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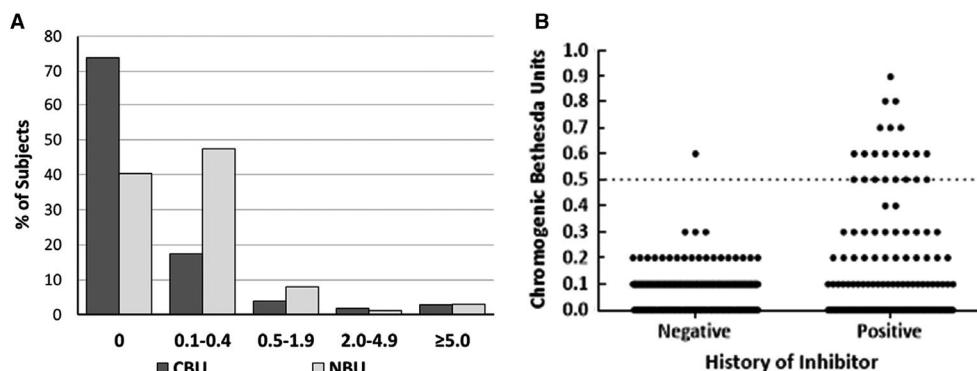
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## The chromogenic Bethesda assay and the Nijmegen-Bethesda assay for factor VIII inhibitors in hemophilia A patients: Are they equivalent?

We previously described in this journal a modified Nijmegen-Bethesda assay (NBA) for factor VIII (FVIII) inhibitors in hemophilia A (HA) that uses preanalytical heat inactivation of infused or endogenous FVIII to allow inhibitor measurement postinfusion<sup>1</sup> and compared that assay with a chromogenic Bethesda assay (CBA) that is identical except for use of an FVIII chromogenic substrate assay (CSA) rather than a one-stage assay (OSA) as the endpoint for inhibitor detection.<sup>2</sup> Our primary focus was on use of the CBA as a confirmatory test for low positive NBA results. Introduction of the non-FVIII treatment product emicizumab (Hemlibra) has brought increased interest in inhibitor assays using CSA because emicizumab interferes with the OSA and

thus with Bethesda assays for FVIII inhibitors using the OSA.<sup>3-5</sup> CSA for FVIII that use bovine factor X (FX) are insensitive to emicizumab,<sup>5</sup> and a CBA using such CSA has been successfully used for inhibitor testing in its presence.<sup>6,7</sup> Clinical laboratories providing inhibitor testing have the option of maintaining two inhibitor assays and choosing the correct one for each patient depending on the product used or switching to a CBA to accommodate testing on all patients. Clinical adoption of a new assay methodology requires demonstration that the new method is equivalent to the old. Recent reexamination of the dataset of paired NBA and CBA results from our original paper<sup>2</sup> revealed differences that may influence such comparisons and that, if not considered, could hinder validation of the CBA for clinical use.

The results reexamined were from 1005 specimens collected from subjects with congenital HA enrolled in the Hemophilia



**FIGURE 1** (A) Distribution of results of 1005 specimens measured in both chromogenic Bethesda units (CBU) and Nijmegen-Bethesda units (NBU). (B) Comparison of results between groups with negative and positive history of factor VIII inhibitor by chromogenic Bethesda assay. The dotted line represents the threshold for positivity previously established for the modified Nijmegen-Bethesda assay<sup>2,8</sup>

Inhibitor Research Study between 2006 and 2012 at 17 US hemophilia treatment centers with previous inhibitor history collected from the enrolling sites.<sup>2</sup> Briefly, the US Centers for Disease Control and Prevention–modified NBA was performed using an FVIII OSA and included heating of patient plasma to 56°C for 30 min and centrifugation before testing.<sup>1</sup> The threshold for positivity was set at  $\geq 0.5$  Nijmegen-Bethesda units (NBU) based on distributions of results on patients with positive and negative history of inhibitor<sup>1</sup> and validated by the frequency of positivity for anti-FVIII antibodies.<sup>8</sup> The CBA was performed by the NBA method, except that FVIII activity was measured using a bovine CSA (Siemens Factor VIII Chromogenic Assay, Siemens, Marburg, Germany).<sup>2</sup> Antibodies binding to FVIII were measured by a fluorescence immunoassay detecting both immunoglobulin (Ig)G and IgM in a subset of 268 specimens.<sup>2</sup> Results were expressed as median fluorescence intensity. The threshold for positivity was set at two standard deviations above the mean median fluorescence intensity of the results obtained for 56 healthy subjects. For this analysis, comparisons between CBA and NBA results were made by nonparametric methods, using the Wilcoxon matched-pairs signed-rank test and the Spearman correlation coefficient ( $r$ ), and by chi-squared test, with significance set at  $p < .05$ , using GraphPad Prism 8.3 (GraphPad Software Inc.).

In our previous paper using these data, we reported excellent correlation between paired NBA and CBA results for specimens with  $\geq 2.0$  NBU ( $n = 42$ ;  $r = .98$ ) and discrepancies between the two assays for positive specimens in the 0.5–1.9 NBU range.<sup>2</sup> We have now found differences in the group with negative titers ( $< 0.5$  NBU,  $n = 883$ ), as well. In that group, the NBA median was 0.1 (interquartile range 0–0.1), and the CBA median was 0 (interquartile range 0–0;  $p < .0001$ ). As illustrated in Figure 1A, the CBA and NBA distributions were significantly different ( $p < .0001$ ). The CBA produced a much larger number of zero Bethesda unit results than the NBA (73.9% vs. 40.4%) with smaller proportions for the CBA than the NBA in the remainder of the negative range (17.4% vs. 47.5%) and in the range of 0.5–1.9 (4.0% vs. 8.0%). Results were similar with the two methods in low-positive (2.0–4.9) specimens at 1.8%

vs. 1.2% and high-positive ( $\geq 5.0$ ) specimens at 2.9% vs. 3.0%. There appears to be a previously unreported shift toward lower CBA results among NBA-negative specimens. Using the CBA, 84.7% of 746 specimens from patients with negative history of inhibitor had zero CBU, whereas only 48.3% had zero NBU.

The observed differences do not alter our previous conclusions that both NBA-negative specimens and NBA-positive specimens with  $\geq 2.0$  NBU are classified correctly by the CBA, with only those with 0.5–1.9 NBU showing classification changes<sup>2</sup>; however, these differences need to be considered when attempting to establish equivalence between the two assays for clinical purposes and might be taken to indicate that the CBA is less sensitive than the NBA. We have recently reported, however, that the limit of detection for the CBA is 0.1,<sup>7</sup> which is lower than the 0.2 calculated for the NBA,<sup>8</sup> indicating that the CBA is slightly more sensitive than the NBA, as we originally showed in dilution studies.<sup>2</sup> Among specimens tested for specific anti-FVIII antibodies, significantly more CBA-positive specimens were antibody-positive than NBA-positive specimens (50/51, 98.0% vs. 83/99, 83.8%;  $p = .012$ ), with the single CBA-positive specimen not showing antibody positivity positive in both NBA and CBA. These results for the CBA in patients receiving replacement therapy are similar to our recent findings in patients receiving emicizumab, which showed 97.6% of 250 CBA-positive specimens to be positive for anti-FVIII IgG<sub>4</sub> antibodies,<sup>7</sup> the most reliable antibody subclass to predict that a functional inhibitor is present, as reviewed.<sup>9</sup> Thus, the lower results in the CBA are not due to lower sensitivity but to greater accuracy at detecting truly negative specimens. The CBA is thought to give fewer false-positive results because lupus anticoagulants, heparin, or nonspecific inhibitors of coagulation affect the OSA but not the CSA, as reviewed.<sup>9</sup>

We also examined two other characteristics of the CBA in this dataset. Our previous suggestion that specimens in the range of 0.5–1.9 NBU had a high frequency of false-positive results and should be confirmed by testing in the CBA and measurement of anti-FVIII antibodies was based on the finding that the frequency of positivity for anti-FVIII antibodies was significantly lower among specimens with

0.5–1.9 NBU than among those with  $\geq 2.0$  NBU (75.4% vs. 97.4%;  $p = .004$ ).<sup>2</sup> A similar analysis for the CBA shows antibody positivity rates in those ranges to be similar at 24/24 (100%) and 26/27 (96.3%), respectively ( $p > .99$ ). Additional antibody testing for confirmation, therefore, is not necessary when the CBA is used as the primary test in patients receiving traditional therapy, as we have shown in those receiving emicizumab.<sup>7</sup>

We also examined the threshold for positivity of the CBA, which was previously assumed to be the same as for the NBA.<sup>2</sup> CBA results plotted by history of inhibitor (Figure 1B) were very similar to the NBA results previously reported.<sup>1</sup> Among 746 negative-history specimens, 745 (99.9%) were below 0.5 CBU; one specimen differed in classification between the two methods with 0.4 NBU and 0.6 CBU. For the 204 positive-history specimens, the distribution was bimodal, as expected because of resolved or treated inhibitors, and similar to that of the NBA, both showing a break at 0.4. Thus, a threshold for positivity of  $\geq 0.5$  appears to be appropriate for the CBA in non-emicizumab specimens, as we have recently confirmed for specimens from patients receiving emicizumab.<sup>7</sup>

The current analysis identified differences among negative results not previously reported, which affect statistical comparison of results but do not affect classification of specimens as positive or negative. It also confirmed that a threshold for positivity of  $\geq 0.5$  is appropriate for the CBA, as previously shown for the NBA,<sup>2,8</sup> when using the US Centers for Disease Control and Prevention–modified methods including preanalytical heat inactivation of patient plasma in patients not receiving emicizumab. The higher rate of antibody positivity among CBA-positive specimens allows a greater degree of confidence in low-titer positive results when tests are performed with the CBA than with the NBA and eliminates the need for additional testing methods to confirm NBA results. In spite of the slight differences observed, the similarity of the CBA to the NBA should allow its use with confidence in patients treated with traditional products as well as those receiving emicizumab.

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## CONFLICT OF INTEREST

Dr. Miller and Mr. Boylan state that they have no real or potential conflicting interests.

## AUTHOR CONTRIBUTIONS

Connie H. Miller planned the study, analyzed results, and wrote the manuscript. Brian Boylan performed research, analyzed results, and wrote the manuscript.

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