CAT
Critically Appraised Topic

Detection of FVIII and FIX inhibitors using Thrombin Generation Assay: a new diagnostic approach

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Date: 12/03/2013

CLINICAL BOTTOM LINE

Introduction
Factors VIII/IX inhibitors are IgG auto- or allo-antibodies that bind functional epitopes and can inhibit the coagulant activity of factor VIII or IX. The Bethesda-Nijmegen assay is commonly used for detection of inhibitors, but is labour intensive and has a poor sensitivity for low titer inhibitors. Thrombin Generation Assay (TGA) is being revived as an overall functional assay of plasmatic hemostasis and allows measuring the influence of inhibitors on global thrombin generation. In our study, the use of the TGA for detection of factor VIII and IX inhibitor antibodies was evaluated.

Material and methods
The study was performed using plasma samples from 40 healthy volunteers and from 45 hemophilia (41 type A and 4 type B) patients. Of these hemophilia samples, eight were diagnosed FVIII inhibitor positive and one FIX inhibitor positive using the Bethesda-Nijmegen method. One patient with acquired hemophilia A showed a residual FVIII activity of 79% on Bethesda-Nijmegen assay with suspected presence of an inhibitor. Plasma samples were heated at 56°C to inactivate all clotting factors, whereas IgG antibodies are heat resistant leaving the potential inhibitor levels unchanged. Heat inactivated plasma samples were mixed in equal parts with commercial pooled plasma (CryoCheck™). TGA was performed on those samples and results were expressed in endogenous thrombin potential (ETP) ratio. This ratio was obtained by dividing ETP results of clinical sample mixtures with ETP results of reference plasma. This reference sample was composed by mixing FVIII deficient control plasma and normal pooled plasma in a 1:1 dilution.

Results
ETP ratio values were between 0.63 and 0.92 (mean 0.76±0.07) for healthy volunteers with normal hemostasis (n=40) and between 0.52 and 1.06 (mean 0.77±0.09) for hemophilia patients without inhibitors (n=35). ETP ratio values were between 0.13 and 0.78 (mean 0.36±0.14) for the hemophilia patients with presence of an inhibitor (n=10) detected using the Bethesda-Nijmegen assay. The patient with acquired hemophilia A exhibited an ETP ratio of 0.42.

Conclusion:
Our preliminary results demonstrate the use of TGA for detection of factor VIII and IX inhibitors in mixing experiments. TGA is also able to detect weak inhibitors or an inhibitory effect of antibodies with complex kinetics. Complementary studies should be performed to evaluate the influence of different variables such as pH.
Factor VIII (FVIII) and factor IX (FIX) inhibitors are IgG antibodies that bind functional epitopes on the factor molecule and inhibit the functionality of the protein. These inhibitors can be of autologous or allogenic origin.

- **Autologous inhibitors:**

Autologous FVIII inhibitors have been described in individuals with previously normal haemostasis and no prior history of a bleeding disorder^1^. They may present clinically with unexpected moderate to catastrophic bleeding episodes by deregulation of the immune system. Acquired hemophilia has an estimated prevalence of 1.48 cases per million per year, and a reported mortality between 9% and 22%. Age at onset is distributed in a biphasic pattern, with a small peak in young individuals and a major peak in those aged 60-80 years. Autologous FVIII inhibitors in elderly people are frequently associated with an autoimmune disease or a malignancy, but can also arise without an underlying associated disease.

- **Allogenic inhibitors:**

Hemophilia A is caused by an inherited deficiency in FVIII, requiring replacement therapy with the deficient factor. Allogenic FVIII inhibitors may develop in hemophilia A patients after treatment with FVIII concentrates^2_. The prevalence of these inhibitors has been estimated to be between 3 and 13%. Patients with mild/moderate hemophilia are at lower risk of inhibitor development than severely affected patients. The development of FVIII usually occurs shortly after replacement therapy has been initiated (within < 30 exposure days) and is most frequent in young hemophils. An explanation for increased risk at early age is that the immature immune system is more susceptible than the mature system, but these findings have not been confirmed^1_. These hemophilic patients may be clinically recognized by excessive bleeding, frequently in unusual parts of the body. This results in a strong need for FVIII supply due to a low recovery and half-life of infused FVIII products. The reason why only a fraction of the patients experience this side-effect is not known.

- **Kinetics:**

The mechanism of inhibition of FVIII activity by autoantibodies versus alloantibodies is quite dissimilar^3_. Allo-antibodies in FVIII-treated hemophils, result typically in a complete neutralization of FVIII activity, following a so-called linear type I kinetics pattern. In contrast, most auto-antibodies are unable to completely inhibit FVIII activity and interact with FVIII via a nonlinear, complex pattern of type II kinetics. Because of this linear nonsaturable reaction process, patients with auto-antibodies may have catastrophic bleeding episodes as if they had no FVIII activity, although their residual low levels of FVIII activity may be detectable in laboratory assays.

![Figure 1. Kinetics of type 1 and type 2 inhibitors against factor VIII.](image)
- Laboratory tests:

The most common laboratory finding is a prolongation of the activated partial prothrombin time (aPTT) with a normal prothrombin time (PT), thrombin time (TT) and platelet count. The Bethesda and the Nijmegen assays are commonly used for the measurement of inhibitor levels in suspected patients. These recording systems measure the inactivation of FVIII molecules in mixtures of test plasma and exogenous FVIII, e.g. normal pooled plasma. In practice, several different dilutions of patient’s plasma are added to a normal plasma sample and incubated for 2h at 37°C, at the end residual content of FVIII:C is measured for all samples. A positive result is noted when a sample causes a significant loss of FVIII:C of normal plasma at 50% of the content of control plasma. In the Nijmegen modification, the sensitivity and specificity of the assay were further improved by substituting FVIII deficient plasma for a buffer in the control mixture, and the normal plasma was also buffered. This method was shown to be more reliable at low concentrations.

Factor VIII inhibitor residual activity is expressed by Bethesda Units. The “Bethesda Unit” of FVIII inhibitory activity was defined as the amount of inhibitor that gives 50% reduction of FVIII. When the residual FVIII activity of undiluted sample is below 25%, retesting of more diluted samples is recommended because of non-linearity of inhibitor concentration and residual FVIII activity with high inhibitor titres. Dilutions have to be made with FVIII-deficient plasma.

The inter-laboratory coefficient of variation in external quality surveys is high (40% of more), with a high number of false-positive and false-negative results, probably caused by a lack of standardization of the assay. However, the early detection of FVIII inhibitors is very important for the safe and accurate treatment of patients. The earlier an inhibitor is detected; the better may be the outcome of the therapy. Although the Nijmegen Assay shows improved specificity and intra-and interlaboratory variation, it is still associated with several limitations related to assay characteristics (pH, temperature, and time of incubation), nature of control sample, and the von Willebrand content of the assay mixture. Epitope specificity plays an important role in the reliability of functional assays because inhibitors against the C2 domain are more difficult to quantify compared with inhibitors against the A2 domain. Finally, interfering factors for the FVIII assay (lupus anticoagulants, heparine) can influence inhibitor assays, resulting in aberrant results. Further investigations are required to develop more sensitive and specific methods. The aim of our study is to develop a new diagnostic tool for detection and titration of FVIII inhibitors by use of the thrombin generation assay.

SEARCH TERMS

5) UpToDate Online version 12.2 (2004)
1. Thrombin generation assay

Clotting times (PT, aPTT, activated whole blood clotting time) do not indicate increased coagulability and are insensitive to mild bleeding disorders (e.g. carriers of hemophilia, mild liver disease). The clotting time is not always a good indicator of the amount of thrombin that is formed. The discrepancy between clotting time and thrombin generation can be explained by the fact that the mechanism responsible for the clotting time is different from the mechanism responsible for bulk thrombin formation. The clotting time is the lag-time of thrombin formation. During the lag time of thrombin formation feedback activation plays a limited role. The full thrombin generation mechanism only gets started when there is enough thrombin formation. The mechanism of thrombin formation during the lag-time therefore is only a subset of the complete mechanism and not necessarily representative of the whole.

For over a century, the generation of thrombin in clotting blood or plasma has been used to assess the coagulation system, but only recently have technical developments brought it into reach of the non-specialized laboratory. That it never has been introduced as a routine test to probe haemostatic function in the clinics, is due to the fact that with the conventional (subsampling) techniques, one curve requires about one man-hour of skilled laboratory work. The complete thrombogram is traditionally obtained by subsampling from clotting blood or plasma at timed intervals and measuring the concentration of thrombin in each sample. It became obvious that automated techniques were needed if measurement of thrombin generation were to be adapted in clinical use. Hence, Hemker et al. invented calibrated automated thrombin (CAT) generation method based on fluorescence measurements.

1.1. Properties of a thrombogram

The thrombin generation assay demonstrates the concentration of thrombin in platelet rich plasma (PRP) or platelet poor plasma (PPP) by monitoring the splitting of a fluorogenic substrate and comparing it to a constant known thrombin activity in a parallel, non-clotting sample. The use of a slow-reacting fluorogenic substrate makes it possible to measure thrombin generation in fibrinogen-containing media in a continuous manner which enables to use PRP and to test the influence of platelets on thrombin generation.

Figure 2: The parameters of the thrombogram

There are different parameters of TG, which characterize the TG curves.
- Lag phase: information about the start of the thrombin formation. Fibrin appears as soon as the first traces of thrombin form and the end of the lag time is the moment at which the clotting occurs.
- Peak thrombin: highest thrombin concentration reached during the time course of thrombin formation and inhibition.
- Endogenous thrombin potential (ETP): the amount of thrombin that is formed altogether within a certain period, e.g. 60 min (area under the TG curve)
- Peak time: the velocity of the progress of thrombin formation
- Start tail: time at which thrombin generation has come to an end. This value is less absolute than the other parameters, sometimes it is hard to tell where thrombin generation really stops. Especially in PRP of under conditions of strong inhibition.

The exact calculation of the thrombin potential makes necessary the correction for the amidolytic activity of α2-macroglubulin-thrombin complex for substrate consumption and for the inner filter effect. The calculation of the thrombin potential is, therefore, more complicated than that of the peak thrombin (maximum amidolytic activity), lag phase and peak time.

The thrombogram is a broad function test of the haemostatic-thrombotic mechanism of the blood\(^{14}\). The thrombogram in platelet poor plasma (PPP) measures:

- Hypocoagulability:
  - Hemophilias and efficacy of Factor Eight Inhibitor Bypassing Activity (FEIBA\(^{TM}\))\(^{15,16}\)
  - Oral anticoagulants
  - Heparins (-likes)
  - Direct thrombin inhibitors

- Hypercoagulability:
  - AT deficiency
  - Prothrombin hyperexpression
  - Protein C and S deficiency
  - Factor V Leiden
  - Oral contraceptives

In platelet rich plasma (PRP) the thrombogram is diminished in:

- Congenital and acquired platelet related disorders (e.g. Glanzmann’s thrombosthenia, von Willebrand disease)
- By antibodies blocking GPIIb-IIIa or GPIb
- By antiplatelet drugs (aspirin and clopidogrel)

**1.2. The chromogenic and fluorescent measurement**

Continuous monitoring of thrombin concentration has been attempted through adding a suitable thrombin to the clotting sample and monitoring the time course of appearance of the amidolytic split product. The use of chromogenic substrates implies removal of fibrinogen and blood platelets, although they are essential components of the clotting system that influence the course of thrombin formation. Since the use of a substrate that yields a fluorescent product, is not heavily influenced by the turbidity of the emerging clot, fibrinogen-containing plasma and PRP can be used. Also the presence of platelets in coagulation does not disturb the measurement. This makes it possible to study the role of platelets in coagulation in large series of samples\(^{17}\).

Using fluorogenic substrates raises the problem how to relate the velocity of fluorescence change to thrombin concentration\(^{18}\). Constant thrombin activity does not cause a constant increase in the output signal, and thus the output signal (OD, fluorescence) produced does not directly reflect the amount of thrombin present\(^{14}\).

The two phenomena that cause this non-linearity:
1. **Substrate depletion**  
The reaction is dependent upon substrate concentration which decreases in time, because of the enzyme activity itself. This means that towards the end of the measured curves, the amount of thrombin would be greatly underestimated if no correction would take place. This correction of amount of thrombin can amount up to 40%. The effect can be diminished by adding an excess of substrate but to a certain limit only. The more substrate is present, the more thrombin molecules are occupied by the substrate and the less are they available for inactivation by natural antithrombins. In a method meant to assess the area under the curve from the total amount of substrate converted, extra antithrombin needs to be added in order to make the experiment practically possible.

2. **Inner filter effect**  
In fluorescence measurements, the concentration of fluorophore (amino-methyl-coumarin, AMC) is not linear with the amount of signal, because fluorescent molecules absorb the light from other product molecules. This effect extends by increasing substrate concentrations, as required for limiting the effect of substrate consumption.

In practice, the effect of substrate consumption cannot be overcome by adding the substrate in excess. As a solution, the activity of a fixed known amount of calibrator was measured in a parallel non-clotting sample and during the whole time course of the experiment. This calibrator is measured in the software and compared to the measurement of fluorescence in another well containing the same plasma.

### 1.3. Calibration

The need for a calibrator

1. **Donor-to-donor variability**  
   This variability is due to two separate effects, the first one is the actual difference in their thrombin generation, and the other one is inherent to fluorescence measurement. The fluorogenic substrate is converted into a certain amount of AMC, which is the fluorophore that produces the signal. When the same amount of AMC is added to different plasmas the amount of signal is not necessarily the same. This is due to the variability in the light absorption characteristics of the plasmas.

2. **Variability caused by the instrument**  
   Another variability caused by measuring the different machines, using different brands of 96-well plates and ageing of the filters and/or lamp. This causes a change of signal in time or a change after replacement of the halogen lamp. Usage of the Thrombin Calibrator and frequently setting the default calibrator fixes the problem.

3. **Inner filter effect and substrate depletion**  
   The relation between the amount of signal and the concentration of the fluorophore is not a straight line. The measured fluorescence is not exactly proportional to the amount of thrombin, thigh concentrations are underestimated. Also, during the course of thrombin formation the concentration of fluorescent substrate goes down, this also causes underestimation at high thrombin concentrations.

The calibration “constant” is not in reality a constant but shows variation in the magnitude of the fluorescent signal (be it not linearly). At every level of fluorescence, a unique calibration constant gives the relation between the velocity of signal increase and thrombin concentration. As the calibrator consumes the substrate, it increases the fluorescence level and thus the Kc can be measured over a range of fluorescence levels. As a calibrator, the α2microglobulin-thrombin complex is used, that splits the signal substrate like thrombin does but that is not affected by natural plasma inactivators.
Thrombin disappears from plasma by two pathways. The bulk of thrombin is captured by anti-thrombin (AT) which disables the active site of thrombin. Another part of the thrombin binds to alpha2-macroglobulin (a2M). This a2M-thrombin complex has no physiological function anymore but is still able to convert the small fluorogenic substrate. This means that during the reaction, the substrate conversion will continue although all thrombin went back to zero. Therefore the curve is analyzed by the program to see where thrombin generation has stopped. Over the whole length of the curve the a2M-thrombin activity is then mathematically removed. The Thrombin Calibrator contains a compound that is able to give a calibrated amount of amidolytic activity when added to plasma. When thrombin generation is measured of a certain plasma in one well, the thrombin calibrator should be added to the same plasma in another well. From the information provided by this calibrator curve a correction can be made for the problems described above. The calibrator is added to another set of wells than the wells in which thrombin generation takes place.

1.4. Normal values and variability

The variation measured in identical parallel experiments in one 96-well plate is low (3-5%). The intraindividual coefficient of variation (CV) was measured by making thrombograms of 4 healthy subjects once in 9 consecutive weeks. The CV of the endogenous thrombin potential (ETP) for each individual was around 8%. The interindividual variability determined based on the means of the 9 individuals experiments was 17.5%. The variation in the population is much higher than the day-to-day variation per individual (table 1).

Table 1: Normal values and variability

<table>
<thead>
<tr>
<th></th>
<th>Average (n = 36)</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>intra-individual</td>
</tr>
<tr>
<td>PPP</td>
<td>ETP, nM·min</td>
<td>1,879</td>
<td>284</td>
</tr>
<tr>
<td></td>
<td>Peak, nM</td>
<td>458</td>
<td>60</td>
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<tr>
<td></td>
<td>Time lag, min</td>
<td>3.1</td>
<td>1.4</td>
</tr>
<tr>
<td>PRP</td>
<td>ETP, nM·min</td>
<td>1,648</td>
<td>315</td>
</tr>
<tr>
<td></td>
<td>Peak, nM</td>
<td>118</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>Time lag, min</td>
<td>8.1</td>
<td>1.9</td>
</tr>
</tbody>
</table>

The intra-individual day-to-day variation was measured on 9 different days in 4 individuals. Experimental CV was determined on 36 identical samples measured simultaneously.

2. Material and Methods
2.1. **Within-run and between-run imprecision**

Within-run and between-run imprecision were determined using commercial normal pooled plasma (Cryocheck™).

2.2. **Assay principle**

All FVIII inhibitor assays are based on a universal method of measuring the effect of a decrease of clotting factor activity in a mixture of normal pooled plasma (CryoCheck™) and the inhibitor plasma. A reference test value was performed by mixing FVIII deficient control plasma (Pool HI) and normal pooled plasma in a 1:1 dilution

- Patient plasma is heated at 56°C for 20 minutes to inactivate all clotting factors
- To remove the debris caused by the heating process, the samples have to be centrifugated at 4500 rpm for 10 min
- Pool HI (FVIII deficient plasma) heated at 56°C for 1 hour.
- Reference mixture: 40µL CryoCheck + 40µL Pool HI

Heating the test and control plasma at 56°C will completely inactivate all clotting factors, whereas immunoglobulins are heat-resistant leaving the inhibitor levels unchanged. For all patients, endogenous thrombin potential (ETP) was measured in a 1:1 mixture of inactivated patient plasma and normal pooled plasma. Results were expressed in ETP Ratio (ETP 1:1 mix/ETP reference value). Afterwards, different dilutions (1/2, 1/5, 1/10) of the inactivated plasma with deficient control plasma were made. ETP Ratio was determined for all different dilutions in a 1:1 mixture by the normal pooled plasma

In a 96-well round bottom plate, 20 µL of a reaction mixture (PPP Reagent Low) is added to one well and 20 µL of Thrombin calibrator, i.e. 785 nM alpha2M-thrombin complex to another; 80 µL of platelet poor plasma is added to both wells. All experiments were carried out in duplicate. Ca++ is added together with the substrate at zero time and readings are done in a microtiter plate fluorometer (Thermolabsystems Oy, Helsinki, Finland) at 37°C. When the experiment is started, the instrument dispenses 20 µL of FluCa solution in each well at zero time. Fluorescence intensity was detected at wavelengths of 390/460 nm (excitation/emission filter). A dedicated software program enabled the calculation of thrombin activity and displayed thrombin activity with time.

2.3. **Influence of pH**

FVIII stability in normal pooled plasma is compromised by pH shift and reduced protein concentration resulting from dilution. This may lead to FVIII inactivation and falsely positive Bethesda titers. The pH-level has also an influence on inhibitor kinetics. This problem has been improved by the Nijmegen modification of the Bethesda assay. The Nijmegen modification increased the specificity by buffering the normal pooled plasma, used in patient and reference mixtures, to pH 7,4 with imidazole buffer and using FVIII-deficient plasma in the reference mixture and for patient dilutions. Both adjustments serve to maintain the pH of the reaction mixtures for the 2h incubation period and thereby stabilize FVIII in the normal pooled plasma. In our experiments using thrombin generation assay, imidazole buffered pooled plasma was used in both test and control mixtures. Heated test plasma as well as factor-deficient control plasma are mixed with equal volumes of imidazole buffered normal pooled plasma pH 7,4.

2.4. **Patients**

Forty-five hemophilia patients (41 hemophilia A and 4 hemophilia B) with FVIII:C between <1 and 28 IU/dL were included in our study. Of these samples, 9 FVIII inhibitor positive samples and one FIX inhibitor positive sample were diagnosed by Bethesda-Nijmegen modified method for evaluation of the inhibitor activity. One patient with acquired hemophilia A showed a residual FVIII activity of 79%
on Bethesda-Nijmegen assay with suspected presence of an inhibitor. TGA was also performed in 40 persons with normal haemostasis.

3. Results

3.1. Within-run and between run

Within-run CVs ranged from 3,2% - 5,9% for ETP (table2). Between-run CVs for ETP were between 10 and 13% (table 3).

<table>
<thead>
<tr>
<th>Table 2: Within-run imprecision</th>
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</thead>
<tbody>
<tr>
<td>Sample</td>
</tr>
<tr>
<td>Cryocheck</td>
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<tr>
<td>Cryocheck</td>
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<tr>
<td>Cryocheck</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 3: Between-run imprecision</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
</tr>
<tr>
<td>Cryocheck</td>
</tr>
<tr>
<td>Reference sample</td>
</tr>
</tbody>
</table>

3.2 Patient results

To find the variation of ETP values in healthy individuals, we performed a TGA with blood from 40 healthy volunteers with normal hemostasis (aPTT, PT, fibrinogen and thrombin time). A variation of ETP ratio values in persons with normal hemostasis is seen. Thus, some individuals generate more thrombin than others. The interindividual coefficient of variation was 12% for ETP and 9% for ETP ratio. Two earlier studies showed a similar interindividual variation in plasma ETP: 17.5% and 23%.

ETP ratio values were between 0,63 and 0,92 (mean 0.76±0.07) for persons with normal hemostasis (n=40) and between 0,52 and 1,06 (mean 0.77±0.09) for hemophilia patients without inhibitors (n=35).

<table>
<thead>
<tr>
<th>Table 4. ETP values, ETP ratio and coefficient of variation of healthy volunteers (n=40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test</td>
</tr>
<tr>
<td>ETP</td>
</tr>
<tr>
<td>ETP ratio</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 5. ETP values, ETP ratio and coefficient of variation of hemophilia patients without inhibitor (n=35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test</td>
</tr>
<tr>
<td>ETP</td>
</tr>
<tr>
<td>ETP ratio</td>
</tr>
</tbody>
</table>
ETP ratio values were between 0.13 and 0.78 (mean 0.36±0.14) for the hemophilia patients with presence of an inhibitor (n=10) detected using the Bethesda-Nijmegen assay. The patient with acquired hemophilia A exhibited an ETP ratio of 0.42.

Table 6. ETP values, ETP ratio and coefficient of variation of hemophilia patients with inhibitor (n=10)

<table>
<thead>
<tr>
<th>Test</th>
<th>Mean</th>
<th>Range</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETP</td>
<td>250</td>
<td>95-531</td>
<td>54%</td>
</tr>
<tr>
<td>ETP ratio</td>
<td>0.36</td>
<td>0.13-0.78</td>
<td>55%</td>
</tr>
</tbody>
</table>

Mean ETP ratio values and ETP ratio ranges of the 3 different study groups (patients with normal hemostasis, hemophilia patients without and with inhibitors) are shown in table 7. The mean ETP ratio (0.36) of the hemophilia patients is lower than the mean ETP ratio of the two other groups (0.77 and 0.82 respectively).

Table 7. Comparison of mean ETP ratio and Range ETP ratio between different study groups

<table>
<thead>
<tr>
<th>Study group</th>
<th>No</th>
<th>Mean ETP Ratio</th>
<th>Range ETP ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemophilia with inhibitors</td>
<td>10</td>
<td>0.36</td>
<td>0.13-0.77</td>
</tr>
<tr>
<td>Hemophilia without inhibitors</td>
<td>35</td>
<td>0.77</td>
<td>0.52-0.97</td>
</tr>
<tr>
<td>Normal hemostasis</td>
<td>40</td>
<td>0.76</td>
<td>0.63-0.92</td>
</tr>
</tbody>
</table>

Figure 3 shows the distribution of test results in the 3 the different study groups. The ETP ratio of healthy volunteers with normal hemostasis ranges between 0.60 and 1. When we determine the cut-off between presence or absence of inhibitors at 0.60, two outliers can be found. One in the group of hemophilia patients without inhibitors (ETP ratio value of 0.52) and one in the group of hemophilia patients (ETP ratio of 0.77). Figure 4 shows the kinetic of thrombin generation in different study groups (hemophilia A with inhibitor, hemophilia B with inhibitor, hemophilia A without inhibitor and persons with normal hemostasis).

Figure 3. Distribution of ETP ratio values in the 3 different study groups
Figure 4. Kinetics of thrombin generation in different patients

4. Lupus anticoagulants

Presence of lupus anticoagulants (LA) may give false-positive results using the Nijmegen modification of the Bethesda assay. LA are a heterogenous group of immunoglobulins against negatively charged phospholipids. LA may be associated with thrombosis and fetal loss. LA prolongs phospholipid-dependent clotting tests by interfering with phospholipid clotting factor binding. LA can mimic the effect of FVIII inhibitors in the inhibitor assay, resulting in false-positive results. Differentiation between LA and anti-FVIII/FIX neutralizing inhibitors may be relevant because of clinical and therapeutic implications. FVIII inhibitors are associated with bleeding problems, whereas LA is associated with an increased risk of thrombosis. Unfortunately, few tests are available that can discriminate between the two types of antibodies. The dilute Russell viper venom test has a high specificity for LA detection because it bypasses the FVIII pathway. Interference of LA with the FVIII inhibitor assay can be overcome by analyzing FVIII with chromogenic substrates because these tests are not influenced by LA.

In order to evaluate the interference between LA and our TGA method for detection of FVIII and FIX, eleven patients with positive lupus anticoagulants were tested for the presence of inhibitor by thrombin generation results. ETP ratio results for these 11 patients were between 0.64 and 0.97 (figure 5). In these preliminary results, no interference effects of LA were seen. Further testing is required to confirm these results.
4. Conclusion

The TGA is increasingly being used as a diagnostic tool in the field of thrombosis and hemostasis. It is well recognized that the TGA in clotting plasma is a better determinant of the overall function of hemostasis than clotting-time-based assays, e.g. PT and aPTT. These preliminary results suggest the assay is of potential interest for detection of inhibitors in hemophilia patients.

TO DO/ACTIONS

1) Including more patients in the "lupus" population assay to rule out interference between LA and FVIII/FIX detection on thrombin generation assay
2) Additional samples of hemophilia patients with inhibitors should be tested for further validation
3) Comparing the results of inhibitor titration between Nijmegen-Bethesda method and the newly developed TGA assay
4) Studying the impact of type 2 inhibitors with complex kinetics on our TGA
**Attachment 1: Kinetic mechanisms of thrombin activation and inactivation during blood coagulation**

![Diagram of blood coagulation mechanisms]

**Attachment 2: Patient characteristics and ETP results of the hemophilia patients with inhibitors (n=10)**

<table>
<thead>
<tr>
<th>No</th>
<th>Hemophilia</th>
<th>FVIII-coagulant</th>
<th>Inhibitors</th>
<th>ETP</th>
<th>ETP REF</th>
<th>ETP Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>&lt;1%</td>
<td>305UB</td>
<td>107</td>
<td>838</td>
<td>0,13</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
<td>&lt;1%</td>
<td>8UB</td>
<td>95</td>
<td>698,5</td>
<td>0,14</td>
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<tr>
<td>3</td>
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<td>&lt;1%</td>
<td>7UB</td>
<td>128,5</td>
<td>698,5</td>
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<td>4</td>
<td>A</td>
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<td>7UB</td>
<td>188,5</td>
<td>698,5</td>
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<td>5</td>
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<td>5UB</td>
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<tr>
<td>1</td>
<td>B</td>
<td>3%</td>
<td>0,8UB</td>
<td>232</td>
<td>639</td>
<td>0,36</td>
</tr>
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<td>7</td>
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<td>28%</td>
<td>1UB</td>
<td>298,5</td>
<td>689,63</td>
<td>0,43</td>
</tr>
<tr>
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<td>1601UB</td>
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<td>689,63</td>
<td>0,43</td>
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<tr>
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<td>4UB</td>
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</tr>
<tr>
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<td>1,1UB</td>
<td>531</td>
<td>689,63</td>
<td>0,77</td>
</tr>
</tbody>
</table>
Attachment 3: Thrombogram obtained at different plasma dilutions (non-diluted, 1/10, 1/50, 1/100, 1/250, 1/500 and 1/1000) in a hemophilia A patient with inhibitor.

Attachment 4: Thrombogram obtained at different plasma dilutions (non-diluted, 1/2, 1/5 and 1/10) in an hemophilia A patient without inhibitor.
REFERENCES

1. Lee et al. Textbook of hemophilia. 2011
3. Kasper C. Diagnosis and management of inhibitors to factors VIII and IX. Treatment of hemophilia 2004; sept:34