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Original Article

Pathology Section

Evaluation of Factor VIII Inhibitor Titre in Haemophilia A Patients: A Cross-sectional Study from a Tertiary Care Institute in Eastern India

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ABSTRACT

Introduction: Haemophilia has a high disease burden in India and development of inhibitors/antibodies to the Factor VIII (FVIII) replacement therapy is a major problem leading to treatment failure, poor quality of life and high financial burden. Identifying risk factors associated with inhibitor production may lead to prevention and plan future treatment approach modification.

Aim: To identify risk factors associated with development of inhibitors and correlation of inhibitor titres with symptom progression.

Materials and Methods: The present study was a institution-based cross-sectional study which was conducted on 52 previously diagnosed Haemophilia A patients, who were being treated with either FVIII, or Fresh Frozen Plasma (FFP) or both, in a tertiary care Institute in West Bengal from January 2012 to June 2013. Mixing study of activated Partial Thromboplastin Time (aPTT) was done. For those patients where mixing study showed presence of inhibitor/antibody, titre of inhibitor was calculated by Nimjegen modification of Bethesda assay. Titre was compared with parameters like age at first exposure, duration of therapy, type of product used etc. Statistical analysis was done using Statistical Package for the Social Sciences (SPSS) version 31.0 using Fisher's exact test, odds ratio, confidence interval etc.,

Results: Patients with inhibitors in inherited Haemophilia A had an earlier age at diagnosis of Haemophilia A (<2 year) (OR=3.18, CI: 0.59-17.1) and also, an earlier age at first exposure to FVIII (<2 years) OR=26.0, 95% CI: 4.2-161.6; Fisher's exact p-value <0.001. Patients receiving FVIII only as replacement therapy had a greater tendency to develop inhibitors(OR=26.0, 95% CI: 4.2-161.6); and within 60 days of exposure (OR=26.0; 95% CI: 7.3-924.6). Fisher's-exact p-value <0.001; compared to combination of FVIII with FFP and cryoprecipitate. When compared between patients with High Titre Inhibitors (HTI) and Low Titre Inhibitors (LTI), HTI patients were found to have more severe bleeding episodes, lesser number of bleeding episodes, earlier age at first exposure to FVIII (OR=13.6), those who received FVIII only (OR=2.5) and fewer number of exposure days to FVIII (16.2 days for HTI compared to 30 days for LTI). The incidence of various transfusion transmitted infections in haemophiliacs was quite low (3.9%) with a comparatively higher incidence of Hepatitis C.

Conclusion: Chances of FVIII inhibitor increases with earlier age at diagnosis, earlier age of first exposure and patients receiving FVIII only. Inhibitors increase the risk of life-threatening bleeding episodes, and higher the titre, more were the chances of severe bleeding.

Keywords: Bethesda titre, Bleeding episodes, Clinicopathological correlation

INTRODUCTION

Haemophilia A is X-linked recessive disorder of Factor VIII (FVIII) deficiency affecting intrinsic pathway of coagulation. Haemophilia A in India has a very high disease burden (prevalence of 0.9 in 1 lac population) and majority of them are undiagnosed at birth [1]. In a developing country, most of them were treated by replacement therapies, leading to generation of a high prevalence of FVIII inhibitors. Inhibitors in haemophilia A are acquired, mostly alloantibodies and IgG in nature. Most inhibitors are directed against A2, A3, and C2 domain of activated FVIII, which is a trimer. Inhibitors can also occur in non haemophiliacs albeit in high concentrations. Older age, autoimmune diseases and malignancies predispose to inhibitor production. Loss of memory cells, failure of immune tolerance, Th1 and Th2 subset of CD4+ T cells are other possible hypothesis. There are two main types of antibodies, i.e., Type I antibodies - seen in classic haemophiliacs who follow linear kinetics, whereas Type II antibodies are the autoantibodies and they exhibit a more complex pattern of inhibition. Antibodies are identified by their ability to neutralise FVIII at 37° C after incubation for 2-3 hours. The highest incidence of FVIII Inhibitors was seen in South India (13.04%). The highest incidence of 20.99% was observed in Chennai, followed by Hyderabad (13.33%), Jammu (9.90%) and Guwahati (8.51%), respectively, with respect to the samples analysed. The other regions showed an inhibitor incidence <8% [2]. Worldwide prevalence of inhibitors in haemophilia-A is reported as 5-7% and when limited to patients with severe disease the prevalence is much higher at 12-13%. Disease severity is defined as factor VIII level, i.e., Severe (<1%), Moderate (1%-5%) and Mild (>5%-<40%) [3]. Potential risk factors for inhibitor development in haemophiliacs are varied, like severity of disease, age of first infusion of factor VIII. The number of exposure days to FVIII, type and purity of coagulation factor concentrates used for treatment, dose and frequency of clotting factor administrations, number, type and severity of haemorrhages (joint, muscle or intracranial), Factor VIII gene mutation, family history, race etc., [4]. The presence of FVIII inhibitors not only complicates the management of haemophilia A patients, it also worsens their quality of life, increases financial burden, increase risk of relapses. So it is essential to screen the haemophilia A patients for inhibitors and quantify the titres in inhibitor positive patients, so that management can be directed accordingly. Also, it is essential to identify factors that lead to the development of these inhibitors and correlate the inhibitor titre with the severity of clinical manifestations,

so that future efforts may be directed to reduce the incidence of these inhibitors.

Objective:

1. Prevalence of FVIII inhibitors in patients with moderate or severe haemophilia A.
2. To identify factors associated with the development of FVIII inhibitors.
3. To compare the severity of clinical manifestations with the presence/absence of FVIII inhibitors, and with the FVIII inhibitor titres in inhibitor positive patients.

MATERIALS AND METHODS

This institution- based cross-sectional study was conducted on 52 patients presenting to Haematology OPD/Medicine/pediatric IPD in a Tertiary care Institute in Eastern India over a period from January 2012 to June 2013. Ethical clearance was taken from Institutional ethical committee (IEC/MCK/35 dated 15.12.2011) and Informed consent was taken from participants/guardians. The study was done in accordance with the Helsinki declaration.

Inclusion criteria: Previously diagnosed cases of moderate or severe haemophilia patients who have received FVIII alone or in combination were included in the study.

Exclusion criteria: Mild haemophilia A, secondary coagulopathies (DIC, acute leukemia, vWD) were excluded from the study.

When comparing High Titre Inhibito (HTI) with Low Titre Inhibitor (LTI), no control population was necessary. Randomisation or blinding was not necessary as inclusive sampling was done using specific inclusion criteria.

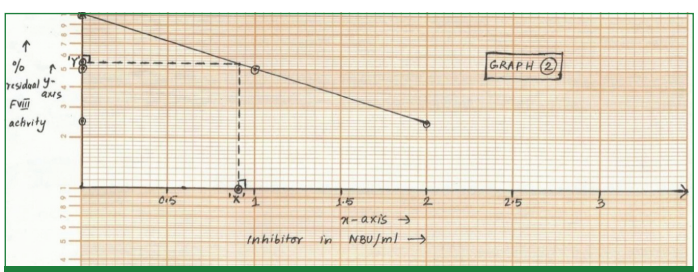
Sample size: Sample size was calculated using the formula:

n= (Z^2 * p * (1-p))/e^2. Here, n is the required sample size, Z is the Z-score for the desired confidence level (e.g., 1.96 for 95%), p is the estimated prevalence of inhibitors (3.5% in West Bengal population [2]), and e is the desired margin of error (5% or 0.05). Hence

$(1.96)^2 \times 0.035 \times (1-0.035)/(0.05)^2 = 51.9$

Study Procedure

History, relevant clinical details, factor VIII level, age at diagnosis, age at first exposure, type of product received, duration of treatment, number of bleeding episodes, number of severe life-threating bleeding, if any etc., were collected and mixing study by aPTT was done using 50:50 mixture of normal plasma and patient plasma, immediately and after two hour incubation under 37°C. If inhibitors were found, estimation of titre was done by Nimegen modification of Bethesda assay [5]. Mild haemophilia patients were excluded as literature suggests, presence of inhibitors are almost nil in them. Inhibitor titre was calculated from standard curve [Table/Fig-1]. One Bethesda unit (BU) was calculated as the amount of antibody that will neutralise 50% of FVIII in a 1:1 mixture of the patient's plasma and normal plasma (after 2 hour incubation at 37°C). Those with <5 NBU/mL were defined as LTI and those higher than 5 NBU/mL were termed HTI. Findings were entered in tables and graphs.



[Table/Fig-1]: Log-linear plot showing percentage residual FVIII activity (y-axis) against inhibitor titre expressed in Nijmegen Bethesda Units (NBU) (x-axis, logarithmic scale), used for calculation of inhibitor strength.

STATISTICAL ANALYSIS

Statistical analysis was done by Fisher's exact test, odds ratio, Student t-test, Mann whitney test in SPSS version 31. A p-value <0.05 was considered as statistically significant.

RESULTS

A total of 52 patients with haemophilia A were included in the study, of whom 10 (19.2%) had moderate disease and 41 (78.8%) had severe disease. One patient had acquired haemophilia A, while the remaining 51 cases were congenital. Acquired case was excluded. All the following tests/statistics are applied on 51 cases. After performing mixing studies, it was observed that none of the patients with moderate haemophilia A and nine patients with severe haemophilia A (21.9%) had inhibitors.

According to age at diagnosis [Table/Fig-2], seven of the nine patients with inhibitors (77.8%) were diagnosed with haemophilia A before the age of one year. In contrast, among patients without inhibitors, 22 of 42 patients (52.3%) were diagnosed before one year of age. The difference in proportions was 25.5%, with an odds ratio (OR) of 3.18 (95% CI: 0.59-17.1). This indicates that patients with inhibitors tend to have an earlier age at diagnosis compared to those without inhibitors.

Age at diagnosis	Without inhibitor (n=42)	With Inhibitor (n=9)	Strength of association
≤6 months	13 (30.9%)	4 (44.5%)	Odds ratio is 3.18. Confidence interval is 0.59-17.1
>6 months, ≤1 year	9 (21.4%)	3 (33.3%)	
>1 year, ≤2 years	6 (14.3%)	0	
>2 years, ≤5 years	11 (26.2%)	1 (11.1%)	
>5 years	3 (7.2%)	1 (11.1%)	

[Table/Fig-2]: Age distribution of patients with inhibitors.
*ODDs ratio and confidence interval calculation done in SPSS

[Table/Fig-3] shows that six of nine patients with inhibitors (66.7%) had received FVIII alone as replacement therapy, while 33.3% had received FVIII in combination with other blood products. No inhibitors were observed in patients who had not received FVIII concentrate at all. In contrast, among patients without inhibitors, only three of 42 patients (7.2%) had received FVIII alone, 71.4% had received FVIII in combination with other products, and 21.4% had received non-FVIII products or no replacement therapy. None of the patients in this study received plasma-derived FVIII.

Type of product	Without inhibitor (n=42)	With inhibitor (n=9)	Strength of association
FVIII* only (recombinant)	3 (7.2%)	6 (66.7%)	OR=26.0, 95% CI: 4.2-161.6; Fisher's-exact p <0.001
FVIII + FFP**+cryoprecipitate	9 (21.4%)	2 (22.2%)	
FVIII + FFP	21 (50%)	1 (11.1%)	
FFP only/FFP+cryoprecipitate/ FFP+blood/ not yet given	9 (21.4%)	0	

[Table/Fig-3]: Presence of inhibitors according to nature of product received as replacement therapy.
*FVIII: Factor VIII; **FFP: Fresh frozen plasma; #odds ratio and confidence interval calculation done in SPSS; p-value calculated by Fishers-exact test

Patients receiving FVIII alone had a significantly higher risk of inhibitor development compared to those receiving FVIII in combination with other blood products or non-FVIII therapy (OR=26.0; 95% CI: 4.2-161.6; p-value <0.001), indicating a strong association between exclusive FVIII exposure and inhibitor formation. Thus, patients receiving FVIII alone showed a greater tendency to develop inhibitors, whereas those receiving FVIII with other products had a lower tendency, and those not receiving FVIII did not develop inhibitors.

[Table/Fig-4] shows that five of nine patients with inhibitors (55.6%) had ≤40 exposure days to FVIII. With increasing exposure days, the proportion of inhibitors declined. No inhibitors were observed in patients with no FVIII exposure or in those with more than 60 exposure days. Early exposure to FVIII (≤60 exposure days) was

strongly associated with inhibitor development. Patients exposed within the first 60 exposure days had significantly higher odds of developing inhibitors (OR= 26.0; 95% CI: 7.3-924.6; p-value <0.001). Similar trends were observed in patients receiving combination therapy (FVIII with FFP/cryo), where inhibitor development declined with increasing exposure days, and no inhibitors were seen beyond 50 exposure days.

Choice of therapy	No. of exposure days	No inhibitor (n=42)	With inhibitor (n=9)	Strength of association
Combo of factor VIII+FFP*/cryo**	1-50	6 (20%)	3 (100%)	OR=26.0; 95% CI: 7.3-924.6; Fisher's exact p <0.001
	51-100	10 (33.3%)	0	
	>100	14 (46.7%)	0	
Factor VIII only	1-20	0	3 (50%)	
	21-40	0	2 (33.3%)	
	41-60	1 (8.3%)	1 (16.67%)	
	>60	2 (16.67%)	0	
Only FFP+Cryo+PRBC	No exposure	9 (75%)	0	

[Table/Fig-4]: Distribution of cases according to duration of therapy.
*FFP: Fresh frozen plasma; **Cryo= Cryoprecipitate; #ODDs ratio and confidence interval calculation done in SPSS; p-value calculated by Fishers-exact test

[Table/Fig-5] shows that six out of nine (66.6%) patients with inhibitors were exposed to FVIII concentrate within two years of age, compared to 15 out of 42 (35.7%) patients without inhibitors. Early exposure (≤2 years) was associated with higher odds of inhibitor development, although this did not reach statistical significance (OR=3.6; 95% CI: 0.8-16.7; Fisher's-exact p-value=0.12). The difference in proportions (66.6% vs 35.7%) was 30.9%. Thus, patients exposed to FVIII within two years of age showed a greater tendency to develop inhibitors.

Age at 1st exposure to FVIII	Without inhibitor (n=42)	With inhibitor (n=9)	Strength of association
≤6 months	3 (7.1%)	2 (22.2%)	OR=3.6; 95% CI: 0.8-16.7; Fisher's-exact p=0.12
>6 months, ≤1 year	7 (16.7%)	2 (22.2%)	
>1 year, ≤2 years	5 (11.9%)	2 (22.2%)	
>2 years	18 (42.9%)	3 (33.4%)	
No exposure	9 (21.4%)	0	

[Table/Fig-5]: Distribution of cases according to age at starting replacement therapy.
*#ODDs ratio and confidence interval calculation done in SPSS. p-value calculated by Fishers-exact test

Up to the beginning of the study, eight out of nine (88.8%) patients with inhibitors had ≤60 bleeding episodes, while only single patient (11.2%) had more than 60 episodes. In contrast, among patients without inhibitors, 21 patients (50%) had ≤60 bleeding episodes and 50% had more than 60 episodes. The difference in proportions was 38.8% (p-value=0.0781). Hence, patients with inhibitors experienced fewer overall bleeding episodes compared to patients without inhibitors.

However, life-threatening bleeding episodes (CNS bleed, GI bleed, or uncontrolled bleeding after trauma) were more common in patients with inhibitors; three out of nine cases (33.3%) than in those without inhibitors; 11 out of 42 cases (26.2%).

Patients without inhibitors 31 (73.8%) experienced a higher proportion of serious but non-life-threatening bleeding episodes (such as haemarthrosis, muscle bleeds, soft-tissue bleeds, oral and nasal bleeding, haematuria, etc.,) compared to patients with inhibitors 6 (66.7%). This difference was not statistically significant (p-value=0.9807, Chi-square test). Although patients with inhibitors had a higher proportion of life-threatening bleeding episodes, the association was not statistically significant.

The presence or absence of a family history of haemophilia A did not significantly influence inhibitor development in this study, as five out of nine (55.5%) patients with inhibitors and 21 out of 42 (50%) patients without inhibitors reported a positive family history.

[Table/Fig-6] shows that 100% of patients with LTI experienced serious bleeding episodes, whereas among patients with HTI, four patients (57.1%) had serious bleeding and three patients (42.9%) had life-threatening bleeding (p-value=0.7768, Chi-square test). No difference was observed in the odds of life-threatening bleeding between HTI and LTI patients (OR=1). However, patients with HTI tended to experience more life-threatening bleeding episodes than those with LTI.

Among patients with LTI, 50% had ≤40 bleeding episodes and 50% had more than 40 episodes (one each). In contrast, five patients (71.4%) had ≤60 bleeding episodes, while two (28.6%) had more than 40 episodes. The difference in proportions was 21.4% (p= 0.7763). Despite higher inhibitor titres, HTI patients did not show a higher number of bleeding episodes in this cohort (Mann-Whitney U test; small sample size).

All patients with LTI were exposed to FVIII concentrate after two years of age, whereas six out of seven (85.7%) of HTI patients were exposed within two years, and only one (14.3%) after two years. The difference in proportions was 85.7% (p-value=0.1565), indicating that HTI patients had an earlier age at first exposure to FVIII (OR=13.6).

Fifty percent of patients with LTI had ≤35 exposure days to FVIII±FFP, while the remaining 50% had >35 exposure days. In contrast, six out of seven (85.7%) of HTI patients had ≤35 exposure days, and one patient (14.3%) had >35 exposure days (p-value=0.9150). Comparing mean exposure days, HTI patients had substantially fewer exposure days (16.2 days) compared to LTI patients (30 days), suggesting earlier inhibitor development in HTI patients.

One out of two patients with LTI received FVIII alone, whereas five out of seven patients with HTI received FVIII alone; the remainder received combination therapy with FVIII, FFP, and cryoprecipitate. The odds ratio for developing HTIs with exclusive FVIII therapy was 2.5, indicating that patients receiving FVIII alone had 2.5 times higher odds of developing HTI compared to those receiving combination therapy.

Serial no. of patient	Type of bleeding	No. of bleeding episodes	Exposure days to FVIII±FFP**	Age at 1st exposure to FVIII	Inhibitor titre	Low titre inhibitors
1	Serious	19	15	33 y	3.5 NBU*/mL	(<5 NBU/mL) (LTI) (n=2)
2	Serious	60	41	20 y	4.5 NBU/mL	
3	Life-threatening	60	34	1.5 y	7.4 NBU/mL	(>/=5NBU/ml) High Titre Inhibitors (HTI) (n=7)
4	Life-threatening	27	10	2 y	12.6 NBU/mL	
5	Serious	100	52	10 mnth	14 NBU/mL	
6	Serious	40	8	1.5 mnth	16 NBU/mL	
7	Serious	25	23	6 mnth	23.4 NBU/mL	
8	Life-threatening	34	3	7 mnth	32 NBU/mL	
9	Serious	12	1	38 y	256 NBU/mL	

[Table/Fig-6]: Comparison of inhibitor titres with clinical behaviour and replacement therapy received.
*NBU: Nijmegen bethesda unit; **FFP: Fresh frozen plasma; #Mann-whitney U test (small 'n' number)

Overall, these observations suggest that patients with HTI tend to have more severe bleeding episodes, fewer total bleeding episodes, earlier age at first FVIII exposure, and fewer exposure days to replacement products compared to patients with LTIs.

Two patients were found to have Transfusion-Transmitted Infections (TTIs)- one with Hepatitis C Virus (HCV) infection and one co-infected with HIV and HCV. The latter was treated at an out-of-state district hospital. The proportion of patients with TTIs was 3.91%.

DISCUSSION

In this study, the proportion of inhibitors in moderate and severe haemophilia A was evaluated. Inhibitors were found in 21.9% of patients with severe haemophilia A. Similar observations have been reported by Singh A et al., Wang H et al., Thornburg CD et al., Nayak J et al., and Mansour A et al., [1,4,6-8]. However, no inhibitors were detected in patients with moderate haemophilia A in the present study.

The patient with acquired haemophilia A differed from the other nine patients with inherited haemophilia A with inhibitors in several aspects, including onset of symptoms after 40 years of age, absence of haemarthrosis, and no prior exposure to replacement therapy. Additionally, inhibitors in acquired haemophilia A followed complex kinetics and exhibited both immediate and delayed inhibition. These findings are consistent with the observations reported by Waldman Radinsky L et al., [9].

In the present study, Nijmegen modification of the Bethesda assay was chosen over the classical Bethesda assay. Valke LLFG et al., demonstrated that adding imidazole to the normal control mix conferred stability to factor VIII (FVIII) and reduced the number of spuriously positive assay results. It also helped reduce the number of LTIs and showed higher recorded titres [5]. The present study similarly showed only two LTI (20%), while a larger proportion were HTI (80%).

We observed that 66.6% of patients with inhibitors were exposed to FVIII concentrate as replacement therapy within the first two years of life. This finding corroborates studies by Cornier M et al., who demonstrated that early intensive treatment during inflammation in infancy generated more inhibitors, often within 50 days of first exposure [10]. Hermans C et al., introduced the concept of exposure days versus danger days (higher dose over a shorter duration) [11]. Abdi A et al., concluded that intensive FVIII treatment remains a risk factor for inhibitor development in non-severe haemophilia A even after more than 50 exposure days [12].

Previous literature by Kohar K et al., Hassan S et al., and van Stam LE et al., has shown that patients receiving recombinant FVIII (rFVIII) develop inhibitors more frequently than those receiving plasma-derived FVIII [13-15]. The present study also demonstrated that patients receiving FVIII alone as replacement therapy had a higher tendency to develop inhibitors compared to those receiving FVIII along with FFP, cryoprecipitate, or PRBCs, who showed a reduced tendency. Patients who did not receive FVIII at all did not develop inhibitors. These observations may be explained by the higher immunogenicity of rFVIII, which contains higher concentrations of FVIII and tends to induce antibody formation in patients lacking tolerance to endogenous FVIII, as seen in severe haemophilia A. When rFVIII is administered along with products such as FFP and cryoprecipitate, inhibitor formation may decline, possibly due to the presence of von Willebrand Factor (vWF), which promotes immune tolerance.

Formation of inhibitors was observed to decline with increasing exposure days to FVIII, with or without FFP and cryoprecipitate. Hermans C et al., showed that intensive treatment with larger doses generates more inhibitors than prophylaxis with continuous low doses or gradually increasing doses over a longer duration, which promotes immune tolerance induction [11]. This phenomenon can be explained by immune tolerance induction mechanisms, whereby elimination of FVIII-specific memory B cells by high doses of FVIII contributes to a decline in inhibitor levels. These findings corroborate observations made by Hassan S et al., Sarmiento Doncel SS et al., Peyvandi F et al., and Xi M et al., [14,16-18].

The literature shows a strong association between a family history of inhibitors and inhibitor development [16,19]. However, in this study, a family history of inhibitors could not be elicited from any patient, as most participants were from areas lacking adequate screening infrastructure. When an association between family history of haemophilia A and inhibitor development was evaluated, no statistically significant association was found.

Patients with inhibitors had a lower number of bleeding episodes compared to patients without inhibitors. Although this finding appears counterintuitive, similar observations have been reported by Young L et al., and Haghpanah S et al., [20,21]. Batran RA et al., observed that almost all high responders reported a low incidence of bleeding episodes- less than one per patient-month- with 19% reporting no bleeding episodes at all [22].

Despite fewer bleeding episodes overall, patients with inhibitors experienced more life-threatening bleeding events, such as Central Nervous System (CNS) and Gastrointestinal (GI) bleeds, compared to patients without inhibitors. Similar findings were reported by van Velzen AS et al., who noted increased complications, treatment costs, and disability in these patients [23].

These observations may be explained by the fact that fewer bleeding episodes lead to fewer exposure days to replacement products, thereby increasing the likelihood of inhibitor development. Thus, the lower frequency of bleeding may paradoxically contribute to inhibitor formation. However, when bleeding occurs in patients with inhibitors, it cannot be effectively controlled with conventional replacement therapy, resulting in a higher incidence of severe and life-threatening bleeding episodes.

Additionally, present study observed that the two patients who developed TTIs were the oldest participants in the study. They had been diagnosed with haemophilia A many years earlier and had received multiple units of whole blood and Packed Red Blood Cells (PRBCs) during their early years, when FVIII concentrates, FFP, and cryoprecipitate were not widely available or accessible. These patients may have received blood products during a period when donor screening and viral inactivation were not strictly implemented. The incidence of TTIs, particularly HCV, has been associated with the number of blood units transfused, which was higher in these two patients compared to others. Zhubi B et al., also reported hepatitis C as the most common TTI [24].

Limitation(s)

As the population sizes of the HTI and LTI subgroups were small, the statistical power was limited. Studies with larger sample sizes are needed to better assess the significance of individual risk factors and to narrow confidence intervals in strength-of-association analyses. Additionally, due to the cross-sectional design, follow-up of patients with inhibitors was not possible to determine whether inhibitors were transient or persistent. Two important hypothesised factors- genetic mutations in the FVIII gene and family history of inhibitors- could not be evaluated due to lack of infrastructure.

CONCLUSION(S)

The likelihood of FVIII inhibitor development increases with earlier age at diagnosis, earlier age at first exposure, and use of FVIII alone as replacement therapy. The presence of inhibitors significantly increases the risk of life-threatening bleeding episodes, and HTI are associated with a greater risk of severe bleeding.

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