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The Use of Genetic Diagnostic Tests to Identify Factor VIII Inhibitors in Patients with Hemophilia A: an Integrative Review

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Abstract. Hemophilia A is an X chromosome-linked bleeding disorder that leads to the complete absence or decrease of clotting factor VIII. People with hemophilia A may experience spontaneous bleeding events. Accurate diagnosis of hemophilia is essential to determine appropriate management. Prophylactic treatment with coagulant FVIII (factor FVIII) is well established as the standard of care for the treatment and prevention of bleeding episodes. However, some patients being treated with prophylaxis may develop inhibitors, which, in hemophilia, refer to IgG antibodies that neutralize clotting factors. Confirmation of the presence of an inhibitor and quantification of the titer is performed through genetic tests to identify factor VIII inhibitors. In this work we list the differences between the types of genetic tests: Nijmegen-Bethesda Assay (NBA), Bethesda Chromogenic Assay (CBA), Fluorescence Immunoassay (FLI) and Enzyme Immunoassay (ELISA). This study was carried out following elements of the systematic review methodology, in order to analyze the differences between the test characteristics and their sensitivities. The study analyzed 10 articles, which suggest that the results of these tests can be more assertive when combined with each other: The NBA as a standard method, associated with the Fluorescence Immunoassay (FLI), which can predict potential inhibitors, or the enzyme immunoassay (ELISA), which can have greater sensitivity to low-titer inhibitors. Therefore, studies that analyze these combinations between different techniques are necessary, and that help to find a standardization of clinical trials.

Keywords: Hemophilia, systematic review, genetic tests, diagnosis of hemophilia, clinical trials.

1 Introduction

Hemophilia A is an X chromosome-linked bleeding disorder that leads to the complete absence or decrease of clotting factor VIII [1]. The deficiency is the result of mutations in the respective clotting factor genes [2]. People with

hemophilia A may experience spontaneous bleeding events (including life-threatening ones) and develop joint damage (arthropathy) as a result of recurrent bleeding [3]. The World Federation of Hemophilia (WFH) estimates that 1 in 10,000 people are born with hemophilia A, with approximately 400,000 people affected worldwide [4].

Accurate diagnosis of hemophilia is essential to inform appropriate management [2]. Hemophilia should be suspected in patients with a history of: easy bruising in infancy, "spontaneous" bleeding (bleeding for no apparent or known reason), particularly into joints, muscles, and soft tissue and excessive bleeding after trauma or surgery [2]. A definitive diagnosis relies on factor testing to demonstrate FVIII or FIX deficiency [2].

Prophylactic treatment with FVIII coagulant (FVIII factor) is well established as the standard of care for the treatment and prevention of bleeding episodes and in surgeries [3]. This is supported by extensive clinical and real-world evidence demonstrating safety and effectiveness in preventing bleeding and, above all, preserving joint health. In this way, the prevention of bleeding events improves the quality of life of people with hemophilia and allows for greater participation of these individuals at school, work and social activities [3].

However, some patients being treated with factor VIII prophylaxis may develop inhibitors, which, in hemophilia, refer to IgG antibodies that neutralize clotting factors [2]. In the current era, when clotting factor concentrates have undergone appropriate viral inactivation, FVIII or FIX inhibitors are considered the most serious treatment-related complication in hemophilia [2]. The presence of a new inhibitor should be suspected in any patient who is clinically unresponsive to clotting factors, especially if he has previously responded [2]. In this situation, the expected recovery and half-life of the transfused clotting factor are severely shortened [2].

Inhibitors are found more often in people with severe hemophilia compared to those with moderate or mild hemophilia [2]. The cumulative incidence (in other words, lifetime risk) of inhibitor development in severe hemophilia A is in the range of 20–30 % and approximately 5-10 % in moderate or mild disease [2]. In severe hemophilia A, the median age of inhibitor development is 3 years or less in developed countries. In moderate/mild hemophilia A, it is close to 30 years of age and is often seen in combination with intense exposure to FVIII with surgery [2].

The measurement of factor VIII inhibitors (FVIII) in the US was standardized in 1975 at a meeting in Bethesda, Maryland, which produced a method named after the conference site [5]. A Bethesda Unit (BU) is defined as the amount of inhibitor producing a residual activity of 50 % [6]. Confirmation of the presence of an inhibitor and quantification of the titer is performed in the laboratory [2]. A low-response inhibitor is defined as a level of inhibitor that is persistently less than 5 BU mL-1, whereas a high-response inhibitor is defined as a level of greater than or equal to 5 BU mL-1 [2].

In this work analyzed four types of genetic assays (tests) that detect and validate the presence of factor VIII inhibitors: Nijmegen-Bethesda Assay (NBA),

Bethesda Chromogenic Assay (CBA), Fluorescence Immunoassay (FLI) and Enzyme Immunoassay (ELISA).

Therefore, the different techniques have differences between their sensitivity for detecting and validating factor VIII inhibitors, and these differences can impact the diagnosis, and consequently the choice of appropriate treatment and the clinical outcome of patients. Thus, this work seeks to understand how these diagnostic tests behave in the detection of factor VIII inhibitors, through analyzes of studies and articles that address the experience with the different tests for the detection and validation of the development of factor VIII inhibitors, as well as analyze the differences in sensitivity between them. The aim of this study was to list the characteristics of different diagnostic genetic tests and to analyze whether differences in test sensitivity impact the detection of factor VIII inhibitors.

2 Materials and Methods

This study is characterized as an integrative systematic review of the literature, guided by the following question: *"What are the diagnostic methods of factor VIII inhibitors in Hemophilia A?"*. The data collection took place in the first quarter of 2022, and included articles published in the last twenty years on the topic (2002 to 2022). The scientific databases used in this review were PubMed and SciELO (Scientific Electronic Library Online), using the English descriptors: "factor VIII inhibitor; hemophilia; diagnosis; assay", organized by test type: "Chromogenic Bethesda (CBA)", "Nijmegen Bethesda (NBA)", "Fluorescence Immunoassay (FLI)" and "ELISA".

The selection of articles considered the following inclusion criteria: studies that addressed methods of diagnosing factor VIII inhibitors for hemophilia; articles that evaluated current diagnostic methods for hemophilia; full articles and human studies. As for exclusion criteria, the following parameters were adopted: articles published more than twenty years ago or that did not present new diagnostic methods, articles that aimed to talk about treatment and management of inhibitors, articles that addressed Acquired Hemophilia A (AHA), non-public articles, articles that did not specifically address the diagnosis of factor VIII inhibitors, and articles in languages other than English.

This work was done using a specific auxiliary tool for systematic reviews: Covidence [7], following elements of the systematic review methodology [8]. The data extraction protocol sought to identify: study identification data (study name, study authors, study ID, study year and country where the study was developed), study design data (study type, number of participants, study population and study objective), description of the methodology used, data on outcome analysis (what the studies say about the diagnostic tests used to monitor inhibitors factor VIII) and the conclusion.

3 Results

The search carried out in the databases found the following results in PubMed and sciELO, using the different descriptors in English: "Factor VIII inhibitor" and "hemophilia" and "diagnosis" and "assay" and "[type of test]", where the field [test type] varied among the four evaluated in this study: Nijmegen-Bethesda, Bethesda Chromogenic, ELISA. This search resulted in 88 articles organized by relevance. Of these 88 articles, 16 showed greater affinity with the topic and were considered for in-depth analysis in Covidence [7]. 10 of these articles met the inclusion criteria, and were considered in this study. The 10 articles were separated into four different categories, which illustrate each of the types of tests analyzed, as follows: 5 studies on the Nijmegen-Bethesda Assay (NBA), 3 studies on the Bethesda Chromogenic Assay (CBA), 2 studies on the Immunoassay (FLI) and 3 ELISA studies. 3 articles were considered in more than one category. The 10 articles analyzed address the diagnostic techniques used to detect the level of factor VIII and its inhibitors, according to data described bellow:

1. Bethesda Chromogenic (CBA):
   Castellone D. D. et. al. 2017 [9]: Chromogenic assays use a longer incubation time and are therefore more sensitive to these mutations. Chromogenic findings also more closely reflect the bleeding symptoms in these patients. Most laboratories perform a one-stage FVIII assay (NBA). Two-stage testing is still performed in some centers, however, due to the long incubation time and the various steps involved, it is more difficult to automate. There are no commercial kits available for the two-stage assay. Few laboratories routinely perform the chromogenic assay. However, one-stage assays (NBA) are more capable of detecting very low levels of FVIII (< 1 IU/dL%) and demonstrate greater accuracy.
   Kershaw G. et. al. (2009) [10]: CBA sometimes gives false positives, in the range of 0.5 to 1.0 BU/mL, a situation that was largely resolved in 1995 with the Nijmegen-Bethesda (NBA). The NBA universal is one way in which inter-laboratory variability can be reduced, especially at the clinically important level of weak inhibitors of <2 BU/mL. Individual laboratories may perform assays consistently, but they still have differences in results from one another due to the specific reagents and instruments used.
   Miller C.H. et. al. 2013 [11]: Chromogenic assays are attractive for the detection of FVIII inhibitors, as they have been shown to be insensitive to heparin, lupus anticoagulants, and other nonspecific inhibitors of coagulation. Two-step chromogenic assays rely on activation of FVIII by a standard amount of thrombin and generation of FXa in an artificial system, while one-stage coagulation assays (NBA) rely on thrombin generation and formation of a fibrin clot in a system containing many plasmas.

2. Enzyme Immunoassay (ELISA):
   Kim S.Y. et. al. 2010 [12]: The ELISA technique showed greater sensitivity than the Bethesda assay in detecting FVIII inhibitors in samples that were

subjected to freezing and thawing procedures, and proved to be an efficient technique for the detection of low titer inhibitors.

Verbruggen B. et. al. 2009 [13]: Advantages of ELISA over coagulation method include the use of small volumes (fingerstick method) and serum samples instead of citrated blood. This test has a wider window, reducing the need for repeat testing and many dilutions. In addition to the less intensive fluid handling requirements, and the absence of interference from lupus anticoagulants and heparin.

Sahud M.A et. al. 2007 [14]: The ELISA method allows for rapid, batch analysis of multiple samples or multiple dilutions of a sample to detect the presence of anti-FVIII antibodies. Results can be readily available within 4-6 h, whereas the Bethesda assay may require more than 6 h of technical time alone, especially if inhibitor titers are high. Additional advantages of performing an ELISA are that only small volumes are needed and serum samples can be used if citrated plasma is not available. In addition, several samples with low inhibitor titers according to the Bethesda assay showed a strong antibody signal by ELISA.

3. Fluorescence Immunoassay (FLI):

   Miller C.H. et. al. 2013 [11]: The described FLI is more sensitive than NBA or CBA, and it is not surprising that it detects antibodies in samples without detectable inhibition of FVIII in clotting assays. Overall, the FLI, which was the most sensitive method tested, showed better agreement with the CBA than the NBA. It is possible, however, that differences in epitope specificity of individual inhibitors could cause differences in the rate of generation of FVIIIa, to which CBA is more sensitive, or differences in reactivity to bovine proteins, leading to false-negative results.

   Boylan B. et. al. 2015 [15]: Examination of FLI results in plasma samples from these seven patients revealed that five of them harbored one or more classes of anti-FVIII Igs in samples before developing an inhibitor detectable by the NBA. All five of these patients were positive for anti-FVIII IgG 1 prior to their conversion from NBA negative to NBA positive. These data provide a rationale for future clinical studies designed to monitor the dynamics of the anti-FVIII antibody profiles of patients with AH, in order to assess their value as predictors of the future development of clinically relevant inhibitors and to determine the utility of αFVIII FLI as a supplement to traditional inhibitor testing methods.

4. Nijmegen Bethesda (NBA):

   Miller C.H. at. al. 2012 [6]: The data support the use of $\geq$0.5 NBU to define a positive inhibitor when the method is used. Experience demonstrates that the NBA can be standardized to be within acceptable limits for clinical trials and can be used for national surveillance. However, it has been suggested that the sensitivity of the inhibitor assay does not extend below 0.4 BU. The limit of the range used for calculation in the original Bethesda assay is that

any inhibitor titer <0.4 BU should be considered negative, and this can make interpretation of low titer inhibitor readings difficult.
Batty P et. al. 2021 [16]: International guidance advises the use of the NBA as the 'gold standard' FVIII inhibitor test. Just under half of the labs self-reported using the NBA in this research. A range of inhibitor assay cutoffs (0-1 BU) was reported similarly to previous studies. This range is potentially problematic in patients with low titer inhibitors (eg, 0.6-0.9 BU), where some centers would define them as positive and others as negative, which affects bleeding treatment choices.
Dimichele D.M., 2006 [17]: The Nijmegen assay was adopted as the official method of choice for the quantification of inhibitors by the FVIII/FIX Subcommittee of the ISTH in 1996, but this assay has problems with accuracy as inter-assay variability is ongoing and it is believed that are related to the inherent imprecision of the one-stage clotting assay.
Verbruggen B. et. al. 2009 [13]: Although the results of the Nijmegen assay demonstrate greater specificity when compared to Bethesda, the results of the ECAT surveys, for example, show a very high coefficient of inter-laboratory variation of 30% for the Nijmegen assay and >40% for the original Bethesda method. The main determinants of test variability are variations between operators in handling liquids.
Miller C.H. et. al. 2013 [11]: Only 4% of the specimens showed disagreement between the NBA and CBA results. Agreement was excellent between specimens negative for NBA (99.7%) and those with NBU>2.0 (100%). The hypothesis that CBA is simply a less sensitive method than NBA is not supported by the dilution curve data. NBA and CBA kinetics appear to be similar for proven inhibitors.

4 Discussion

Evaluating inhibitor testing methods is difficult because there is no gold standard against which to compare them. In practice, laboratory and clinical evidence is used to determine whether a patient has an inhibitor. A significant proportion of patients with prior inhibitors test negative because they have been successfully treated, either by ITI (Immune Tolerance Induction) or by using alternatives to factor replacement [6]. Considering this scenario, the present study suggests that:

4.1 Bethesda Chromogenic (CBA)

Chromogenic assays use a longer incubation time and are therefore more sensitive to mutations that affect factor VIII levels. This test has also been shown to be insensitive to heparin, lupus anticoagulants, and other non-specific clotting inhibitors that may affect the test result. Although this test is still performed in some centers, due to a long incubation time and the various steps involved, it is more difficult to automate, and can generate greater interlaboratory variability.

This test also proves not to be as assertive in detecting low titer inhibitors (<2 BU/mL). There are already other tests with greater ability to detect very low levels of FVIII (< 1 IU/dL %) more accurately.

4.2 Enzyme Immunoassay (ELISA)

The ELISA technique is more sensitive than the Bethesda assay in the detection of FVIII inhibitors in samples that were subjected to freezing and thawing procedures, and it proved to be an efficient technique for the detection of low titer inhibitors. Considering that the tests are generally not performed in hospital laboratories, but in specialized laboratories, which analyze patient plasma samples that are frozen and transported, this technique demonstrates good performance in tests performed to detect FVIII inhibitors even after thawing. Sample. Another advantage of using ELISA is the volume required for the test, and serum samples can be used if citrated plasma is not available. Previous studies report on the potential utility of a capture ELISA assay as an alternative to conventional functional inhibitor assays [18] [19]. These studies noted assay variability that correlated with the source of FVIII. In this study, high purity FVIII increased the sensitivity of the test; alternatively, FVIII bound to von Willebrand factor significantly decreased the sensitivity of the assay [18]. But the potential of these assays to detect rising low-titer inhibitors or non-neutralizing antibodies is currently unclear [19].

4.3 Fluorescence Immunoassay (FLI)

FLI is more sensitive than the NBA or CBA assay, and detects antibodies in samples without detectable FVIII inhibitors in clotting assays. This assay showed the most sensitive method tested and showed better agreement with the CBA than the NBA. Its greatest benefit is its ability to predict the future development of clinically relevant inhibitors, and it can be used as a supplement to traditional inhibitor testing methods.

4.4 Nijmegen Bethesda (NBA)

The Nijmegen Bethesda test can be standardized to be within acceptable limits for clinical testing and can be used for national surveillance. And because it is a one-phase trial, it ends up having a lower associated cost. But the problems related to the Bethesda method and its variants (such as the NBA) point to the lack of precision in identifying low titers of inhibitors, and for high titers, the method requires repeated assays, with dilution processes [12]. That is, studies suggest that the NBA assay has high sensitivity, but still lacks greater specificity.

Methods of detection and quantification of FVIII inhibitors have improved in recent decades, but they still lack sensitivity and specificity, and interlaboratory variation is still very high. Low titer inhibitors still cannot be detected properly and, therefore, the clinical significance of these inhibitors cannot be evaluated

without constant monitoring, which exposes the patient to a debilitating process, especially when we talk about pediatric patients, who may present greater difficulty of venous access. There is a need for better methods or improvement of existing methods and the development of standards and controls to reach a stage of reliable inhibitor testing and comparable laboratory data. However, it is understood that there is an access limitation related to the resources needed to pay for the inputs and the structure of protocols that combine and perform different tests on a routine basis.

5 Conclusion

The Nijmegen Bethesda (NBA) method is considered the standard method for identifying and monitoring factor VIII levels and also for detecting inhibitors, as well as tracking these levels during prophylactic and ITI treatments. But the results of this test can be more assertive when associated with analyzes by other techniques, such as the Fluorescence Immuno Assay (FLI), which can predict potential inhibitors, and the Enzyme Immuno Assay (ELISA), which can have greater sensitivity for inhibitors of low title, which is one of the weaknesses of the NBA. Therefore, studies that analyze these combinations between different techniques are necessary, to help finding a standardization of clinical trials.

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