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ORIGINAL ARTICLE

In silico and in vitro evaluation of the impact of mutations in non-severe haemophilia A patients on assay discrepancies

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Abstract

Haemophilia A (HA) is caused by a lack or reduced amount of factor VIII protein (FVIII). About one-third of patients with non-severe HA carrying specific missense mutations show discrepant results between FVIII activity (FVIII:C), measured by one-stage or chromogenic two-stage assays. The aim of this study was to elucidate the mechanism underlying the assay discrepancy in vitro and in silico. Thirteen missense mutations in the *Factor 8*-gene associated with discrepant results in patients were transiently expressed. FVIII:C of the mutations was determined using two one-stage assays (FVIII:C_{1st}, FVIII:C_{Bonn}) and a two-stage chromogenic assay (FVIII:C_{chr}). Furthermore, thrombin generation test (TGT) and in silico analysis were performed to investigate the haemostatic potential as well as the structural impact of the variants, respectively. For the majority (9/13) of the analysed mutations, the discrepancy was confirmed. Moreover, we established a modified TGT protocol for in vitro characterization of FVIII. Hence, TGT parameters were significantly impaired in the group of variants associated with higher chromogenic values. Additionally, in silico analysis revealed the impact of the mutations on FVIII protein structure leading to assay discrepancy. Moreover, the data shows that also among one-stage clotting assays, assay discrepancy is observed. Our results show that for the majority of mutations, application of a global assay like TGT method could help to improve diagnosis or correct assessment of the severity of HA.

Keywords FVIII:C · One-stage assay · Chromogenic assay · Discrepancy · Haemophilia A

Introduction

Haemophilia A (HA) is a hereditary disorder affecting 1 in 5000 males worldwide which is caused by quantitative or qualitative deficiency of coagulation factor VIII (FVIII) [1]. The severity of the disease correlates with FVIII activity (FVIII:C), which is determined by means of FVIII activity measurement assays. The correct assessment of FVIII:C levels is an important step in the haemophilia care as it is not only essential for the diagnosis, but also for the evaluation of the treatment. Based on the measured FVIII:C levels, HA is classified into three degrees of severity: severe (FVIII:C < 0.01 IU/ml), moderate (0.01–0.05 IU/ml) and mild (0.06–0.40 IU/ml) [2–4].

Depending on the number of reaction phases (or stages) involved in the assay principle, FVIII:C assays are categorized into one-stage or two-stage assays. The one-stage FVIII clotting assay is based on assessment of activated partial thromboplastin time (aPTT) which is prolonged in the absence or deficiency of FVIII [5]. Simplicity, wide availability of the reagents, lower costs and ease of automation make the one-stage assay the most commonly used type of assay [6, 7]. The traditional two-stage clotting method [8], however, is not frequently performed and is largely replaced by the chromogenic assay (FVIII:C_{chr}) in which a chromogenic substrate is used in the second stage, which measures the levels of FXa that are generated proportionally to activity of FVIII in the first stage [9, 10]. In the majority of HA patients, the quantified FVIII:C level is equivalent when measured by both assays. However, one-third of

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non-severe HA patients have been reported to have discrepant results between the one-stage and chromogenic assay [6, 11–15]. Based on the order of difference in the results, two types of discrepancy are described. The classical discrepancy is when the FVIII:C one-stage assay is higher than the two-stage assay, which is the case for most of the reported discrepancies. In fewer cases, inverse discrepancy (FVIII:C one-stage < FVIII:C two-stage) is reported. Both discrepancies lead to an incorrect diagnosis and/or assessment of the severity of disease.

The discrepancy phenomenon has been associated to specific missense mutations in the *F8* gene. Recently, our group reported a number of discrepant mutations showing both the classical and inverse discrepancy [16]. In the current study, we present the data on in vitro and in silico characterization of these mutations by assessing FVIII:C using three different FVIII measurement assays. Moreover, functional characterization of FVIII variants was done, using the thrombin generation test (TGT) to determine whether their haemostatic capacity is altered.

Materials and methods

Construction and in vitro transient expression of FVIII mutations

We analysed 13 mutations previously reported to be associated with a discrepancy between one-stage and two-stage assays [16]. Desired nucleotide exchanges in the full-length *F8* cDNA were introduced via site-directed mutagenesis into the pCIneo-FVIII plasmid according to the instructions of the iProof High-Fidelity PCR Kit (Bio-Rad Laboratories, Inc., Munich, Germany) using primers listed in Supplementary Table 1. Recombinant FVIII variants were transiently expressed in COS-1 cells with 4 μg of each plasmid using Lipofectamine™ 2000 (Thermo Fischer Scientific) according to the manufacturer's instructions as previously described [17]. Data were collected in triplicates from three different experiment ($n = 9$). Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Thermo Fischer Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Thermo Fischer Scientific) or in OptiMEM (Thermo Fischer Scientific) supplemented with 1% BSA (bovine serum albumin, Thermo Fischer Scientific). Conditioned media were collected 72 h after transfection, centrifuged and stored at − 80 °C until used. Cell lysates were prepared using RIPA lysis buffer (Thermo Fischer Scientific) containing protease inhibitor cocktail (Roche Diagnostica, Indianapolis, IN, USA) and stored at − 80 °C until used.

Measurements of FVIII activity and antigen

Activity of expressed FVIII mutations was measured by three different assays: a two-stage chromogenic (FVIII:C_{chr}) and two one-stage clotting assays (FVIII:C_{1st} and FVIII:C_{Bonn}). Both automated commercial assays (FVIII:C_{1st} and FVIII:C_{chr}) were performed on a Behring Coagulation System® (BCS, Dade Behring, Marburg, Germany) using reagents from Siemens Healthcare Diagnostics (Eschborn, Germany) according to the manufacturer's instructions. The in-house one-stage assay (FVIII:C_{Bonn}) is an aPTT-based assay that was performed on a KC 10A coagulometer (Trinity Biotech, Lemgo, Germany) as previously described [16]. The $FVIII_{chr}/FVIII_{1st}$ ($FVIII_{Bonn}$) or the reverse ratio (ΔFVIII:C) was calculated, and results were considered discrepant if ΔFVIII:C ≤ 0.6. FVIII antigen levels (FVIII:Ag) were measured using the VisuLize® Faktor VIII Antigen Kit (Hemochrom Diagnostica GmbH, Essen, Germany), according to the kit instructions.

Calibrated automated thrombography—thrombin generation test

CAT-TGT was performed on a Fluoroskan Ascent™ Microplate Fluorometer (Thermo Fisher Scientific) using reagents from Diagnostica Stago (Düsseldorf, Germany). Protocol was followed as described by Hemker et al. [18] with few modifications. Prior to analysis, collected media were supplemented with 15 mM sodium citrate (NaAc, to be comparable with citrated plasma) and the final protein content of all variants was adjusted to 0.05 IU/ml based on the FVIII:Ag levels. Samples were diluted 1:1 in FVIII-deficient plasma (Siemens Healthcare Diagnostics), and the initiation of thrombin generation was triggered either by 4 μM phospholipids (PL) (Hemochrom Diagnostica, Essen, Germany) or by PPP reagent.

In silico modelling of FVIII mutations

The structural and functional impact of the mutations reported in this study was analyzed using in silico tools. Four PDB files corresponding to the structure of human FVIII, two modelled i.e. PMDB (protein modelling database) ID: PM0076116 (non-active FVIII lacking B domain) and PM0076119 (activated FVIII) and two crystal structures i.e. PDB ID: 3CDZ (resolution 3.98 Å) and 2R7E (resolution 3.98 Å) (both non-active FVIII lacking B domain) were downloaded from the PMDB (Protein modeling database; http://mi.caspur.it/PMDB/main.php) and PDB (Protein database; https://www.rcsb.org/pdb/home/home.do) [19–21]. In order to remove steric clashes and improve rotamer geometry, each downloaded PDB file was optimized by a short solvated molecular dynamic simulation refinement run of 500 ps (force field used: Yamber03, a variant of AMBER force fields). The lowest energy structure in this simulation run was used for further structural analysis. The wild-type residues were replaced with the specific mutant residues in the respective FVIII PDB files and optimized for the best possible

rotamer (using SCRWL libraries). The local environments of the wild type and the mutant residues were inspected to make a comparative assessment. Hydrogen bonds and potential clashes for the high-resolution structures were detected by the standard visualization option of YASARA. All image rendering, structural analysis and visualization were performed on YASARA 17.4.17 [22].

Results

For all mutations, ΔFVIII:C was determined by measuring FVIII:C using three FVIII measurement assays. For the $FVIII_{WT}$ construct, ΔFVIII:C was in the range of 0.9–1.3 ($n = 9$). Of the 23 previously reported mutations [16], the in vitro expression of 13 variants led to production of measurable amounts of FVIII:Ag, which were used for further characterization (Table 1).

Group 1: mutations resulting in FVIII:C one-stage assay higher than FVIII:C chromogenic assay

For variants p.T294I and p.P1844S, the in vitro results were in line with the discrepancy observed in patients. For the p.T294I mutation, a ΔFVIII:C of 0.5 was obtained for FVIII:$C_{chr/1st}$ and 0.6 for FVIII:$C_{chr/Bonn}$. Similarly, ΔFVIII:C of ≤ 0.6 for both assays (FVIII:$C_{chr/1st} = 0.4$ and FVIII:$C_{chr/Bonn} = 0.5$) were observed for the p.P1844S mutation.

The analysis of the p.R550H mutation revealed different results when comparing the two one-stage assays. The in vitro analysis shows a ΔFVIII:C of ≤ 0.6 only when $FVIII_{1st}$ was compared with the $FVIII_{chr}$ (FVIII:$C_{chr/1st} = 0.5$), while the FVIII:$C_{chr/Bonn}$ ratio was not discrepant (ΔFVIII:C = 0.7). Interestingly, discrepant results were also observed between the two one-stage assays (ΔFVIII:C = 0.6). The analysis of the remaining three mutations (p.Y133C, p.V682A and p.R1768H) revealed no discrepancy between the chromogenic and one-stage assays. However, the p.V682A showed a discrepancy between the two one-stage assays (ΔFVIII:C = 0.6; Table 1).

Group 2: mutations resulting in FVIII:C one-stage assay lower than FVIII:C chromogenic assay

According to the affected functional sites, the mutations are classified into four different groups: mutations affecting thrombin cleavage sites (2-I), the von Willebrand factor (VWF) binding (2-II), the coagulation factor IX (FIX) binding (2-III), and the mutations localized outside the functional regions of FVIII protein (2-IV).

Group 2-I mutations are located in close vicinity (p.E739K) or directly at a thrombin cleavage site (p.R1708H). For the p.E739K, similar to the in vivo data, higher FVIII:C_{chr} values were observed compared with both one-stage assays (FVIII:$C_{1st/chr} = 0.3$ and FVIII:$C_{Bonn/chr} = 0.2$). In line with the patient data, FVIII:C levels in the chromogenic assay were similar to the wild type (29 to 32% in $FVIII_{WT}$). The p.R1708H mutation also represented a ΔFVIII:C of ≤ 0.6 for both assays (FVIII:$C_{1st/chr} = 0.5$ and FVIII:$C_{Bonn/chr} = 0.2$). Similar to patient reports, normal FVIII:Ag was observed for the p.E739K and the p.R1708H mutations. Remarkably, for the p.R1708H mutation also, the results of the two one-stage assays were discrepant (ΔFVIII:C = 0.4).

Table 1 Summary of the functional tests performed for the variants. The discrepant results of the measurement assays and the differences of TGT parameters to $FVIII_{WT}$ are in italics. Data are mean values for $n = 9$ measurements

Hemostatic parameters			FVIII:Ag (IU/dl)	FVIII:C_{1st} (IU/dl)± SD	FVIII:C_{Bonn} (IU/dl)± SD	FVIII:C_{Chr} (IU/dl)± SD	FVIII:$C_{1st/Bonn}$	FVIII:$C_{chr/1st}$	FVIII:$C_{chr/Bonn}$	Lag-Time (min)	ETP (nM min^{-1})	Peak (nM)	TTP (Min)
WT			29	26,5 ±0,5	25,3 ±0,2	32,1 ±0,9	1,0	1,2	1,3	6	2737	282	11
Group 1 (one-stage>two-stage)													
Group 1	pHGVS	Domain	FVIII:Ag (IU/dl)	FVIII:C_{1st} (IU/dl)± SD	FVIII:C_{Bonn} (IU/dl)± SD	FVIII:C_{Chr} (IU/dl)± SD	FVIII:$C_{Bonn/1st}$	FVIII:$C_{chr/1st}$	FVIII:$C_{chr/Bonn}$	Lag-Time (min)	ETP (nM min^{-1})	Peak (nM)	TTP (Min)
	Y133C	A1	10	10,7 ±0,4	8,1 ±0,6	7,7 ±0,2	0,8	0,7	1,0	6	2308	*204*	*14*
	T294I	A1	12	12,2 ±0,5	11 ±0,4	6,1 ±0,5	0,9	*0,5*	*0,6*	6	*2015*	*128*	*20*
	R550H	A2	19	7,2 ±0,3	4,6 ±0,7	3,3 ±0,2	*0,6*	*0,5*	0,7	*8*	2422	*203*	*15*
	V682A	A2	25	8,6 ±0,9	5,5 ±0,2	6,9 ±0,7	*0,6*	0,8	1,3	*8*	2358	*173*	*17*
	R1768H	A3	21	11,4 ±0,6	8,2 ±0,5	10,5 ±0,4	0,7	0,9	1,3	7	2638	259	12
	P1844S	A3	8	13 ±0,3	12 ±0,2	5,8 ±0,4	0,9	*0,4*	*0,5*	7	*2129*	*145*	*18*
Group 2 (two-stage>one-stage)													
Group 2	pHGVS	Domain	FVIII:Ag (IU/dl)	FVIII:C_{1st} (IU/dl)	FVIII:C_{Bonn} (IU/dl)	FVIII:C_{Chr} (IU/dl)	FVIII:$C_{Bonn/1st}$	FVIII:$C_{1st/chr}$	FVIII:$C_{Bonn/chr}$	Lag-Time (min)	ETP (nM min^{-1})	Peak (nM)	TTP (Min)
I	E739K	a2	29	10 ±0,2	7 ±0,3	29 ±0,6	0,7	*0,3*	*0,2*	*9*	2522	261	*15*
	R1708H	a3	35	6,5 ±0,4	2,4 ±0,4	12,5 ±0,7	*0,4*	*0,5*	*0,2*	*10*	2464	264	*16*
II	Y1699F	a3	10	20,2 ±0,8	14,2 ±0,3	15,8 ±,4	0,7	1,3	0,9	*10*	2724	268	10
	S2138Y	C1	9	12,7 ±0,2	6,3 ±0,4	10,7 ±0,4	*0,5*	1,2	*0,6*	6	2769	266	10
	D2150N	C1	12	16,6 ±,5	16,5 ±0,7	20,3 ±0,7	1,0	0,8	0,8	6	2748	279	10
III	R546W	A2	25	5,9 ±0,4	3,5 ±0,3	6,9 ±0,7	*0,6*	0,9	*0,5*	*8*	2401	*208*	*15*
IV	V697L	A2	27	6,9 ±0,3	4,5 ±0,2	12,8 ±0,4	0,7	*0,5*	*0,4*	*9*	2538	255	13

Group 2-II mutations affect the VWF binding site. Among the analysed mutations, the p.S2138Y showed a ΔFVIII:C of ≤ 0.6 only when $FVIII_{chr}$ assay was compared with the $FVIII_{Bonn}$ assay ($FVIII:C_{Bonn/chr} = 0.6$). For this mutation also, the ΔFVIII:C of the one-stage assays was discrepant ($FVIII:C_{Bonn/1st} = 0.5$). For the mutations p.Y1699F and p.D2150N, no discrepancy between the assays was observed.

Group 2-III comprises mutations that are located in close vicinity of the FIX binding site. The mutation p.R546W was the only variant of this group with sufficient antigen levels for analysis. The mutation was discrepant when the $FVIII_{chr}$ assay was compared with the $FVIII_{Bonn}$ assay ($FVIII:C_{Bonn/chr} = 0.5$); however, results were not discrepant when compared with $FVIII:C_{1st}$ ($FVIII:C_{1st/chr} = 0.9$). When comparing the two one-stage assays, discrepant results between the two tests were observed ($FVIII:C_{Bonn/1st} = 0.6$).

Among the mutations of the fourth category (group 2-IV), expression of only the p.V697L mutation led to measurable antigen/activity levels. Similar to the patient data, higher $FVIII:C_{chr}$ values were observed in comparison to both one-stage assays ($FVIII:C_{1st/chr} = 0.5$ and $FVIII:C_{Bonn/chr} = 0.4$).

Thrombin generation potential of the FVIII variants

Due to the insensitivity of the TGT protocol to FVIII using 1 pM tissue factor (TF) (Supplementary Figure 1A), the assay was modified as described in Supplementary Methods. Accordingly, the analysis of in vitro expressed FVIII variants was performed using the following conditions: FVIII antigen levels were measured, and all variants were adjusted to 0.05 IU/ml. Later, the media were supplemented with 15 mM NaAc and mixed with 4 μM PL and subsequently diluted 1:1 in FVIII-deficient plasma and assessed by TGT. Thrombin generation of $FVIII_{WT}$ was initiated after 6 min; peak thrombin (282 nM) was reached after 11 min, and the endogenous thrombin potential (ETP) was 2737 nM min. The thrombin generation curve (Fig. 1) illustrates three distinct phases: initiation of the coagulation (lag time) followed by the formation of large amount of thrombin in the propagation phase which results in culminating a peak thrombin concentration, and termination of the pro-coagulant response. ETP (area under the curve) represents the amount of thrombin formed over 60 min. Clot formation would occur at the end of the lag time; therefore, this parameter corresponds to the clotting time.

Among the six studied variants from the group 1, the amplification phase of coagulation, as reflected by the lag time, was prolonged for R550H and V682A variants (25% increase compared to the $FVIII_{WT}$). Except for the p.R1768H, all variants showed an increase in the time to peak (TTP) with the highest effect observed for p.T294I and p.P1844S variants (45% and 39% increase compared to the $FVIII_{WT}$). Interestingly, p.R1768H showed a similar thrombin

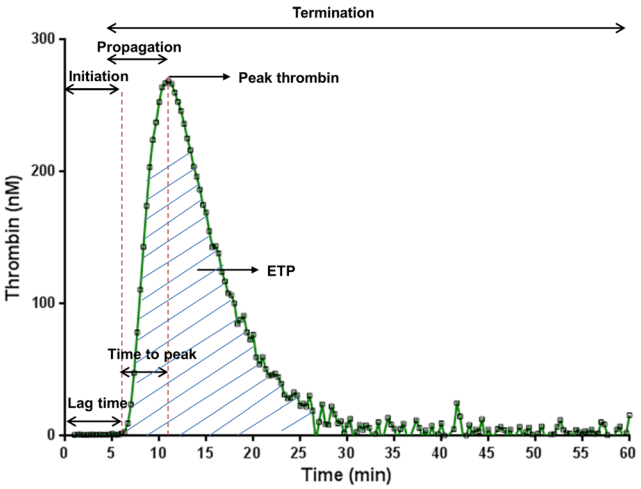

Fig. 1 Parameters of the thrombin generation curve using the calibrated automated thrombogram (CAT) method. ETP (Endogenous thrombin potential) represents the area under the curve

generation profile as the $FVIII_{WT}$, while a significant reduction of peak height was observed for all the other variants with the lowest peak height observed for the p.T294I, p.P1844S and V682A mutations (55%, 49% and 39% decrease) (Fig. 2).

All variants of the group 2 showed similar ETP values comparable to the $FVIII_{WT}$ indicating a normal thrombin generation potential in TGT. Both mutations which are located close to or on the thrombin cleavage sites (2-I) and the p.R546W (2-III) showed increased lag time (50%, 67% and 67% for p.E739K, p.R1708H and p.R546Q, respectively) and TTP values (36%, 45% and 36% for p.E739K, p.R1708H and p.R546Q, respectively). For the p.R546W variant, in line with the lowest activity results according to one-stage and chromogenic assays, the peak height was also decreased (26%). These values reflect the initiation and propagation phases of coagulation, indicating a defect in maintaining the normal rate of these phases of thrombin generation. Among the variants in the close vicinity of VWF binding site (2-II), only the p.Y1699F variant showed an increase in the lag time (67%, Fig. 3). The p.V697L variant (2-IV) showed only an increase in lag time (50%); the remaining TGT parameters were similar to the $FVIII_{WT}$.

In silico analysis of discrepant mutations

Mutations in A1, A2 and A3 domains

The Tyr133 residue in the A1 domain resides on a disordered coil like 25 amino acid long region. This disordered part is stabilized by the coordination of a copper ion (Cu^{2+}) with multiple residues within this region. This residue is also close to a calcium binding site that involves the Cys2019 residue (A3 domain). Although the backbone of Tyr133 residue and Cys2019 residue are not close enough to form a disulphide

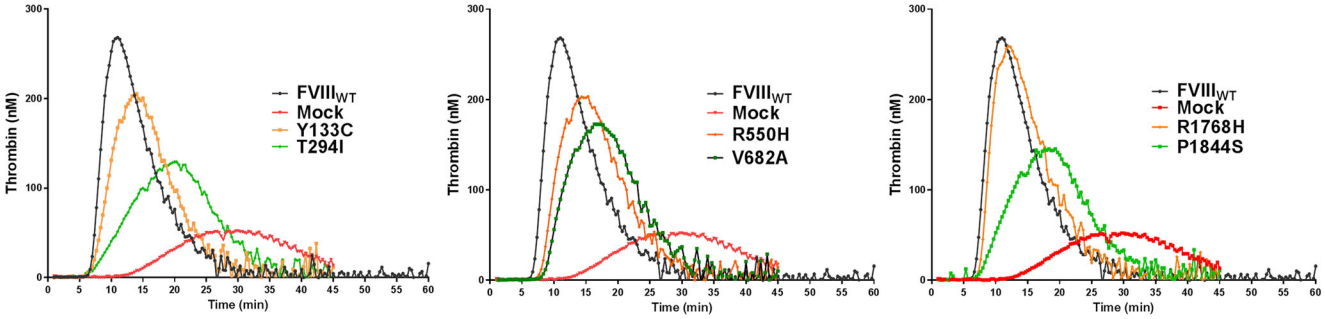

Fig. 2 Thrombin generation test curves of the FVIII mutations associated with one-stage higher than chromogenic assay. Collected media from FVIII$_{WT}$, non-transfected cells (Mock) and mutants associated with FVIII:C$_{1st>chr}$ were supplemented with 15 mM NaAc, adjusted to 0.05 IU/ml and diluted 1:1 in FVIII-deficient plasma. Thrombin generation was triggered with 4 μM PL. Each curve is the average of three independent experiments performed in duplicates. Representative thrombin generation curves are shown

bond i.e. in the event of a Tyr to Cys substitution, the flexible nature of this region might permit the formation of a native disulphide bond during the folding of this region. This could result in bringing these two regions from the A1 and A3 domains closer and thus disrupting the Cu^{2+} and Ca^{2+} binding sites which in turn leads to functional implications (Fig. 4a).

The Thr294, Arg550 and Arg546 residues lie in a region of A1 and A2 domains that form the only weak inter-domain interactions between these two domains (Fig. 4b). Changes in this region could affect the strength of the inter-domain interaction. The A2:A3 interface has three ion-pair interactions due to a clustered network of charged residues (Arg759:Glu397/Glu1830, Lys1852:Asp685/Tyr683, Glu626:Arg1919/Ala1920). The mutated residues Val682 and Pro1844 lie close to the second cluster/ion pair, possibly affecting the magnitude of the A2:A3 interaction (Fig. 4c).

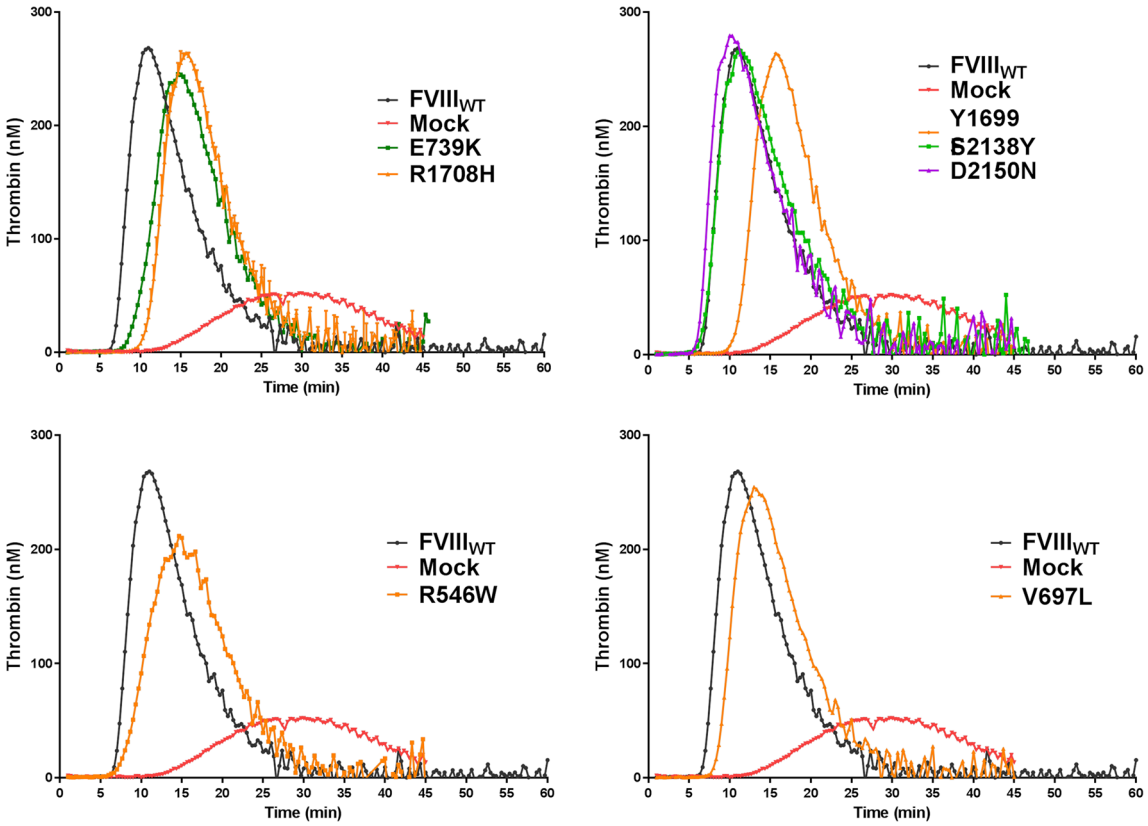

Fig. 3 Thrombin generation test curves of the FVIII mutations associated with one-stage lower than chromogenic assays. Collected media from FVIII$_{WT}$, non-transfected cells (Mock) and mutants associated with FVIII:C$_{1st<chr}$ were supplemented with 15 mM NaAc, adjusted to 0.05 IU/ml and diluted 1:1 in FVIII-deficient plasma. Thrombin generation was triggered with 4 μM PL. Each curve is the average of three independent experiments performed in duplicates. Representative thrombin generation curves are shown

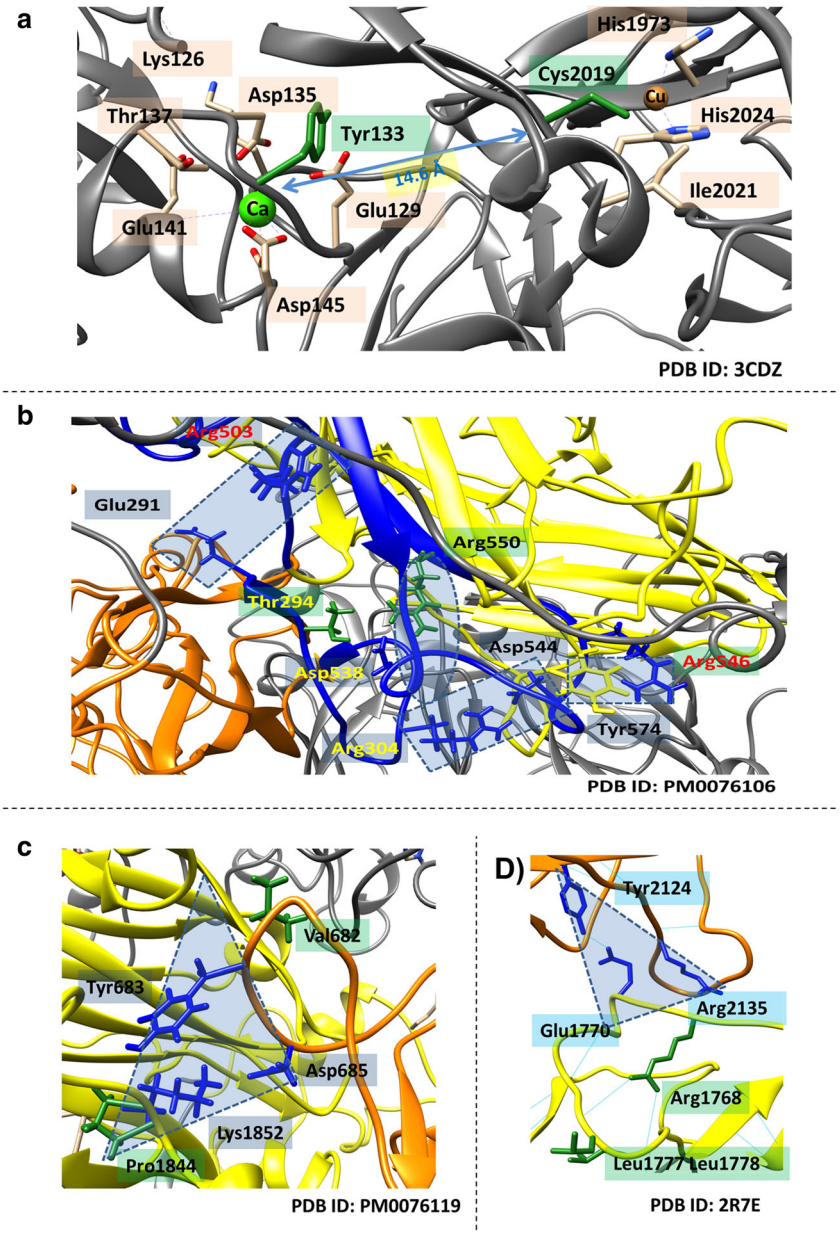

Fig. 4 Close-up molecular views of the A1, A2 and A3 domain residues on which mutations have been reported. This figure is split into four panels. In all panels, the protein backbone is depicted in differently coloured ribbon format. The residues on which the mutations have been reported are depicted in green stick format while other proximal residues of interests are also depicted in stick format but coloured differently depending on the domain. Heteroatoms are depicted as ball models. **a** The ribbon format backbones of the A1 and A3 domains are both depicted in grey colour. The heteroatoms of calcium and copper are coloured green and orange, respectively. **b** The domains A2 and A3 ribbon backbones are depicted with orange and yellow colours, respectively. A small stretch of residues that form the interface between A2 and A3 has been depicted with blue ribbon format with critical ion-pair interaction residue on this stretch represented as blue-coloured stick models. **c**, **d** The domain backbone and residue depiction follow the colour and format code of **b**

The C1 and C2 domains cover a large portion of the A1, A2 and A3 domain surfaces. However, the physical inter-domain interactions observed between these domains are far and few. One of these is the ion-pair cluster of Glu1770 of the A3 domain with the Arg2135 and Tyr2124 residues of the C2 domain. The Glu1770 residue is located on a long disordered coiled region (Fig. 4d). In order to stabilize the outward orientation of the Glu1770, this region requires stabilization by three hydrogen bonds. Two of them are formed by Arg1768 with neighbouring Leu1777 and Leu1778 residues. According to the crystal structure 3CDZ, Arg1768 does not appear to be capable of forming these bonds, whereas the structure 2R7E displays the contrast, which is evident for the flexibility of this disordered region. These H bonds are formed with the terminal hydrogens (NH1 and NH2) of Arg1768. Therefore, the long side chain, essential for forming these H bonds, are lost in case of the substitution of Arg with His, despite retaining the same charge. This leads to the destabilization of the Glu1770 and of the A3:C2 inter-domain interface.

Mutations in C1 domain

The C domains have limited stabilizing interactions with A domains [19]. The C1 domain contains one glycosylation site at the N2137. Among the five glycosylation sites on FVIII that

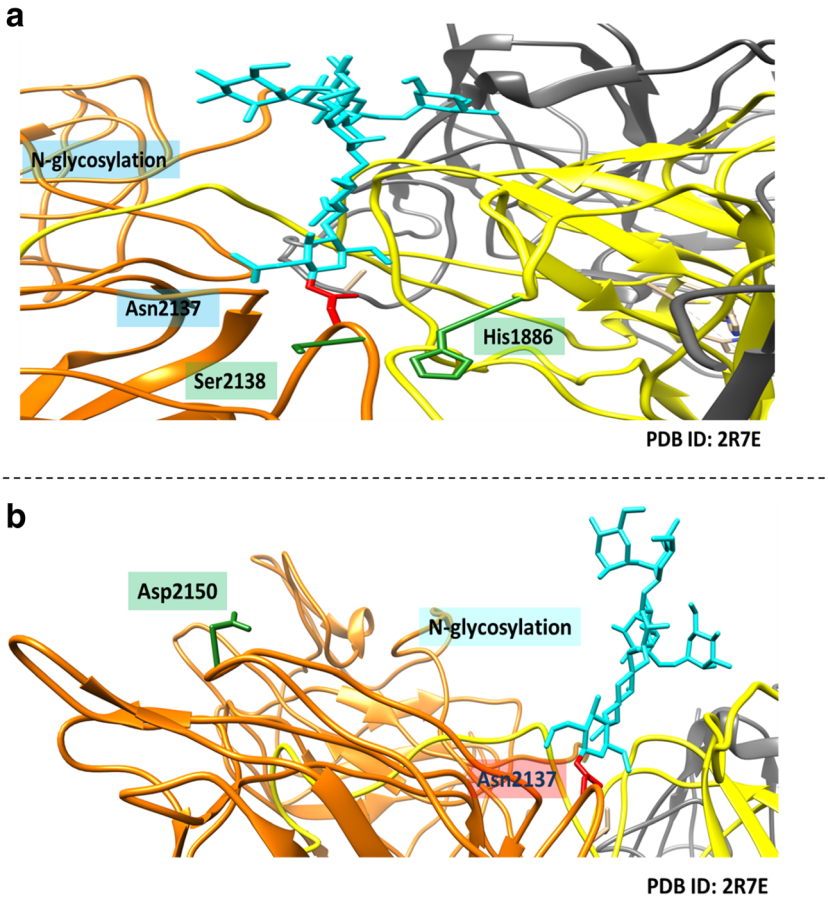

Fig. 5 Close-up molecular views of the C1 domain residues on which mutations have been reported. This figure is split into two panels. **a**, **b** The backbones of domains A3 and C1 are depicted in yellow- and orange-coloured ribbon format, respectively. The residues on which the mutations have been reported are depicted in green stick format while the glycosylated Asn residue is coloured red with the carbohydrate part coloured cyan

do not occur on the B domain, this is the most buried residue. A substitution on the neighbouring S2138 residue to an aromatic Tyr residue might result in aromatic stacking interaction with an oppositely placed His1886 residue (Fig. 5a).

The residue D2150 has significantly better surface exposure than N2137. Therefore, a substitution at this position to a N2150 could lead to an incorrect glycosylation of N2150 instead of N2137, which can in turn affect the correct folding of the protein (Fig. 5b).

Mutations in acidic regions a2 and a3

Two sulphated tyrosine residues 738 and 742 together with aspartic and glutamic acid residues at 731, 736, 739, 740 and 743 generate a high density of acidic cluster with the hairpin-like loop at the C-terminus of the A2 domain. Experimental studies have shown that this region is likely to function as a thrombin exosite binding region during the proteolysis of Arg391-Ser392 and/or Arg759-Ser760 peptide bonds [19]. Therefore, a substitution at the Glu739 residue to a neutral leucine residue might reduce the binding affinity of this region for thrombin (Fig. 6a).

Two mutations that are located in the a3 acidic region on and close to the third thrombin cleavage site of FVIII showed interesting structural implications. The mutation, p.R1708H, is exactly on the P1 residue of this cleavage site. Thrombin cleavage sites in different thrombin substrates show a very strong conservation of the P1 arginine residue [17, 23, 24]. This is owing to the positively charged side chain of this residue that forms contacts with residues in the thrombin catalytic cavity that bear a strong negative electrostatic potential (usually attributed to the existence of the negatively charged Asp189 residue of thrombin). These interactions are critical for burying the cleavage site within the thrombin catalytic cavity close to the nucleophilic Ser195 residue of thrombin. The R1708 residue is structurally resolved only in the PDB file 2R7E in which it is completely exposed on the surface of the protein which further aids in its interaction and insertion into the thrombin active site cleft. The mutation p.R1708H does not show a remarkable change in antigenic stability as observed for some of the other mutations, but it does show a functional loss in activity indicating that, while this mutation does not change the overall fold of the protein, it directly interferes with the catalytic efficiency of the protein (Fig. 6b). Although the mutation is to a histidine, the presence of

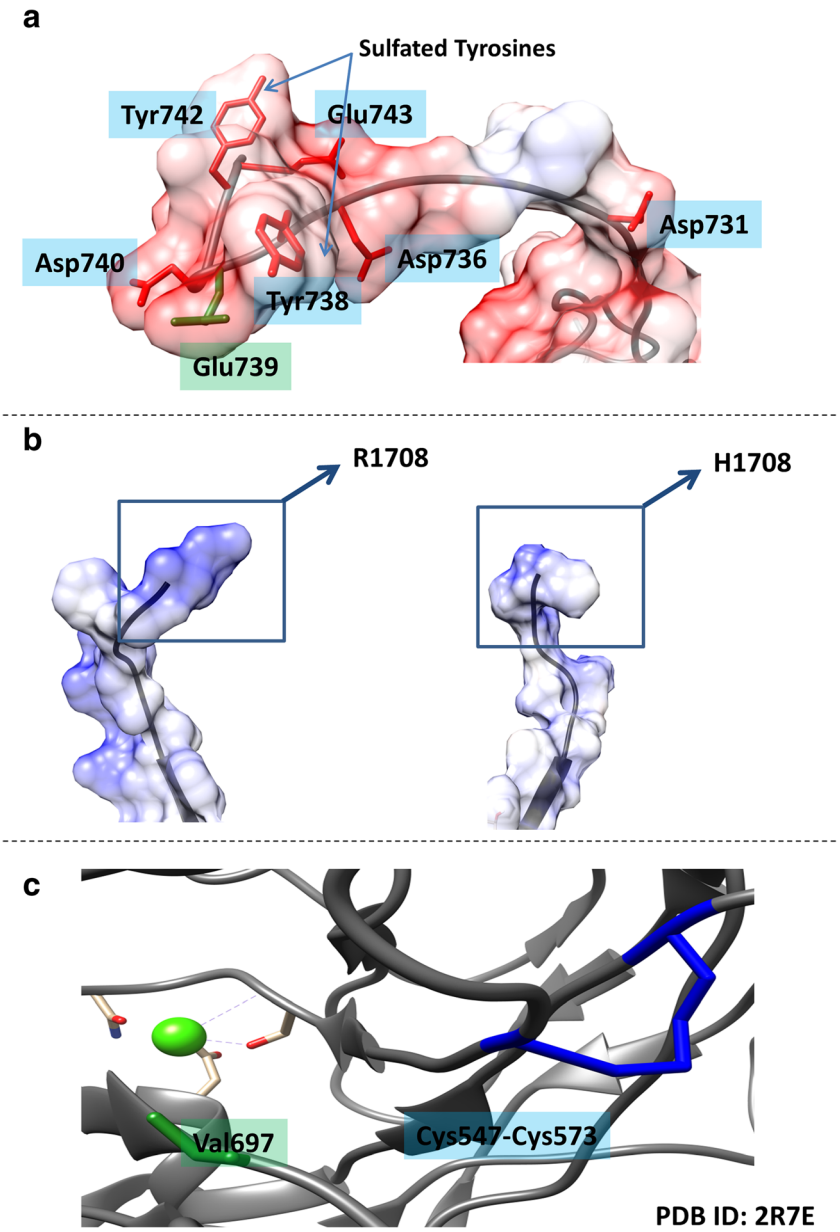

Fig. 6 Close-up molecular views of the A2 and A3 domain acidic region residues on which mutations have been reported. The figure is split in three panels. In all panels, protein backbone is depicted in grey coloured ribbon format. **a**, **b** The electrostatic surfaces of the two regions are depicted and coloured based on columbic charges (blue: positive and red: negative). **a** The residue on which the mutation has been reported is depicted in green stick format while other proximal negatively charged residues of interests are depicted in red-coloured stick models. **c** The residue on which the mutation have been reported is depicted in green stick format and a proximal disulphide bond also depicted in stick format with the participating cysteine blue-coloured

positive charge on this residue will depend on the protonation state of the molecule [25].

The mutation p.V697L occurs close to the disulphide bond between residues Cys547 and Cys573. The substitution here is with a residue that is very similar to the wild-type amino acid in almost all respects except for a bulkier side chain. Both the mutated residue and the proximal disulphide bond reside on disordered regions. FVIII has a total of eight disulphide bonds, many of which occur on disordered regions and as a result of bonded constraints lend stability to them. Earlier studies found that seven out of these eight disulphide bonds were critical to the structure of FVIII [25]. The Cys547–Cys573 disulphide bond is one of the indispensable disulphides within FVIII. The bulky nature of the substituted leucine residue might disturb the disulphide configuration next to it thereby disturbing the overall fold of the domain and protein (Fig. 6c).

Discussion

The diagnosis of HA is based on the assessment of FVIII levels. The most commonly used methods for measurement of FVIII:C are the one-stage clotting assay and the two-stage chromogenic assay. Choosing which assay to use arises concern in two phases of routine HA care, during the initial diagnosis, and while monitoring the treatment. This is particularly critical when a discrepancy is observed between the two FVIII

measurement methods. Both structural modifications in the recombinant FVIII products (i.e. PEGylation, Fc fusion proteins, B domain deleted products) and missense mutations in the *F8* gene could lead to assay discrepancy. Such missense mutations are reported in about 30% of non-severe HA patients. Recently, the assay discrepancy was also reported in haemophilia B [26] which underlies the potential impact of protein modifications caused by mutations or modification of recombinant protein on the assay results. To decide which method gives the clinically relevant FVIII:C levels is a serious challenge. In particular, it is not apparent which assay best reflects the in vivo FVIII cofactor ability. In this study, we analysed the impact of 13 mutations on quantity and quality of the expressed FVIII protein. Moreover, the structural impact of amino acid exchanges on FVIII protein was investigated based on an in silico modelling.

For the mutations in group 1, where the one-stage assay is higher than the two-stage assay, the assay discrepancy was only detected in three mutations: p.T294I C, p.R550H and p.1844S. Interestingly, also for two mutations, p.R550H and p.V682A, we observed assay discrepancy between the two clotting assays. These mutations were also showing significant differences in parameters of TGT reflecting different phases of the coagulation cascade (Fig. 1). The mutated residues Thr294 and Arg550 lie on the A1 and A2 domain interface. A number of ion-pair interactions between the two domains define this interface as per a computational study done on non-activated and activated models of B domain deleted FVIII. Among these mutated residues, none participates directly in these inter-domain ion pairs; however, they are proximal to some of these ion-pair residues and their own inter-residue interactions contribute to the networking and stabilization of the other ion pairs (i.e. Arg550:Asp538 ion-pair and Arg546:Tyr574 backbone hydrogen bonds indirectly stabilize the inter-domain interaction between Arg304:Asp544 while the Thr294 is critical for the stabilization of the Arg503:Glu291 inter-domain ion pair). All, irrespective of their specific molecular mechanism, results in a misfolded unstable molecule [27]. In particular, the mutation p.R550H has been shown to reduce the stability of FVIII and FVIIIa due to an increased rate of dissociation of A1/A2 subunits as well as an accelerated spontaneous inactivation prompted by inter-domain affinity alterations [11, 28, 29]. A computational study has shown that the activation of FVIII leads to diminished interaction between A2:A3 domains which might account for the A2 domain dissociation upon activation and its reduced half-life [19]. Mutations of Val682 and Pro1844 could exaggerate the effect of dissociation by weakening the proximal interface interactions. Many studies have associated the variants located in the interface of A1, A2 and A3 domains, with instability of FVIIIa [16, 30, 31]. The low peak thrombin and increased TTP values for p.V682A speaks in favour of affected stability of only FVIIIa and not the heterodimer FVIII. Accordingly, higher incubation time after initiation of the coagulation in chromogenic assay could explain lower activity results.

In the group 2 mutations, consistent with the previous reports, discrepancy was seen for both mutations of group 2-I. Interestingly, for both mutations, according to ETP and peak height, TGT was in line with the results obtained with chromogenic assay and only the lag time and TTP was extended. The extended lag time could be due to the localization of these residues in the vicinity (R759) and at the thrombin cleavage site (R1708) which is masked in the chromogenic assay due to the high thrombin concentration, and the long incubation time reflects the effect assessed by the clotting assay [17]. Moreover, the pKa assigned to the mutated H1708 residue is lower than 7.4 (pH of blood). This suggests that for this histidine residue, most likely the deprotonated state dominates, which then would not be able to interact and stabilize the cleavage site in the thrombin active site cleft as effectively as an arginine or even a lysine would. This mutation was the only mutation showing discrepancy between the results based on all three methods, and even TGT analysis revealed significant differences to the $FVIII_{WT}$.

The S2138 residue was the only discrepant mutation of the group 2-II. Here, TGT was not showing any differences to $FVIII_{WT}$. This residue is neighbouring the N2137 residue, one of the five Asn glycosylation sites in FVIII (N60 and N258 in A1 domain, N601 in A2, N1829 in A3 domain, and the N2137 in C1 domain). The aromatic stacking interactions arising from the substitution on the neighbouring S2138 could serve to bury the N2137 deeper into the A3:C1 interface cleft making the glycosylation of this residue difficult and most likely affecting the folding of this variant. Moreover, the C domains have limited stabilizing interactions with A domains which involves the S2138 residue (Ala1885:Ser2138) [32]. From this group, the p.Y1669F was showing an extended lag time in TGT, which is also close to the R1708 cleavage site. This residue is currently unresolved in all structures so far modelled or crystallized for FVIII. The p.R546W mutation showed discrepant results in assay assessment, and also TGT parameters were severely affected. The peak thrombin was the lowest among all variants of group 2. Taking to the account that the two latter mutations are the most recurrent mutations in non-severe patients in our centre [33] highlights once more the importance of the selection of the assay for the first diagnosis of HA. Among the mutations of the fourth category (2-IV), expression of only p.V697L mutation led to measurable activity levels. This mutation is localized in the A2 domain of FVIII protein. Similar to the patient data, higher $FVIII:C_{chr}$ values were observed in comparison to both one-stage assays suggesting a higher rate of A2 subunit dissociation in the chromogenic assay after activation by thrombin.

Interestingly, our analysis revealed discrepancy between the two one-stage clotting assays for mutations clustered in

Table 2 Differences between components and test procedures. DP: deficient plasma, ?: concentration unknown, 1: estimated value as the contact activated serum used in the in-house method is also a source of FIX, 2: as the aPTT reagent used for the test is suitable for lupus antibody detection, the concentration is assumed to be low. 3: The time which it takes from the moment the test sample is placed in the device for initial steps of the assay, until the start of the incubation time step before recalcifying

	Assay	FVIII:$_{1st}$	FVIII:$_{1st/Bonn}$	FVIII:$_{chr}$	TGT
Concentration	FIX	0.63–2.50 μg/ml	0.45–1.82 μg/ml (0.91–3.64 μg/ml)$_1$	5.5 μg/ml	0.83–3.33 μg/ml
	Phospholipid	Low^2	?	20 μM	2.7 μM
	Ca^{2+}	6.25 mM	5.5 mM	10 mM	?
	VWF activity in DP	43	107.6	0	–
Incubation time	Incubation time before recalcifying (preparation time)^3	185 s (2 min)	240 s (5–10 min)	0 (2 min)	0 (5–10 min)
	Incubation time after recalcifying (measurement time)	0 (up to 60 s)	0 (up to 60 s)	90 s (60 s)	0 (60 min)
Dilution factor	DP dilution in total volume	4×	5.5×	No DP	3×
	Sample dilution in total volume	40×	11×	120×	3×
Volume	DP per microliter of sample	10×	2×	No DP	1×
	Sample volume (total assay volume)	5 μl (200 μl)	50 μl (550 μl)	1.25 μl (150 μl)	40 μl (120 μl)

both groups. This could be explained by differences in the assay components and the test procedure as listed in the Table 2. This result emphasizes that the assay discrepancy is not just a matter of assay stages but also concentration of other clotting factors, the incubation lengths and the initial dilution might affect the results. The number of laboratories in the field of haemostasis that use the chromogenic assay is far too low in comparison with those using the one-stage assay (6.1% vs. 90.2% [34]). Noteworthy, in this study, more than 16 different aPTT reagents were used for the one-stage assay and 4 different chromogenic assays in different laboratories were applied, bringing even more possible discrepancy for FVIII:C activity.

The discrepancy between FVIII:C results of the one-stage/two-stage assay in patients bearing specific point mutations in mild/moderate phenotypes has crucial influence on diagnosis of HA. The risk of misdiagnosis is especially greater for mutations that displays normal activity if measured only by a single assay, since the lower activity correlating with their actual phenotype can be overlooked. Precise measurement of FVIII:C is also substantial for monitoring of therapy with FVIII concentrates for patients during their treatment. Moreover, accurate labelling of FVIII concentrates by manufacturers in a standardized manner is required for determination of correct dose necessary for the treatment. Therefore, assay discrepancies are significantly challenging the clinical management of all patients with HA suggesting performing both one- and two-stage assays as first line screening of patients with non-severe HA phenotype. The results of this study also underline that in the era of the new modified therapeutic FVIII products, the results obtained from the available FVIII measurement assays need to be considered with caution when comparing trough levels and pharmacokinetics by product switch in patients. Thus, a more standardized assay is needed overcoming all the variables influencing the test to correctly assess FVIII cofactor activity.

Author contributions BP, JO and MG designed the study; MG and ACB performed research; BP and MG analysed the data; AB performed in silico analysis; AP and JO revised the manuscript; and BP and MG wrote the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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