Emicizumab Impact on Factor VIII Inhibitor Determination in Plasma Samples from Persons with Hemophilia A (PwHA) Using a New Kit for Modified Nijmegen-Bethesda Assay (MNBA)

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Background/Objectives

- An inhibitor response via antibody development to Factor VIII (FVIII) replacement therapies is the most significant complication of hemophilia A treatment today.
- An accurate diagnosis of FVIII inhibitors is essential in guiding patient management. However, due to use of different methods and reagents (e.g. plasma sources, absence of or variations in heat deactivation, buffered or non-buffered plasma, etc.) across laboratories, the inhibitor titer varies as high as 50% between laboratories in international proficiency studies.[1]
- Emicizumab, a novel bispecific antibody with tenase restoring effect **(Figure 1)**[2] approved in the US and Europe for bleeding prophylaxis in PwHA with FVIII inhibitors, interferes with clot-based Bethesda assays.[3]
- Chromogenic endpoint assays have better specificity than one-stage clot-based assays[4] since the latter depends on fibrin clot formation which is impacted by the presence of heparin, lupus anticoagulants, and inhibitors of coagulation factors other than FVIII as well as emicizumab.
- Thus, chromogenic MNBA has potential for standardization and improvement of the FVIII inhibitor assay.

Conclusions

- We have previously shown that the chromogenic MNBA has superior reproducibility over the clot-based MNBA.[9]
- Here we have demonstrated that the bovine-based chromogenic MNBA is suitable for FVIII inhibitor measurement in plasma samples containing emicizumab.
- Thus, the new MNBA kit shows promise for laboratories seeking a standardized FVIII inhibitor assay.

References

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Methods

- To eliminate FVIII depleted plasma as a potential source of variation and to standardize inhibitor titer measurement, a kit for MNBA was developed with these components:
  - IB-PNP: Imidazole Buffered Pooled Normal Plasma (pH = 7.4, 100 mM Imidazole, FVIII 95-105%) to provide a source of native FVIII and prevent pH change during incubation.
  - IB-BSA: Imidazole Buffered Bovine Serum Albumin (pH = 7.4, 4% w/v BSA in 50 mM Imidazole) to replace FVIII depleted plasma in the Nijmegen assay.
  - POS-Ctrl: Positive FVIII inhibitor control (~1 BU/mL, polyclonal anti-human FVIII antibody in a buffered human FVIII depleted plasma).
  - NEG-Ctrl: FVIII inhibitor-free human plasma (buffered).
- The MNBA kit components were frozen and stored at < -70 °C until use **(Figure 2)**.
- Frozen citrated plasma samples were collected from PwHA with a history of FVIII inhibitors in a global phase 3 clinical study (HAVEN 1 ClinicalTrials.gov number, NCT02622321). Patient consent and approval by a medical ethics committee were obtained.
- In total, 30 paired de-identified plasma samples from 15 PwHA pre- and post-emicizumab administration were thawed, heat deactivated, and centrifuged. The supernatant was drawn off and stored at < -70 °C until testing **(Figure 3)**.
- The heat deactivation step was incorporated in sample preparation to dissociate antibody-FVIII complexes and to eliminate remaining FVIII activity in plasma samples from PwHA, thus preventing the likelihood of false negative results.[5,6] Antibodies, such as FVIII inhibitors and emicizumab, remain after heat deactivation.
- **Chromogenic and Clot-based MNBA:** after thawing the heat deactivated plasma samples and FVIII Inhibitor Kit controls (Test Samples), a 1:1 mixture of IB-PNP, with either undiluted or IB-BSA pre-diluted Test Samples, were prepared (Test Mix, 400 µL).
- A 1:1 Control Mix was prepared with IB-PNP:IB-BSA (400 µL).
- The Control Mix and Test Mixes were incubated for 2h at 37 °C in a water bath followed by a 10 min. incubation on ice.
- After incubation, FVIII activities in the mixed samples were determined on a Siemens BCS® XP analyzer using Siemens' products: a bovine-based Factor VIII Chromogenic Assay and a one-stage clot-based assay using Pathromtin® SL aPTT reagent.
- The Test Mix dilution with residual FVIII activity closest to 50% and within the accepted range of 25-75% was used to calculate the FVIII inhibitor titer in Bethesda Units **(Figure 4)**.
- **Anti-FVIII Antibody ELISA:** after thawing, the heat deactivated plasma samples[7] and the ELISA kit-provided serum controls were tested in duplicate according to the manufacturer's instruction using a qualitative solid-phase indirect FVIII ELISA (Immucor FVIII Antibody Screen), to detect IgG antibodies against a full-length recombinant human FVIII (plate coated with Kogenate FS).

Results

- In plasma samples collected from 15 PwHA prior to administration of emicizumab, complete agreement in inhibitor detection was obtained between chromogenic and clot-based MNBA with a strong correlation in FVIII inhibitor titer **(Figure 5)**.
- However, in plasma samples from these same 15 PwHA collected post-administration of emicizumab, the clot-based MNBA consistently yielded false negative FVIII inhibitor results **(Figure 6)**. The presence of inhibitors in these samples was still measurable by the chromogenic MNBA but could not be measured by the clot-based MNBA **(Figure 6)**.
- Comparison of the anti-FVIII antibody ELISA results with the chromogenic and clot-based MNBA results showed complete agreement between all three methods for the 15 plasma samples collected pre-administration of emicizumab **(Table 1)**. Post-emicizumab administration, however, the 13 samples from PwHA that were positive by all three methods pre-emicizumab, remained positive by both anti-FVIII antibody ELISA and the chromogenic MNBA but were all negative by the clot-based MNBA.
- The lack of emicizumab interference with the bovine-based chromogenic MNBA was further demonstrated by the full recovery of inhibitor titer of the MNBA kit POS-Ctrl spiked with emicizumab (final concentration 100 µg/mL). The same spiked sample when tested with the clot-based MNBA was found to be falsely negative for FVIII inhibitor. Addition of emicizumab to the MNBA NEG-Ctrl had no impact on FVIII inhibitor detection.

Figure 5

Correlation between MNBA results pre-emicizumab administration for 15 plasma samples from PwHA

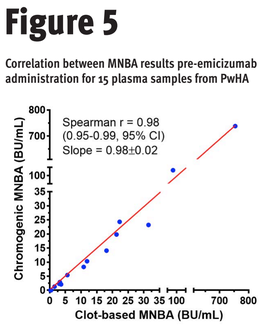

Figure 6

Comparison of chromogenic vs. clot-based MNBA in 15 plasma samples from PwHA before and after emicizumab administration

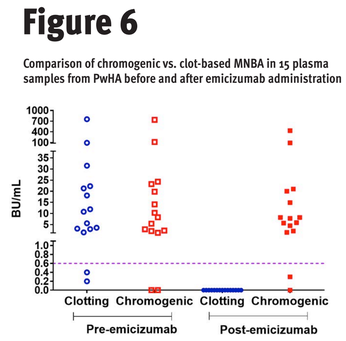

Table 1

Comparison of Chromogenic and Clot-based MNBA* results with ELISA results pre- and post-emicizumab administration**

FVIII Inhibitor detection in plasma samples from PwHA (N=15)		Pre-Emicizumab Administration		Post-Emicizumab Administration	
		Immucor anti-FVIII ELISA		Immucor anti-FVIII ELISA	
		Positive	Negative	Positive	Negative
Chromogenic MNBA	Positive	13	0	13	0
	Negative	0	2	2	0
Clot-based MNBA	Positive	13	0	0	0
	Negative	0	2	15	0

* ≥0.6 BU/mL cutoff (a consensus recommendation by ISTH-SSC)[8]
** >0.321 OD_{405nm} cutoff (determined by a lot specific cutoff control provided in ELISA kit)

Figure 1

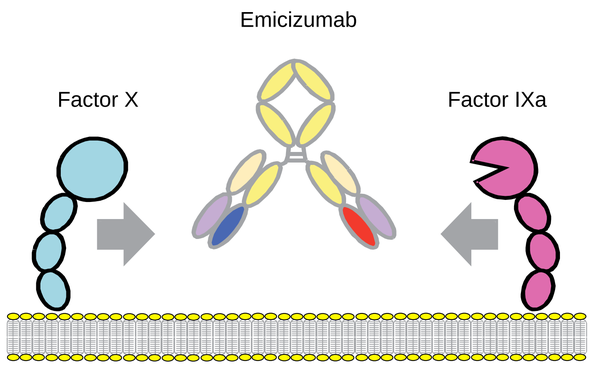

Figure 2

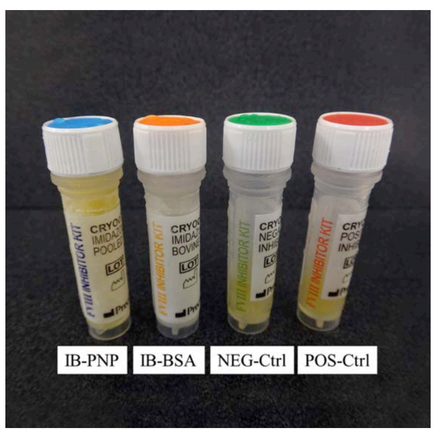

Figure 3

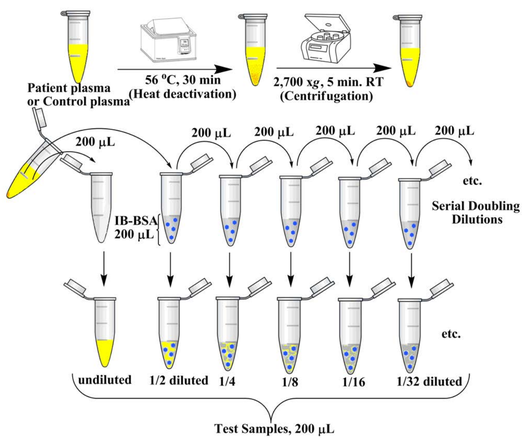

Figure 4