.38
c.5918A>T	*p.His1973Leu	5	-	67	21	3.19
c.5954G>A	p.Arg1985Gln	2	-	30	15	2.00
ND	unknown	2	-	14	5	2.80
LOWER ONE-STAGE FORM						
c.1094A>G	*p.Tyr365Cys	13	1	26	74	0.35
c.1804C>G	p.Arg602Gly	1	-	13	26	0.50
c.6956C>T	p.Pro2319Leu	1	-	4	10	0.40

Table 3. Rotational thromboelastometry results shown for 27 non-discrepant, 5 lower two-stage discrepant and 9 lower one-stage discrepant patients using two reagents, commercially produced INTEM and an in-house low tissue factor modification.

Parameter	Non-discrepant n=27		Lower two-stage n=5		Lower one-stage n=9		Normal range N=40
	Median (SD)	Abnormal ROTEM	Median (SD)	Abnormal ROTEM	Median (SD)	Abnormal ROTEM	
INTEM							
CT (s)	219 (39.3)	13	208 (17.1)	2	184 (20.8)	0	136-215
CFT (s)	91.5 (53.7)	3	79 (14.7)	0	72 (15.3)	0	71-127
α-angle (°)	72 (6)	2	74 (3.1)	0	76 (2.7)	0	67-75
MCF (mm)	57 (8.2)	3	61 (2.9)	0	61 (4.0)	0	49-61
Low TF modification							
CT (s)	424 (120.5)	10	456 (60.0)	0	480 (90.8)	1	291-513
CFT (s)	184 (93.3)	2	160 (33.1)	0	166 (41.6)	0	118-271
MCF (mm)	51 (7.3)	2	54 (4.4)	0	54 (5.5)	0	40-57
AUC (mmx60)	5056 (689.9)	2	5353 (383.9)	0	5412 (522)	0	4050-5703
MaxV (mm/min)	7 (3.2)	7	8 (3.1)	0	9 (2.3)	0	4-10
MaxVT (s)	586 (181.3)	9	651 (87.5)	0	584 (112.6)	2	345-646

Median results are shown for each parameter with 1SD in brackets. Male ranges are quoted, the equivalent ranges for females, where different, are for INTEM: CFT 59-99s, α-angle 71-79°, MCF 53-65mm. and for low tissue factor modification: CFT 104-209s, MCF 45-61s, AUC 4493-6061mmx60, MaxV 6-11s. The number of subjects who had results with abnormal ROTEM parameters is shown as a prolongation to CT, CFT, MaxVT and decrease in α-angle, MCF, AUC and MaxV.

FVIII:C levels when assayed by one-stage and two-stage clotting assays. Two forms of discrepancy are recognized which are linked to specific missense mutations in the *F8* gene. Lower two-stage discrepancy has long been considered the more common of the two forms. More than 15 missense mutations have been linked to the lower two-stage form, which confer instability of the A2 domain of FVIIIa[15] leading to reduced FVIII:C2 and FVIII:CR but higher FVIII:C1 levels. Bleeding in these patients is akin to that in patients with non-discrepant MHA and the FVIII:C2 assay better reflects the clinical severity.

In 2002 a mutation, p.Tyr365Cys, was described which had the reverse effect; a reduced FVIII:C1 and higher FVIII:C2 and FVIII:CR levels[5] and no bleeding problems. The lower one-stage form has been associated with fewer mutations including p.Tyr365Cys (Tyr346Cys), p.Glu340Lys (Glu321Lys) and p.Glu739Lys (Glu720Lys).[15] In our center in the UK, the prevalence of assay discrepancy linked to the p.Tyr365Cys mutation is higher than that of the lower two-stage discrepancy: this may reflect a possible common ancestor for the mutation.[4]

Assay discrepancy has not been uniformly defined in previous publications. We defined assay discrepancy as a 2-fold or greater difference in FVIII:C1 (using Actin FS) and FVIII:C2 levels. Thirty percent of patients in this study had a 2-fold or greater difference in FVIII:C and were classified as assay discrepant; 12% of these had the lower two-stage form. This proportion is in agreement with other reports of the lower two-stage form of assay discrepancy.[2,3] Five percent of our patients with reduced FVIII:C2 (and FVIII:CR), had FVIII:C1 within normal limits. In most laboratories worldwide (in which only the one-stage FVIII assay is employed), these patients, who all have a history of bleeding and impaired thrombin generation, would likely not have been diagnosed as suffering from hemophilia A.

Eighteen percent of patients were identified with the lower one-stage form of assay discrepancy. There have been limited reports of this form of discrepancy since most centers employ the FVIII:C1 assay alone for diagnosis.[4,5,16-19] All but two patients in this group had the p.Tyr365Cys change in the *F8* gene, a mutation which has not been linked to excessive bleeding.[4,5,18] Thirteen patients with lower one-stage discrepancy (p.Tyr365Cys) in our study had a reduced FVIII:C1 but FVIII:C2 within normal limits meaning the diagnosis was made based only on the one-stage method.

The 2001 ISTH Scientific and Standardization Committee on FVIII and FIX defined MHA as FVIII:C >5 and <40 IU/dL[1] but excessive bleeding has been described in hemophilia carriers with FVIII:C levels between 40 and 60 IU/dL.[20] In this study we included five males (p.Arg1985Gln, p.Asp1727Val, p.Ser1799Pro, p.Arg1783Thr and one unknown) and three female carriers of p.Tyr365Cys with either FVIII:C1 or FVIIIC2 between 40 and 50 IU/dL and historical FVIII:C levels below 40 IU/dL. ETP was reduced in all but one male (p.Ser1799Pro) and the thrombin peak was reduced in all five males. Thrombin generation was normal in the three carriers of p.Tyr365Cys.

Genotype

In our subjects, two mutations were commonly found; p.Arg1985Gln (Arg1966Gln), in a total of 17 patients and p.Tyr365Cys in 16 patients from 15 families. p.Arg1985Gln, has been tentatively linked to the lower two-stage form of assay discrepancy[21] but this has not been verified by subsequent studies and p.Tyr365Cys is linked to lower one-stage discrepancy.[5] Other mutations linked to the lower two-stage form of discrepancy (p.Arg550His, p.Arg717Trp and p.His1973Leu), were identified in a smaller number of patients. Assay discrepancy has a genetic basis but the phenotype does not always reflect the genotype, especially in an aging population in which FVIII:C levels may increase or normalize in patients with milder disease. Five individuals with mutations linked to assay discrepancy were phenotypically classified as non-discrepant; two patients with p.Arg717Trp, one with

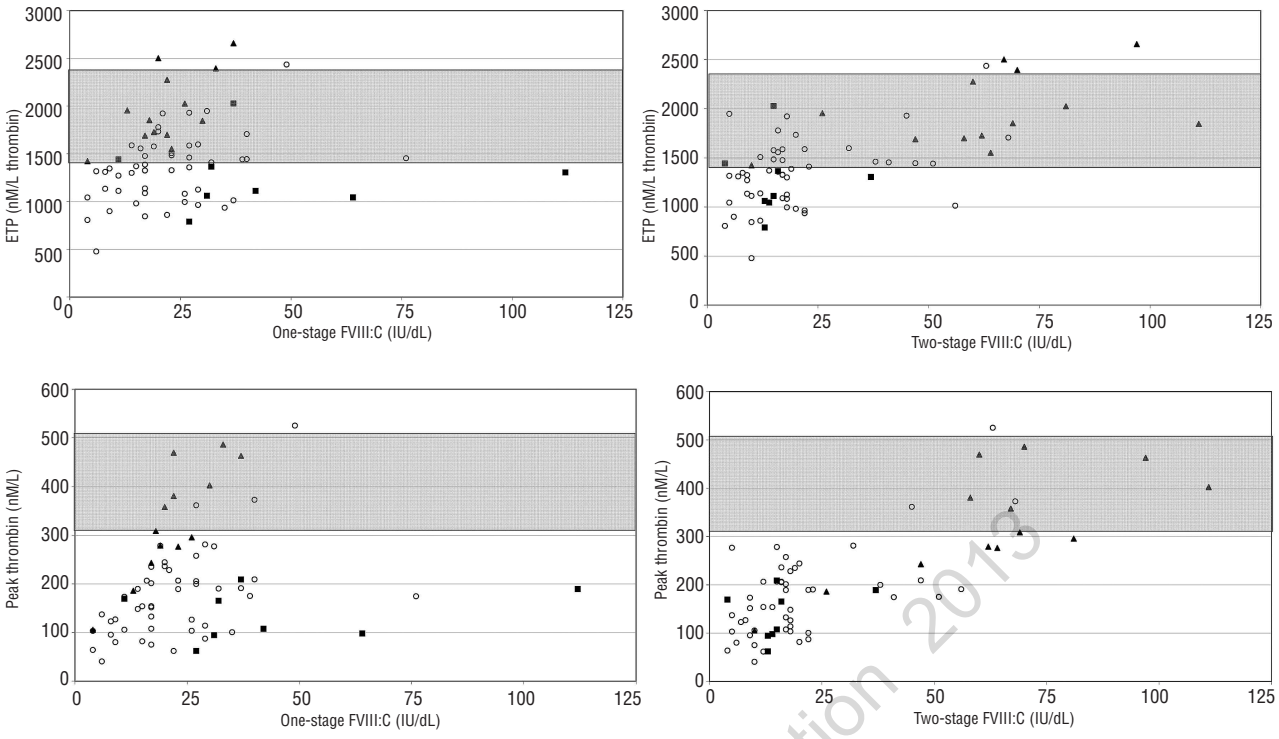

Figure 2. (A-D) CAT results and factor FVIII:C in non-discrepant MHA,[22] lower two-stage discrepancy (■) and lower one-stage discrepancy.[22] Normal reference ranges are denoted by the shaded areas of each graph. The relationship between (A) endogenous thrombin potential (ETP) and one-stage FVIII:C, (B) ETP and two-stage FVIII:C, (C) peak thrombin and one-stage FVIII:C and (D) peak thrombin and two-stage FVIII:C.

p.Arg550His and three female carriers of p.Tyr365Cys from two families (Table 2). In contrast, patients with two mutations not previously linked to assay discrepancy, p.Arg602Gly and p.Pro2319Leu, appear in both the non-discrepant and lower one-stage groups. Both patients in the lower one-stage discrepancy group have higher (and non-discrepant) results by FVIII:C1 using Synthasil reagent (*individual data not shown*) indicating that differences may occur with alternative reagents.

Four carriers of p.Tyr365Cys were included in this study but only one exhibited discrepant FVIII:C levels so it is likely that ratio calculations for discrepancy will not work in all carriers of haemophilia due to the mix of normal and mutant FVIII.

Prolongation of activated partial thromboplastin time

Many chance diagnoses of MHA are the result of investigations of an unexpected, prolonged APTT, irrespective of a history of bleeding symptoms. In contrast, some undiagnosed MHA patients with a positive bleeding history have a normal APTT which may curb further investigations, leaving the patient at risk of significant bleeding after surgery or trauma. The current Clinical and Laboratory Standards Institute guidelines (H47-A2)[22] recommend that a suitable APTT reagent should be prolonged in plasma samples which contain less than 30 IU/dL FVIII but the sensitivity of different reagents has been shown to vary significantly.[23] A normal APTT was demonstrated in 11% of our cohort of patients, distributed among the three groups; in each case the FVIII:C1 was above 30 IU/dL. This confirms that the APTT cannot be relied upon as a screen for patients with MHA and the personal bleeding history of each patient is a much better indicator of hemostasis even when the APTT is normal. Thrombin generation was tested in eight of nine patients in this group. Reduced ETP and peak thrombin were found in all except two female carriers of p.Tyr365Cys, suggesting that thrombin generation assays are also a better indicator of hemostasis than is the APTT.

Alternative reagents

In this study we categorized patients according to their FVIII:C by a single one-stage assay using Actin FS and our routine two-stage clotting assay, which is rarely performed in other centers. The two-stage clotting FVIII:C assay has been used in our hemophilia center over several decades for the initial diagnosis of all hemophilia A cases whereas the one-stage clotting assay is the most commonly used FVIII:C assay elsewhere. This may confer a selection bias compared to other centers that use the one-stage assay alone. There are many APTT reagents used for the FVIII:C1 estimation worldwide, so our patients were also tested using another popular APTT reagent, Synthasil. Although a statistically significant difference was observed between the results obtained with the two reagents this was not clinically significant. The FVIII:CR assay is used in a small number of laboratories in place of FVIII:C2 and the three commercially available FVIII:CR assays used in the present study gave similar results to the FVIII:C2 assay.

Rotational thromboelastometry

We have previously shown that thromboelastometry discriminates poorly between normal individuals and those with MHA[9] but to the best of our knowledge no studies have investigated the ability of ROTEM to detect FVIII:C assay discrepancy. Some of the patients with MHA

from our center participated in both a previous[9] and the current study so it is not surprising that similar conclusions have been reached. The only frequent abnormality in the non-discrepant group was the prolongation of the CT in both INTEM and the low tissue factor-modified ROTEM and reduced MAXV and prolonged MAXVT in the low tissue factor-modified ROTEM. Overall, however, more than half of all patients had normal parameters.

In this study, corn trypsin inhibitor was not added to the collection tube prior to blood collection to prevent initial contact activation at the low tissue factor concentration. The ISTH working party for thromboelastography/thromboelastometry has suggested the use of corn trypsin inhibitor to improve sensitivity so we acknowledge that this may be a limitation of our study.

Although the number of patients with discrepancy studied was small, our observations indicate that in view of its poor sensitivity, it is unlikely that ROTEM using the assay conditions described will be helpful in identifying patients with discrepant hemophilia A.

Calibrated automated thrombography

Several studies have demonstrated reduced thrombin generation in patients with hemophilia which subsequently normalized after treatment with FVIII concentrate or DDAVP.[24] ETP and peak thrombin generation show the most sensitivity to hemophilia so the results for lag time and time to peak, while shown in Table 4, are not alluded to in this report. The reduction in thrombin generation and the response to treatment at a given FVIII:C level are, however, highly variable. It is this variability in thrombin generation, with some patients with severe hemophilia attaining near normal ETP and others with only a mild reduction in FVIII:C but a clearly reduced ETP, which has given the hope that thrombin generation parameters may correlate better than FVIII:C measurements with clinical phenotype.

An ETP of 20-50% of normal has been reported in patients with hemophilia or rare coagulation deficiencies who demonstrate a severe bleeding tendency.[25,26] There is also a relationship between bleeding and failure to normalize ETP in hemophilia patients with inhibitors treated with inhibitor bypass agents at the time of surgery.[27]

We therefore studied thrombin generation in patients with discrepant factor FVIII:C assays and compared it to that in patients with non-discrepant assays and normal subjects given suggestions in the literature that two-stage assays and chromogenic assays may correlate than one-stage assays with the clinical phenotype.[4] Gilmore *et al.* reported on the effect of three mutations linked to lower two-stage discrepancy (p.Arg550His/Cys and p.Arg717Trp) on CAT, demonstrating impaired thrombin generation in this group similar to non-discrepant patients with the mean ETP being 58-67% of normal and peak thrombin generation being 27-30% of normal. Peak thrombin generation was reduced in all, irrespective of normal FVIII:C1 results. The authors concluded that CAT is a useful adjunct to other laboratory methods investigating patients with lower two-stage discrepancy.[28] This is similar to our results in the lower two-stage discrepancy group. We found that the mean ETP was 66% of normal in the lower two-stage discrepancy group and 69% of normal in the non-discrepant group. Mean peak thrombin was 33% and 43% of normal, respectively.

The relationship between lower one-stage discrepancy and thrombin generation has been investigated in two French families: family X, with p.Ile388Thr mutation and family Y with p.Phe2146Ser. Family X had reduced FVIII:C1 (mean 14 IU/dL) and normal FVIII:CR (mean 90 IU/dL), little bleeding and CAT results close to those in the normal population. Family Y also had reduced FVIII:C1 (mean 10 IU/dL) and normal (but lower) FVIII:CR (mean 47 IU/dL) but reported significant spontaneous or post-operative bleeding and CAT results close to those of non-discrepant patients with MHA. The ETP in the non-discrepant group in that study was 67% of the mean normal and the peak was 42% of the mean normal.[19] Our results in the group with lower one-stage discrepancy (with p.Tyr365Cys) in this study were similar to those of family X. Since these two mutations are located in the same area of *F8*, it is not surprising that a similar mechanism of action and pattern of results were noted. No studies of thrombin generation in patients with p.Tyr365Cys have been reported previously. Our patients with p.Tyr365Cys also did not have a bleeding history and in keeping with this, all patients and carriers of this mutation had a normal ETP and normal or only a mildly reduced peak thrombin generation. Trossaërt *et al.* concluded that the advantage of thrombin generation studies may be in the identification of patients with normal FVIII:CR measurements but a bleeding tendency and reduced thrombin generation. Overall in our study, eight patients had FVIII:C2 levels between 40-60%, of whom five males had a bleeding history and abnormal thrombin generation and three females (with p.Tyr365Cys) did not have a bleeding tendency and demonstrated normal thrombin generation. This confirms an advantage of thrombin generation studies over FVIII:C measurements. Overall, the ETP and particularly the peak thrombin generation reflected FVIII:C2/CR measurements, including those in patients with a normal FVIII:C1 but reduced FVIII:C2 and those with normal APTT measurements. A statistical correlation was found between peak thrombin generation and Actin FS FVIII:C1, Siemens FVIII:CR and Technochrom FVIII:CR but not with FVIII:C2 in contrast to our previous findings.[9] In our previous report we included several patients with severe and moderate hemophilia. Trossaërt *et al.* reported a better correlation between FVIII:CR less than 20 IU/dL and thrombin generation parameters.[19] This may be the reason for our currently contrasting findings when taken in conjunction with the relatively small number of patients in each group.

Conclusion

There remain many unresolved issues in the definition, diagnosis and management of patients with discrepant MHA. The definition of assay discrepancy has not been universally agreed which makes the linking of a particular mutation to assay discrepancy unpredictable. The overall incidence of discrepancy is thus unclear although it has been estimated at approximately 30% of patients. The genotype of a patient with MHA is not an accurate predictor of the degree of assay discrepancy but sometimes correlates to the bleeding diathesis, particularly in patients with the p.Tyr365Cys mutation in whom bleeding is rare.

The APTT, as a screening test, should not be relied upon to estimate FVIII:C activity because a normal result does not exclude the presence of a reduced FVIII:C, particularly in patients with assay discrepancy. At present, the FVIII:C2 assay is rarely performed so it is important to employ FVIII:CR assays that can detect patients with the lower two-stage discrepancy but normal FVIII:C1 in whom the diagnosis of MHA would otherwise be missed.

Of the global assays, the standard and modified ROTEM (using our assay conditions) do not discriminate well between patients with MHA and normal subjects so their use in the diagnosis of assay discrepancy is not practicable. The ETP and peak thrombin generation using CAT, however, show better discrimination between normal subjects and those with MHA. Overall, our results using CAT support the value of using a two-stage or chromogenic FVIII assay. Similar to other studies they also suggest additional value in the ETP and peak thrombin measurements in relation to bleeding tendency. The value of thrombin generation measurements in the management of individual patients does, however, remain uncertain until large prospective studies are performed.

The accurate identification of MHA patients with assay discrepancy is vital for their clinical management and it is clear that a significant number of patients at risk of bleeding will remain undiagnosed as long as laboratories perform only a FVIII:C1 for diagnosis. We, therefore, recommend that both a one-stage and a chromogenic (or two-stage) FVIII assay are included in the investigation of patients with mild bleeding disorders.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. White GC, Rosendaal FR, Aledort LM, Lusher JM, Rothschild C, Ingerslev J. Definitions in haemophilia. Recommendations of the scientific subcommittee on factor VIII and factor IX of the International Society on Thrombosis and Haemostasis. Thromb Haemost. 2001; 85(3):560.
2. Duncan EM, Duncan BM, Tunbridge LJ, Lloyd JV. Familial discrepancy between the one-stage and two-stage factor VIII methods in a subgroup of patients with haemophilia A. B J Haematol. 1994;87(4): 846-8.
3. Cid AR, Calabuig M, Cortina V, Casaña P, Haya S, Moret A, et al. One-stage and chromogenic FVIII:C assay discrepancy in mild haemophilia A and the relationship with the mutation and bleeding phenotype. Haemophilia. 2008;14(5):1049-54.
4. Bowyer AE, Goodeve AC, Liesner R, Mumford AD, Kitchen S, Makris M. p.Tyr365Cys change in factor VIII: haemophilia A, but not as we know it. Br J Haematol. 2011;154(5):618-25.
5. Mumford AD, Laffan M, O'Donnell J, McVey JH, Johnson DJD, Manning RA, et al. A Tyr346 Cys substitution in the interdomain acidic region a1 of FVIII in an individual with FVIII:C assay discrepancy. Br J Haematol. 2002;118(3): 589-94.
6. Over J. Methodology of the one-stage assay of factor VIII (VIII:C). Scand J Haematol 1984;33 (suppl 41):13-24.
7. Pool JG, Robinson J. Assay of plasma antihaemophilic globulin. Br J Haematol. 1959;5(1):17-23.
8. Sørensen B, Johansen P, Christiansen K, Woelke M, Ingerslev J. Whole blood coagulation thrombelastographic profiles employing minimal tissue factor activation. J Thromb Haemost. 2003;1(3):551-8.
9. van Veen JJ, Gatt A, Bowyer AE, Cooper PC, Kitchen S, Makris M. Calibrated automated thrombin generation and modified thromboelastometry in haemophilia A. Thromb Res. 2009;123(6):895-901.
10. van Veen JJ, Gatt A, Cooper PC, Kitchen S, Bowyer AE, Makris M. Corn trypsin inhibitor in fluorogenic thrombin-generation measurements is only necessary at low tissue factor concentrations and influences the relationship between factor VIII coagulant activity and thrombogram parameters. Blood Coagul Fibrinolysis. 2008;19(3):183-9.
11. Hemker HC, Giesen PLA, Al Dieri R, Regnault V, de Smedt E, Wagenvoord RJ, et al. Calibrated automated thrombin generation measurement in clotting plasma. Pathophysiol Haemost Thromb. 2003;33 (1):4-15.
12. Williams IJ, Abuzenadah A, Winship PR, Preston FE, Dolan G, Wright J, et al. Precise carrier diagnosis in families with haemophilia A: use of conformation sensitive gel electrophoresis for mutation screening and polymorphism analysis. Thromb Haemost. 1998;79(4): 723-6.
13. Keeney S. Use of robotics in high-throughput DNA sequencing. Methods Mol Biol. 2011;688:227-37.
14. Hathaway WE, Christian MJ, Jacobson LJ. Variant mild haemophilia: discrepancy in one-stage and two-stage factor VIII assays. Thromb Haemost. 1983;50:357.
15. d'Oiron R, Pipe SW, Jacquemin M. Mild/moderate haemophilia A: new insights into molecular mechanisms and inhibitor development. Haemophilia. 2008 14 (suppl 3):138-46.
16. Roelse JC, de Laaf RTM, Mertens K, van Mourik JA, Oldenburg J, Voorberg J. Altered sensitivity towards factor IXa explains assay discrepancies in plasma of haemophilia A patients with Glu720 Lys substitution in factor VIII. J Thromb Haemost. 1999;1:2722 (abstract 5).
17. Cutler JA, Mitchell MJ, Smith MP, Savidge GF. The identification and classification of 41 novel mutations in the factor VIII gene (F8C). Hum Mutation. 2002;19(3):274-78.
18. Lyall H, Hill M, Westby J, Grimley C, Dolan G. Tyr346 Cys mutation results in factor VIII:C assay discrepancy and a normal bleeding phenotype - is this mild haemophilia A? Haemophilia. 2008;14(1): 78-80.
19. Trossaert M, Regnault V, Sigaud M, Boisseua P, Fressinaud E, Lecompte T. Mild haemophilia A with factor VIII assay discrepancy: using thrombin generation assay to assess the bleeding phenotype. J Thromb Haemost. 2008;6(3):486-93.
20. Plug I, Mauser-Bunschoten EP, Brocker-Vriends AHJT, van Amstel HKP, van der Bom JG, van Dieman-Homan JEM, et al. Bleeding in carriers of haemophilia. Blood. 2006;108(1):52-6.
21. Goodeve AC, Peake IR. The molecular basis of haemophilia A: genotype-phenotype relationships and inhibitor development. Semin Thromb Haemost. 2003;29(1): 23-30.
22. Standards Institute(CLSI) C. One-Stage Prothrombin Time (PT) Test and Activated Partial Thromboplastin Time (APTT) Test; Approved Guideline - Second Edition H47-A2, 2008.
23. Bowyer AE, Kitchen S, Makris M. The responsiveness of different APTT reagents to mild factor VIII, IX and XI deficiencies. Int J Lab Hematol. 2011;33(2):154-8.
24. van Veen J, Gatt A, Makris M. Thrombin generation testing in routine clinical practice: are we there yet? Br J Haematol. 2008;142(6):889-903.
25. Dargaud Y, Beguin S, Lienhart A, Al Dieri R, Trzeciak C, Bordet JC, et al. Evaluation of thrombin generating capacity in plasma from patients with haemophilia A and B. Thromb Haemost. 2005;93(3):475-80.
26. Al Dieri R, Peyvandi F, Santagostino E, Giansily M, Mannucci PM, Schved JF, et al. The thrombogram in rare inherited coagulation disorders: its relation to clinical bleeding. Thromb Haemost. 2002;88(4): 576-82.
27. Dargaud Y, Lienhart A, Negrier C. Prospective assessment of thrombin generation test for dose monitoring of bypassing therapy in hemophilia patients with inhibitors undergoing elective surgery. Blood. 2010;116(25):5734-7.
28. Gilmore R, Harmon S, Gannon C, Byrne M, O'Donnell JS, Jenkins PV. Thrombin generation in haemophilia A patients with mutations causing factor VIII assay discrepancy. Haemophilia. 2010;16(4):671-4.