Discrepancy between one-stage and chromogenic factor VIII activity assay results can lead to misdiagnosis of haemophilia A phenotype

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Keywords
Assay discrepancy, FVIII:C, haemophilia A, factor 8 gene mutations

Summary
Severity of bleeding phenotype in hemophilia A (HA) depends on the underlying mutation in the F8 gene and, ultimately, on the concentration and functional integrity of the factor VIII (FVIII) protein in circulating plasma. Initial diagnosis for HA and monitoring of treatment is typically performed by measuring of FVIII activity by either one-stage assay or chromogenic assay.
We review evidence for why both types of assay do not give comparable results in a significant proportion of patients with non-severe haemophilia A and why the discrepancy in results between both methods segregates with distinct subclasses of known missense mutations causing haemophilia A. The current understanding of the mechanistic basis for how FVIII:C assay discrepancies arise are discussed. Conclusion: We propose that both methods should be used in initial patient diagnosis along with follow-up genetic analysis to avoid potential misdiagnosis and to optimize treatment monitoring of patients with HA phenotypes.

In the contact activation (intrinsic) pathway of coagulation factor VIII (FVIII) functions as a cofactor for FIXa in the proteolytic activation of factor X to FXa. A qualitative and quantitative deficiency of FVIII is associated with the bleeding disorder haemophilia A (HA). The accurate assessment of factor VIII activity (FVIII:C) is important for:
● diagnosis of HA,
● determining the severity of the bleeding phenotype,
● for monitoring the treatment with FVIII concentrates and
● for accurately determining concentrate potency.

A major problem in the correct assessment of the FVIII:C arise from the fact that FVIII activity is measured indirectly. As FVIII is not an enzyme but, rather a cofactor in the activation of factor X by factor IXa, all currently available factor VIII assays measure FVIII:C activity in generating factor Xa in the presence of calcium ions and phospholipids (1, 2). Concomitantly, factor Xa is estimated either directly by spectroscopic absorbance methods, such as the chromogenic assay using a chromogenic substrate, or indirectly by assessment of the formation of fibrin by thrombin (factor IIa) in an in vitro coagulation cascade.

Currently, three types of assay systems exist for the measurement of factor VIII in biological samples:
● one-stage assay (4, 5),
● classical two-stage assay (6, 7) and
● chromogenic substrate assays (8, 9).

The one-stage assay is based on the correction of the prolonged activated partial thromboplastin time (aPTT) of FVIII-deficient plasma by dilutions of FVIII from patient’s plasma. This assay uses the intrin-
sic system in which the reaction is initiated via contact activation and ends with fibrinogen clotting. The simplicity of the method, as well as the wide availability of reagents and the ease of automation, are the main reasons why this assay is widely used in clinical laboratories. Although the aPTT based one-stage assay is the most closely related system to the physiologic conditions with respect to the components concentration, it has been shown that this assay is particularly subject to variability of results and exhibits quite large coefficient of variation.

Alternatively, the two-stage assay system consists of two consecutive steps.

1. FX is activated by FVIII in the presence of of factor IXa, factor V/Va, calcium ions and phospholipids.
2. The activated FX formed is measured in the second step either in a clotting system (fibrinogen conversion to fibrin-the classical two-stage assay) or by the enzymatic cleavage of a chromogenic factor Xa substrate which is photometrically quantified (chromogenic assay).

In most cases the measurement of the FVIII:C by the different assays gives equivalent results, but there have been continuing reports of discrepancies between assay systems. Discrepancy in FVIII:C results have been described in the measurement of the FVIII potency in concentrates and in the assessment of in vivo recovery of FVIII concentrate infusions (10, 11). Several recent reports have observed discrepancies among different measurement methods as a part of a haemophilia phenotype. Haemophilia A is a heterogeneous disorder with various bleeding phenotypes reported for various underlying mutations in the F8 gene (12–16). A number of naturally occurring mutations have been described to be associated with the discrepancy phenomena. Up to 30% of the non-severe HA patients are reported to exhibit such inherited discrepancy (17). The underlying molecular mechanisms of the assay discrepancy have been associated with the differences in the mode of FVIII activation in the various assay systems. In this review, the genetic basis of the observed discrepancy in FVIII:C measured with different assays in patients with HA is discussed.

**Activation of FVIII**

The FVIII procofactor consists of 2332 amino acids and is synthesized as a single-chain polypeptide of approximately 280 kDa with multidomain structure A1-A2-B-A3-C1-C2 (18–20). In plasma, however, FVIII is predominantly present as a cleaved, two chain molecule due to proteolytic processing (20). The A1, A2 and B domains are denoted as the heavy chain, whereas A3, C1 and C2 domains form the light chain of the FVIII heterodimer. Both chains are associated by a monovalent copper ion-dependent linkage between the A1 and A3 domains (21).

In vivo, FVIII activity is regulated by proteolytic activation by thrombin. Thrombin cleaves FVIII at Arg740, Arg1689, and Arg372, generating activated FVIII (FVIIIa), consisting of the metal ion-coordinated heterodimer A1/A3-C1-C2, which is associated with the A2 domain through a weak ionic interaction (22–25). An acidic amino acid-rich region at the carboxyterminus of the A1 subunit most likely interacts with positively charged residues within the A2 subunit to retain the heterotrimeric FVIIIa configuration (26, 27). The FVIIIa heterotrimer exhibits a pH-dependent dissociation of the A2 subunit from the A1/A3-C1-C2 heterodimer that correlates with loss of procoagulant activity. Because the A2 domain easily dissociates from the heterodimer at physiological concentrations, the FVIIIa heterotrimer is a relatively labile activation product (28–30).

These observations highlight the role of A2 dissociation in limiting FVIIIa activity in vitro. However, it is not yet known whether spontaneous A2 subunit dissociation or further proteolysis limits FVIIIa procoagulant activity in vivo.

In the presence of a lipid membrane surface, FVIIIa acts as a cofactor for the enzyme FIXa in the activation of the substrate FX (31, 32). Binding sites for FIXa have been identified in the A2 domain (Ser558 to Gln565 and Asp712) and the A3 domain of FVIII (Glu1811 to Lys1818) (2, 33). Furthermore, recent data indicate that an interactive site for FX is located in the A1 domain (Arg336) of FVIII (34).

**Factor VIII:C higher in one-stage assay**

Recent reports have identified a haemophilia A phenotype in which the patient plasmas exhibit a familial discrepancy in which the FVIII procoagulant activity was at least twofold higher when measured in a one-stage assay compared with a two-stage assay (35–40). All reported patients have mild to moderate haemophilia A phenotype. A common feature of all described cases is that FVIII antigen is presented with considerably high levels, but the activity is significantly reduced, suggesting a protein dysfunction displaying reduced biological activity rather than inefficient secretion (CRM’ mutations) as the pathological mechanism.

Several genetic mutations have been described to be associated with this discrepancy phenomenon. Initially, the molecular mechanism for one of these mutations, a missense mutation Arg531 His, was characterised as resulting from an increased rate of inactivation of FVIIIa due to enhanced A2 subunit dissociation (41, 42). The data showed a loss of charge within the A2 subunit that implied a weakened electrostatic interaction after thrombin activation, manifest as a four-fold increased rate of A2 subunit dissociation. Additionally, in vitro biochemical analysis of a recombinant-derived Arg531 His mutation led to the observation that the thrombin-activated mutant protein had an increased rate of spontaneous inactivation secondary to an increased rate of A2 subunit dissociation. Structural analyses on the FVIII protein molecule demonstrated that this genetic alteration is localised on the A-domain interface. A further study of the same mutation by another group revealed a reduced amounts of heterodimeric FVIII relative to wild-type FVIII, suggesting that the mutation may also reduce the stability of the triplicated A domains even before thrombin activation. This further highlights the role of protein-protein interactions at the A-domain interfaces (43).

Other mutations associated with FVIII:C discrepancy phenomena wherein the one-stage assay results yield greater activities than the two-stage/chromogenic
assay (Ala 284 Glu, Ala 284 Pro, Ser 289 Leu, Arg 527 Trp, Arg 531 His, Arg 698 Leu, Arg 698 Trp, Leu 1932 Phe, Met 1947 Ile, Arg 527 Trp, Arg 531 His, Arg 698 Leu, Arg 698 Trp, Leu 1932 Phe, Met 1947 Ile, His 1954 Leu). (Fig. 1a) have been shown to involve amino acid substitutions that alter charge or hydrophobicity leading to interdomain instability and disruption of protein-protein interaction (36, 44, 45).

Summarising the data for all mutations, where one-stage assay shows higher results than chromogenic, there appear to be a clear clustering of all mutated residues at or very close to the interface of A1-A2, A2-A3, and A1-A3 interfaces. Apparently, these mutations are associated with reduced biological FVIII cofactor activity, as well as decreased stability of the heterodimer and abnormal behaviour in FVIII:C assays.

Insights into the differences between the one- and two-stage assays allow to hypothesise possible mechanisms. In the one-stage assay, FVIIIa is generated only in the final rapid clotting stage when Ca²⁺ ions are added. In this case, any effect of additional instability of the heterotrimer would be minimized.

In contrast, the chromogenic assay, comprising two separate enzymatic steps, requires a prolonged phase during the first step in which FVIII becomes proteolytically activated to form heterotrimeric FVIIIa structure and exert its cofactor function with factor IXa. Thus, if the molecular consequence of this group of mutations is to destabilize the activated heterotrimer, the prolonged incubation time during the first phase of chromogenic assay would favour the higher rate of A2 dissociation and, thus, would tend to decrease FVIII:C activity. The observed high dissociation rate of A2 may be further influenced by the high dilution of the patient’s plasma.

This phenomena becomes clinically significant when one-stage assay represents FVIII:C values within the normal range. In a survey from the United Kingdom 16% of the patients identified with assay discrepancy had an one-stage assay FVIII activities within the normal range, suggesting these patients could have had their hemophilia unrecognized if this was the only method used. (42, 45, 46).

A number of missense mutations including Glu 321 Lys, Thr 346 Cys, Glu 720 Lys, Arg 1639 His, Arg 1689 His, Ile 369 Thr, Phe 2127 Ser have been described to be associated with this discrepancy. All these mutations result in mild haemophilia A phenotype with detectable circulating FVIII antigen, in most cases close to the normal values. Patients with such mutations exhibit normal levels of FVIII cofactor activity when measured by the chromogenic assay method but have reduced levels of procoagulant activity when measured by one-stage assay. Different mechanisms have been proposed to contribute to this type of assay discrepancy.

The first proposed mechanism suggests an impairment of FVIII activation by thrombin. The three residue substitutions (Glu 321 Lys, Tyr 346 Cys and Ile 369 Thr) are located close to or in the interdomain region a1 (aa 337–372). The tyrosine 346 residue has been shown to be sulfated and mutation at this site leads to delayed thrombin activation at Arg 372. Glu 321 and Ile 369 also lie adjacent to this acidic region and mutations at these residues may also interfere with thrombin cleavage at Arg 372. In the one-stage FVIII:C assay, where the physiological concentrations of thrombin activate the physiological concentrations of FVIII, the time between the first appearance of catalytic amounts of thrombin and a measurable clot may be as little as 15–20 s. Under these conditions, the assay will be extremely sensitive to altered thrombin binding or cleavage of FVIII. In contrast, in the chromogenic assay, an excess amount of thrombin activates diluted FVIII in a much longer time compared to the one-stage assay. Thus any altered affinity of thrombin towards FVIII will be overcome by this high thrombin concentration and activate FVIII.

The second proposed mechanism involves binding of FVIII to FIXa. Recombinant purified FVIII bearing Glu 720 Lys substitution was found to require higher

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Hämostaseologie 4/2010
concentrations of FIXα to achieve full cofactor activity (47). Glu 720 is located within a short acidic region between the A2 and B domains and this mutation may result in reduced affinity of FVIIIa for FIXα or in reduced catalytic activity of FIXα presumably by another mechanism. If Glu 720 Lys introduces a defect in the assembly of the tenase complex through impaired binding to FIXα, then the excess of FIXα in chromogenic assay together with the longer required incubation time, could lead to activation of FVIII even when the affinity to FIXα is reduced due to the mutation.

Regarding the haemophilia phenotype, most of the described patients with normal FVIIIC as determined by chromogenic assay, and especially those bearing the Tyr 346 Cys mutation, exhibit a very mild bleeding phenotype (49, 52).

Conclusions

- Most mutations contributing to a higher one-stage FVIII:C value relative to that for the chromogenic assay, are clustered in the A1, A2, and A3 domains, in the regions at the interface between the subunits and, thereby, may exhibit an impaired FVIII II stability.
- The genetic alteration responsible for the opposite FVIII:C discrepancy phenomena, where the chromogenic assay results yield higher FVIII:C values than those of the one-stage assay, affect mainly thrombin activation and FVIII binding to FIXα in assembly of the tenase complex.
- The strong association between the phenotype and the group of mutations demonstrates a genetic basis for the discrepancy between the one-stage and chromogenic assay.

Despite the fact that both assays for measuring FVIII:C have been available for many years, there is still no consensus which method measures more accurately the FVIII cofactor function. In families with discrepancy between one-stage and chromogenic FVIII:C levels, there is ongoing discussion which value reflects the clinical phenotype of the patient, especially assessing the degree of severity of HA.

In order to avoid ambiguities in correct interpretation of results from any single assay method or possibly entirely failing to diagnose a mild HA patient, we recommend the use of both one-stage and chromogenic FVIII:C assays for the initial diagnosis of patients with bleeding complications that may suggest haemophilia.

The molecular genetic analyses of the underlying defect will further assure the diagnosis and lead to better monitoring of ongoing patient therapy.

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