Original Research Article

Study of factor VIII inhibitor and F8 gene mutationsin persons with hemophilia A from Benin

ABSTRACT

Aim: determine the prevalence of a FVIII inhibitors and identify the genetic mutations associated with their development in beninesepersonne with hemophilia (PwH) A.

Study design: this is cross-sectional descrptive study conducted from June 2022 to May 2023 in hemophilia treatment centers in Benin.

Methodology: Inhibitor screening was carried out systematically in all PwHs A receiving FVIII infusion through determination of the circulating anticoagulant index and the Nijmegen-Bethesda assay. The molecular study strategy used for the F8 gene associated with hemophilia A is dependent on the severity of the hemophilia. Other data were collected either from patients' responses to the questionnaire or by studying their medical records and the center's hemophilia registry.

Results: Of the 97 PwHs A followed up, 57 had been treated with FVIII infusion. Of these, 21 had developed inhibitors, representing a frequency of 36.8% of treated PwHs A and 43.75% of severe PwHs. None of the moderate or mild PwHs A had developed anti-FVIII antibodies. PwHs A with inhibitors had a median age of 11 years, ranging from 1 to 66 years. The Nijmegen-Bethesda test revealed 11 high responders and 10 low responders. Mutation analysis of the F8 gene revealed seven cases of intron 22 inversion, seven cases of nonsense mutations, three cases of deletion and one case of missense mutation. Mutations weren't identified in three patients because their DNA did not amplify on long-distance PCR. In terms of therapy, immune tolerance induction wasn't achieved in any of the 21 patients, but they are treated with emicizumab and bypass depending on the context.

Conclusion: Although a cross-sectional study with a limited sample size, this study provides valuable information on beninesePwHs A with inhibitors. The frequency of inhibitors is high in treated PwHs A, and almost all patients who have developed inhibitors have high-risk genetic mutations.

Keywords:*Hemophilia A, Inhibitors, Nijmegen-Bethesda test, F8 gene variants, genetics*

1. INTRODUCTION

A most important complication of replacement therapy in the management of hemophilia A is the development of anti FVIII inhibitors. Thesealloantibodies, againstinfused FVIII mostlyneutralizeits pro-coagulant activity, markedlycompromising the efficacy of antihemophilictreatment [1] and increases the morbidity and mortalityassociatedwithhemophilia A [2]. Typically, inhibitoronsetoccurswithin the initial 10 to 50 days of treatmentwithinfused FVIII [3, 4]. After 50 exposuredays (EDs), the probability of inhibitordevelopmentdiminishessubstantially. Among, the prevalence of factor VIII inhibitors in thesepreviouslytreatedpersonswithhemophilia A (PwHs A) varies geographically, averagingapproximately 30% in severe cases and 5% in moderate to mild cases [5]. The risk of formation of an inhibitorisinfluenced by the genetic and environmentalfactors. The type of genetic mutation in the FVIII gene has a major influence on inhibitor formation. This studyaims to ascertain the prevalence of anti-factor VIII inhibitors and delineate the genetic mutations associatedwiththeirdevelopment in beninesepersonswithhemophilia A.

2. PATIENTS AND METHODS

This cross-sectional descrptive study was conducted from June 2022 to May 2023. It aimed to include all patients with hemophilia A exposed to FVlll products from various manufacturers and of various purities at hemophilia treatment centers (HTC) in Benin. Screening of inhibitors was systematically conducted among all PwHseither receiving infused FVIII as part of this study, upon suspicion of therapeutic inefficacy or following management of a severe hemorrhagic event. Data were collected through a questionnaire administered to patients with hemophilia A aged 18 years and above, or to their parents if they were minors, These data were supplemented by a study of medical records and the hemophilia registry of the center. Collected variables included the severity of hemophilia A, medical history, age at diagnosis of anti-factor VIII inhibitors, age at diagnosis of hemophilia, bleeding complications of hemophilia, type of treatment received, number of EDs and the type of genetic mutation of PwHs..

Screening of factor VIII inhibitors was established using the mixing test activated partial thromboplastin time (APTT) followed by confirmation via Nijmegen-Bethesda assay. The patient’s plasma was mixed with a normal reference plasma (normal control, Siemens). Normal control plasma and patient’s plasma dilutions were made in imidazole buffer; FVIII deficient plasma of Siemens was used. After a 2 hours incubation at 37”C, residual FVlll was measured. One Bethesda Unit (BU) was defined as the amount of antibody inactivating one-half of the factor VIll in the mixture. The positivity threshold for inhibitors was set at 0.6 BU, with a hemophiliac classified as a high responder when the inhibitor titer was greater or equal than 5 BU.

The molecular study strategy used for the F8 gene associated with hemophilia A is dependent on the severity of the hemophilia. The initial approach in severe hemophilia A patients was to test for the presence of intron 22 microinversion using Long Range Polymerase Chain Reaction (LR PCR). If negative, the microinversion of

intron 1 was tested by triplex PCR. In the absence of these two micro-inversions, all 26 exons of the F8 gene and their flanking regions were sequenced, followed if necessary by sequencing of the promoter and 3'UTR region.In cases of moderate or minor hemophilia, the search for genetic mutations is carried out directly by sequencing the 26 exons and their flanking regions. In case of negativity, sequencing of the promoter and the 3'UTR region was performed. Interpretation and nomenclature of the anomalies identified were based on international databases of hemophilia genetic anomalies.

3. RESULTS AND DISCUSSION

Of the 97 PwHs A monitored in HTC in Benin, 57 had been treated with infused factor VIII. The distribution of these patients according to the severity of hemophilia A revealed that 48 patients were severe form, eight were moderate, and one patient had mild hemophilia. Among the 57 treated patients, 21 had developed anti-factor VIII antibodies, resulting in a prevalence of 36.8% of inhibitors among treated hemophilia A patients and 43.75% among severe hemophilia A patients. None of the moderate or mild hemophilia A patients carried anti-factor VIII inhibitors during this study, as shown in Table I.

Table I : Frequency of beninesePwHs A with inhibitors

	Number of PwHs A	**Number of PwHs A treatedwithinfused FVIII**	**Number of patients withinhibitors**	**Frequency of occurrence of inhibitors**
Number of PwHs A	97	57	21	**36,8%**
Number of severePwHs A	65	48	21	**43,75%**
Number of moderatePwHs A	25	08	00	**00%**
Number of mildPwHs A	06	01	00	**00%**

PwHs A with inhibitors had a median age of 11 years, ranging from 1 to 66 years. Their distribution is depicted in Figure 1. They predominantly resided in the departments of Atlantique (8 patients), Littoral (5 patients), and Borgou (3 patients). The departments of Zou (2 patients), Collines (1 patient), and Ouémé (1 patient) had the lowest representation. Regarding ethnicity, 11 patients were Fon, followed by Adja (5 cases), and Yoruba (4 cases). Only one Dendi individual developed anti-factor VIII antibodies.

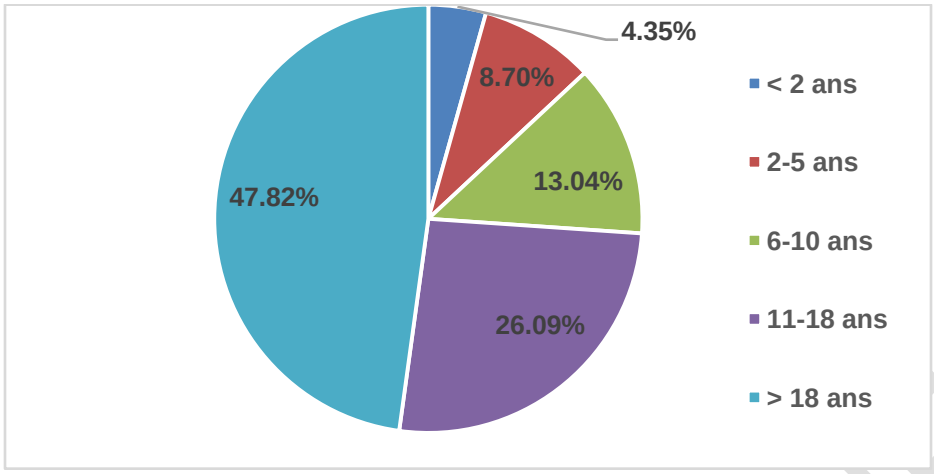

Figure 1: **Distribution of age of PwHs A with inhibitor**

The mean age at the onset of initial bleeding in these PwHs was 3 years 8 months with the range of 3 months and 10 years. However, the diagnosis of hemophilia was established at a mean age of 5 years and 8 months, ranging from 3 months to 21 years. Discovery circumstances were mainly post-traumatic cutaneous-mucosal bleeding (8 cases), post-circumcision bleeding (6 cases), and recurrent hemarthrosis (6 cases). Two patients were identified through family screening. The mean number of annual bleeding rates among these 21 PwH was 34, ranging from 10 to 96 per year. Among these bleeding episodes, four patients had a history of intracranial hemorrhage, and two had a history of psoas hematoma with blood loss and anemia decompensation. All 21 patients had a history of hemarthrosis, and 19 had hemophilic arthropathy at the time of this study. Regarding family history of hemophilia, 10 of the 21 hemophilia A patients who developed inhibitors reported a family history of hemophilia, while this history was not found in four patients. Additionally, seven patients were unaware of the presence or absence of hemophilia in their families. None of the 21 patients were aware of a family history of anti-factor VIII inhibitors. Their distribution according to the discovery circumstances of the inhibitors revealed 12 cases of therapeutic inefficacy, 4 cases of intracranial hemorrhage, and 5 cases of inhibitor detection following the management of post-traumatic bleeding events (road traffic accidents, psoas hematoma, circumcision) during this study. Additionally, all patients were on-demand treatment before inhibitor development. The mean number of days exposure was 22 per PwHs, ranging from 5 to 59 EDs. Two patients out of the 21 had more than 50 EDs at the time of inhibitor discovery during this study.

Inhibitor screening was conducted by the absence of mixing test correction with a circulating anticoagulant index (Rosner index) greater than 15 in the 21 PwHs A. Performing the modified Bethesda assay revealed 11 patients with an anti-factor VIII inhibitor titer > 5 BU, while the other 10 had titers ranging between 0.7 and 4.5 BU.

Among the 21 patients, mutations could not be identified in 3 patients due to the absence of DNA amplification during the search for intron 22 inversion. Of the remaining 18 patients, seven carried intron 22 inversion, seven

had nonsense mutations, and three patients had a deletion in the F8 gene. Among these three, one had an intron 26 + 3'UTR deletion, and two had a single nucleotide deletion. Finally, one out of the 21 PwHs carried a missense mutation and developed anti-factor VIII inhibitors (Table II).

Table II: Frequency of types of mutations in PwHs A with inhibitors

Type of mutations	Effective	Frequency
Intron 22 inversion	7/18	38,9%
Nonsense mutation	7/18	38,9%
Deletion	3/18	16,7%
Missense mutation	1/18	5,5%

In terms of therapy, immune tolerance induction was not achieved in any of the 21 patients, but 10, including all those who developed severe hemorrhagic events, were placed on prophylactic emicizumab treatment, and the other 11 were on-demand activated prothrombin complex concentrate treatment.

4. DISCUSSION

In ourstudy, weinvestigatedinhibitorsamongbeninesePwHs A treatedwithinfused factor VIII and found a frequency of 36.8%. This frequencyishigherthanthatgenerallyobserved in the caucasian population, whichwas 7% in France [6], and rangedfrom 3.6% [7] to 27% [8] in the literature. For studieswithsample sizes of over 500 patients withhemophilia A, reportedprevalences of anti-factor VIII inhibitorsweretypicallylower, rangingfrom 5% to 7% [3], except for twostudiesthatincluded over 1000 hemophilia A patients, whereреportedinhibitorprevalencesexceeded 10% [9,10]. Whenstudiesinvolvesmallerhemophiliacohorts, reportedinhibitorprevalencesappearhigher. For instance, in Turkey, a study of 58 childrenwithhemophilia A by Ören et al. reported 27% prevalence of inhibitors [8], whileAronisfound a prevalence of 26.8% in Greece [11]. Regardingstudiesconducted in sub-SaharanAfrica, sample sizes rangedfrom 22 in Senegal to 54 in Ivory Coast, with respective prevalence rates of 20% [12] and 12% [13]. These data reveal a higherinhibitorfrequency in ourstudy population compared to studiesconducted in the sub-region. This differencecouldbeexplainedeither by the presence of transient inhibitors in some of the patients withhemophilia A included in ourstudy, or by the factthatalmost all PwHstreated in Benin were on-demandtreatment, whichisanotherrisk factor for developing anti-factor VIII inhibitors [14,15]. Additionally, the types of therapeuticfactors and theirorigins (plasma-derived or recombinant) usedvarywithin the samePwHfrom one bleedingepisode to another, whichconstitutes an additionalrisk factor [15]. Furthermore, none of the patients hadundergone immune tolerance induction since the use of factor VIII in Benin. Nevertheless, according to manyauthors, the frequency of inhibitorsishigher in black patients withhemophilia A than in the caucasian population because F8 gene haplotypes differbetween races [16-18]. Indeed, haplotype H1 is more prevalent in the caucasian population (60%) and H2 in Africans (74%), while haplotypes H3 and H5 are exclusivelyfound

in africans. Therapeutic factor VIII products have been producedusing H1 or H2 haplotypes [16], whichenhances the immunogenicity of the therapeutic FVIII protein.

Duringourstudy, a high number of annualbleedingepisodeswererecordedamong the 21 patients, six of whomdevelopedseverehemorrhagic manifestations, and 19 developedhemophilicarthropathy. This confirms the significantmorbidity of hemophilia in patients whodevelopinhibitors and the impairment of theirquality of life [19-21]. Additionally, as thisstudyis the first conducted in our country, all 21 PwHswithinhibitorswereunaware of whethertheyhad a familyhistory of anti-factor VIII inhibitors. The circumstances of inhibitordiscoveryamongtheсePwHsweremainlytherapeuticinefficacyduring the treatment of bleedingepisodes or duringsystematic screening during the study. Indeed, very few hemophiliacs come for routine consultation for their follow-up in Benin either due to lack of financialmeans to pay consultation fees or becausetheyreside far from the hemophiliatreatment center (HTC) in Cotonou, wherespecific tests like inhibitor screening are available. Hence, thereis a need to establish HTC in each of the 12 departments of Benin and to train laboratory personnel.

Giventhatinhibitordevelopmentis a multifactorial process, with the best-characterizedrisk factor being the type of F8 gene mutation, genetic mutations wereidentifiedin 18 out of the 21 patients whodeveloped anti-factor VIII inhibitors, withsevencarrying intron 22 inversion (38.9%), sevencarrying nonsense mutations (38.9%), and three patients having a deletion (16.7%). Indeed, according to the literature, patients withsignificant F8 rearrangementssuch as inversions, nonsense mutations, and large deletions have a high risk of developing FVIII inhibitors [19]. The sameholdstrue for single nucleotidedeletions, as found in two patients in ourstudy, which are alsoconsidered high-risk mutations as they lead to a change in the reading frame and thus a stop codon [22]. Moreover, a case of missense mutation wasassociatedwith a severephenotype of hemophilia A and developedinhibitors. Whilemanyauthorsassociatemissense mutations with a lowrisk of inhibitor occurrence [19], Fodil et al. reported a frequency of 13.51% of severehemophilia A patients withmissense mutations whodevelopedinhibitors in theirstudy [23]. According to theirstudy, the risk of developing anti-FVIII inhibitorsincreaseswhen the substitution results in an aminoacid class change. Indeed, in the missense mutation found in patient HA 56 duringourstudy, arginine wasreplaced by histidine at position 2169. According to the aminoacid distribution, arginine belongs to class 4 (basic), while histidine is polar, neutral, and belongs to class 2 [23]. Therefore, itisnecessary to continue thisstudy on a larger population to betterunderstandthesedifferentmechanisms in the Beninese population.

5. CONCLUSION

Despitebeing a cross-sectional studywith a limitedsample size, thisstudyprovidesvaluable insights into the population of Beninese patients withhemophilia A withinhibitors. It has highlighted the absence of systematic and screening for anti-factor VIII inhibitorsduring the first 50 EDs and of immune tolerance induction. Moreover,

nearly all patients whodevelopedinhibitorscarried high-riskgenetic mutations such as intron 22 inversion, nonsense mutations, and deletions. In addition to thisgeneticrisk factor, otherenvironmentalfactorssuch as on-demandtreatment and changingtherapeutic factor VIII types witheachbleedingepisodecontribute to inhibitordevelopment. Finally, the management of these patients has been facilitated by the introduction of new non-substitutive therapies and bypassing agents.

ETHICAL APPROVAL

This study received approval from the Institutional Committee of Ethics and Research at the University of Parakou (Benin) (Authorization 0464/CLERB-UP/P/SP/R/SA). Prior to the study commencement, participants were provided with detailed information through a newsletter, which included the study's objectives, benefits, and associated risks, in order to obtain informed consent. The confidentiality of research results was maintained by utilizing a unique code for each patient.

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