THE PREVALENCE OF AND RISK FACTORS ASSOCIATED WITH DEVELOPMENT OF FACTOR VIII INHIBITORS IN PATIENTS WITH HEMOPHILIA A SEEN AT THE KENYATTA NATIONAL HOSPITAL

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A research proposal submitted in the Department of Human Pathology in partial fulfilment of the requirements for the award of the Master of Medicine in Human Pathology at the University of Nairobi.

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STUDENT'S DECLARATION

I hereby declare that this is my original work under the guidance of my supervisors and has not been presented to the University of Nairobi or anywhere else for review and approval.

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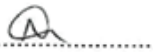
Signature  Date.....1/08/2022.....

SUPERVISOR'S DECLARATION

We confirm that the work reported in this dissertation was carried out by the candidate under our supervision. It is submitted for the award of Master of Medicine Degree in Human Pathology from the University of Nairobi with our approval.

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DEDICATION

To myself, with pride and relief. So glad you are almost done.

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I wish to acknowledge God, for granting me exceptional grace and patience while handling this book.

To my supervisors Dr Kibet, Dr Magutu and Dr Matilda for your guidance and time spent on my work, many thanks.

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LIST OF ABBREVIATIONS

KNH	Kenyatta National Hospital
SPSS	Statistical Package for the Social Sciences
UoN	University of Nairobi
WHO	World Health Organization
FVIII	Factor VIII
FVIII:C	Factor VIII activity
APTT	Activated partial thomboplastin time
$CaCl_2$	Calcium chloride
IU/dl	International units per decilitre
BU	Bethesda units
CANAL	The Concerted Action on Neutralizing Antibodies in Severe Hemophilia A
RODIN	The Research of Determinants of Inhibitors Formation
MIBS	Malmo International Brother Study
APTT	Activated Partial Thromboplastin Time
HIRS	Hemophilia Inhibitor Research Study
QALYs	Quality Adjusted Life years
DALYs	Disability Adjusted Life Years

ABSTRACT

Background

Hemophilia A is a global challenge affecting 1 in 5000 males. Up to 5% of the hemophiliacs on treatment develop inhibitors which neutralize infused factor concentrates reducing the effectiveness of treatment and predisposing the patient to life threatening complications. Kenyatta National Hospital (KNH) is a major treatment centre nationally attending to two thirds of the hemophilia patients registered in Kenya. Currently, routine screening of factor inhibitors is not practiced in Kenya hence the prevalence and associated risk factors are unknown. The aim of this study was to determine the prevalence of and risk factors associated with the development of Factor VIII inhibitors in patients with Hemophilia A at KNH. This will enable early detection and comprehensive management to avert life threatening bleeds, physical disability and reduce morbidity and mortality.

Study Objective

To determine the prevalence of and selected risk factors associated with the development of Factor VIII inhibitors in patients with Hemophilia A seen at Kenyatta National Hospital.

Methodology

This study was cross sectional, done at Kenyatta National Hospital using consecutive sampling. Clinical and socio-demographic data was collected using a questionnaire and blood samples were drawn for inhibitor testing. The study was carried out from January 2021 to January 2022. Data was entered in a secure database prior to exporting to Statistical Package for social Science version 21 for analysis. Prevalence of inhibitors in Hemophilia A patients was reported in proportions and univariate and multivariate analysis was used to analyze the selected risk factors associated with development of FVIII inhibitors in patients with Hemophilia A in KNH.

Results

The prevalence of FVIII inhibitors in the 61 patients tested with Hemophilia A seen at KNH was found to be 19.7% (n=12). All these patients had low titer inhibitors and severe hemophilia. Half of those with inhibitors were between 10 and 19 years. The risk factors associated with the development of inhibitors found that when age at first treatment was less than one year, one was five times more likely to develop inhibitors compared to those who received treatment for the first time when above fifteen years. Of those who had inhibitors 33.3% had received an average dose of 1000IU of factor. Fifty percent of those who had minor surgery developed inhibitors and had

odds of 0.7 times less likely to develop inhibitors. Regarding medical history, 91.7% of those who had trauma and major bleeds were 3.6 times more likely to develop inhibitors. These findings were however not statistically significant.

Conclusions

The prevalence of Hemophilia A inhibitors in KNH patients is high 19.7% (n=12). Several risk factors were found to be associated with the development of inhibitors in this study are exposure to trauma, major bleeds and age at first treatment less than 1 year.

Recommendations

Routine testing of inhibitors to be standard of care in Hemophilia management across Kenya.

Create more awareness on the existence of inhibitors in Hemophilia to avoid possible triggers by use of unnecessary blood products or unplanned surgeries. There needs to be a multidisciplinary approach in management of hemophiliacs to avoid risk of inhibitor development.

Carry out health economic assessments on QALYs and DALYs to determine the actual socioeconomic burden of those with inhibitors to influence policy on affordable care like essential drugs for management of inhibitors in hemophilia A.

1.0 CHAPTER ONE: INTRODUCTION

Hemophilia is a disorder of bleeding caused by deficiency of coagulation of factor VIII (Hemophilia A) and factor IX (Hemophilia B). Its inheritance is X-linked. The most common type is Hemophilia A (Classic Hemophilia) caused by F8 gene mutations resulting in complete lack of or defective factor VIII (FVIII) (1). Due to its inheritance pattern, it occurs predominantly in males and rarely in women. Patients with hemophilia suffer from life threatening bleeds and require constant replacement of the deficient factor which is largely unavailable. A recent meta-analysis by Iorio et al in 2019 shows the global prevalence of the disease at birth to be much higher than previously anticipated at 1,125,000 cases of which 418,000 have severe disease (2). Hemophilia A treatment requires replacement of FVIII using either plasma derived or recombinant factor given intravenously to achieve hemostasis. One of the most serious complications of treatment is development of inhibitors (1).

Inhibitors are antibodies that neutralize infused factor making it insufficient for treatment hence requiring less effective yet more expensive treatment modalities (3). Inhibitor levels are measured using Bethesda units (BU) and classified into high (>5BU) and low titre (<5BU). High titre inhibitors are the commonest at 60%. The documented prevalence of inhibitor formation in all cases of hemophilia A lies between 5-7% with a prevalence of up to 13% when limited to severe disease (4).

There are various risk factors affecting development and detection of inhibitors that can broadly be divided into modifiable and non-modifiable (5). Screening for inhibitors is not routine in most centres in Kenya hence the prevalence and associated risk factors are unknown. This study determined the prevalence of and selected risk factors for development of factor VIII inhibitors in patients with hemophilia A at KNH to enable early detection and comprehensive management to avert life threatening bleeds, physical disability and reduce morbidity and mortality.

2.0 CHAPTER TWO: LITERATURE REVIEW

2.1 Background

Normal individuals have FVIII levels ranging from 5-40 IU/dl for normal hemostasis to occur. Patients with Hemophilia A are deficient in FVIII and are classified based on their levels of residual FVIII activity into mild, moderate and severe. Mild cases have >5% of normal factor activity (usually between 5-40 IU/dl), moderate 1-5% factor activity (1-5 IU/dl) and severe cases have less than 1% of normal factor activity (<1 IU/dl) (6). Residual factor levels determine the phenotypic presentation of hemophiliacs where those with severe type bleed spontaneously and the non-severe types bleed after trauma or procedures (7).

In management of hemophilia A, bleeding is prevented or treated by replacement of factor VIII by giving recombinant or plasma derived factor concentrates. Severe cases may require prophylactic factor replacement to avoid life threatening hemorrhage. In non-severe cases administration of desmopressin which stimulates endogenous FVIII release by inducing release of Von Willebrand factor (VWF) from weibel palade bodies in endothelial cells is used. VWF is a carrier for FVIII (8). Inhibitor formation is a major complication of hemophilia treatment. It results in lack of clinical response from infused factor hence a risk of significant morbidity and mortality.

Inhibitors are antibodies against FVIII specifically, polyclonal high affinity immunoglobulin G (IgG). They are mostly of the IgG_4 subtype and do not fix complement (3). The mechanisms that lead to these inhibitors have been explored over the past decade and shown to be multifactorial involving cytokines, cells and immune regulatory molecules. There are various risk factors postulated for development of inhibitors in various studies. The aim is to find a non-immunogenic hemostatic option in future, but, in the meantime an accurate stratification of those likely to get inhibitors will help in guiding their management while avoiding complications (9).

2.2 Epidemiology

The World Federation of Hemophilia (WFH) Global Survey 2018 documents 4% of those reported to have hemophilia A have inhibitors (10). A systematic review by Witmer and Young in 2013 detailing inhibitor epidemiology in hemophilia A reports an overall prevalence of 5-7% with a higher rate of 12-13% in severe disease. Sixty percent being high titre inhibitors (>5BU) with the rest being low titre (<5BU) (3). There are few studies in Sub-Saharan Africa

regarding inhibitors mostly due to poor access to care and limited screening (11). In 2014 Balagog et al in Cameroon reported a prevalence of inhibitor development of 18.5% in Hemophilia A and 30% in severe disease. Seck et al in Senegal reported a similar prevalence of 20% and in North Africa, 11% was reported (12). According to the WFH Global Survey 2019 only 1.5% of those screened in Kenya were found to have inhibitors (13). These numbers may be much higher than reported as inhibitor testing is not routinely done in the country and those screened were already showing clinical symptoms of inhibitors as evidenced by treatment failure.

2.3 Pathogenesis

To initiate an immune response that results in high affinity polyclonal antibodies to FVIII there are various immunologic steps. Initially, the infused molecule undergoes endocytosis by antigen presenting cells like macrophages, B cells and dendritic cells. This antigen is processed and the peptides presented on the cell surface to a CD4+ T cell. Previously untreated patients use dendritic cells while those previously exposed use B cells as their antigen presenters. Once the CD4+ T cells are activated they stimulate antigen specific B cells to differentiate into plasma cells that secrete antibodies or memory B cells. Additionally, to activate the CD4+ T cells to do the above, some triggers are required. These are termed danger signals and are released through cell death, tissue damage, systemic inflammatory response and stress. They entail interleukins, heat shock proteins, reactive oxygen species, growth factors and adenosine triphosphate. The high affinity polyclonal antibodies to FVIII secreted by the plasma cells are mainly of the IgG_4 subtype. They recognize epitopes located on the A2 and C2 domains of the FVIII and neutralize the factor through steric hindrance or immune complex formation rendering it ineffective (9). Inhibitors bound to these epitopes interfere with FVIII or FIX binding and also block tenase activity. There is increased proteolytic clearance of FVIII (14).

Studies postulate a T cell independent pathway for immune response to FVIII to be responsible for the formation of non-neutralizing antibodies. As to whether they have any clinical significance is still debatable. Many patients develop inhibitors within 9-12 exposure days where one exposure day is one unit of time in which a Hemophiliac is given factor replacement. A study by Witmer and Young in the UK revealed a bimodal peak of inhibitor development risk in early childhood and in elderly people (3).

FVIII inhibitors are classified based on extent of inhibition and kinetics. Type I inhibitors completely neutralize FVIII in a linear fashion depending on the dose. This completely

inactivates FVIII. These occur more in severe Hemophilia (15). Type II inhibitors follow do not completely inactivate factor and occur mostly in mild hemophilia or those without hemophilia but develop an acquired inhibitor of FVIII (5).

2.4 Risk factors for development of inhibitors

There are several hypotheses to what risk factors lead to inhibitor development. This risk varies through life although evidence suggests a majority of inhibitors develop in severe hemophiliacs at an average of 1-2 years after 9-12 treatments. The peak in risk is noted within the first 50 exposures to factor and this risk reduces greatly after 200 treatment days (16). Up to thirty percent of severe hemophiliacs are affected by inhibitors. They develop them between the 5-50th FVIII infusion although can still occur later. An estimated 5-8% of mild to moderate hemophiliacs develop inhibitors (5).

Risk factors can broadly be divided into modifiable and non-modifiable factors. Modifiable factors include the duration and frequency of factor exposure, type of factor product, presence of danger signals and age at first treatment. These factors are environmental and can allow possible intervention and prevention of inhibitor development by eliminating the influence. Non-modifiable factors entail genetic mutations, a positive family history of inhibitors, ethnicity and gene polymorphisms. If these are found early, they are amenable to modified therapy towards more targeted treatment.

2.4.1 Modifiable risk factors

Type of factor product

Choice of factor replacement plays a role in inhibitor development. Recombinant factor is found to have higher risk for inhibitor development over plasma derived product. This increased immunogenicity is proposed to be secondary to changes in posttranslational modifications of FVIII and a lack of binding to Von Willebrand (5). Plasma derived products that contain VWF are said to competitively inhibit inhibitor binding as they both bind to the C2 domain. VWF also increases the half-life of FVIII. A randomized control study by Guelcher et al found 26.8% incidence of inhibitor development as compared to 44.5% for those on recombinant products. When limited to high titre inhibitors, this incidence was 18.6% and 28.4% for plasma derived versus recombinant product respectively (17).

Danger signals

The danger theory may explain the association between development of inhibitor and treatment-related risk factors (18). This theory coined by Matzinger states that factor VIII itself is not enough to activate antigen-presenting cells, but that substances released when tissue is damaged called “danger signals” are required to induce an effective immune response against FVIII. Major bleeds, trauma and surgery cause inflammation that results in release of cytokines that activate` antigen presenting cells which present FVIII as an antigen and upregulate co-stimulation to T lymphocytes. This increases formation of antibodies by B lymphocytes (7). The Concerted Action on Neutralizing Antibodies in Severe Hemophilia A (CANAL) study conducted in multiple hemophilia centres on 366 patients born between 1990 and 2000 found a 3.7 relative risk in those with prior history of surgery to develop inhibitors. Another similar study by Gouw et al showed at 2.7 relative risk for those who have had surgery (19).

Age at first treatment

As regards age at first treatment, several studies show incidence of inhibitor development was higher in hemophiliacs initiating treatment before 6 months old than for older pediatrics. The CANAL study again demonstrated the rate diminished from 41% in those treated before the age of six months to 18% in those on therapy after eighteen months (20). Another study by Gouw et al in 2007 found that the incidence of developing inhibitors when young depends on the treatment intensity where the more intense the treatment the more likely one is to develop inhibitors.

Prophylactic treatment

Prophylactic treatment of hemophilia is associated with a decreased risk of inhibitors when compared to treatment on demand. Early start of this prophylaxis reduced immunological signals and was seen as a form of immune tolerance. A prospective observational study titled The Research of Determinants of Inhibitors Formation (RODIN) composed of 574 previously untreated patients showed that this treatment decreased the risk of inhibitors in patients with low risk F8 mutations (missense, small deletions, insertions and splice site mutations) than those with high risk mutations (20).

2.4.2 Non-modifiable risk factors

Genetic mutations

Genetic mutations as a risk factor for inhibitors can broadly be divided into 2 categories: Severe molecular defects or null mutations where there is complete failure of FVIII protein production. They entail large deletions, intron 22 inversions and nonsense mutations. The prevalence of developing an inhibitor in these null mutations ranges from 21% for intron 22 inversions to 88% for large deletions. Milder molecular defects are those which have some loss of function of the FVIII protein but retain some production. These account for less than 10% incidence (16).

The risk of developing FVIII antibodies strongly relates to severe molecular defects and is less in the milder molecular defects (5). There is however some discordance in patients with similar genetic mutations in F8 gene including siblings hence the importance of factoring in other factors than genetic factor involvement.

Family history

Family history of inhibitor is an established risk factor for developing inhibitors. According to the Malmo International Brother Study (MIBS) done on 113 families where two or more members had severe Hemophilia A in 2005, there is 48% higher risk in families with history of an inhibitor. The CANAL study showed 3 times increased risk in these patients (7).

Ethnicity

As pertains ethnicity, the Hemophilia Inhibitor Research Study done in the US in 2012 regarding national surveillance of inhibitors shows twice the risk in Blacks and Hispanics in the United States of America. The MIBS study showed the risk in Africans to be 56% and that in Caucasians 27%. The H3 and H4 haplotypes were a proposed reason as these were only found in Africans though the exact cause is still under investigation (5).

Gene polymorphisms

According to the MIBS study, gene polymorphisms in production of cytokines (Interleukin 10, TNF α and CTLA-4) are implicated in higher risk of inhibitor development. One's major histocompatibility complex phenotype is codependent and predicts if an immune response will be mounted to the foreign FVIII molecule.

2.5 Lab assessment of inhibitors

Inhibitors are suspected when there is prolonged bleeding in a patient with no prior history of bleeding diathesis, failure of factor replacement to correct the bleed or prolonged activated partial thromboplastin time (APTT). Lab assessment of this bleeding patient entails the routine blood counts to assess platelets. This is followed by a coagulation screen where the APTT is mostly prolonged then a mixing test. The suspicion of FVIII inhibitor is sustained by a slow acting (60-120 min) non-correction when incubated at 37 degrees Celsius. This rules out lupus anticoagulant as an inhibitor due to its immediate non-correction upon mixing (15). This then gives way to specific factor inhibitor titres.

Methods for detection and quantification of inhibitors include Bethesda Assays or the Nijmegen-modified Bethesda assay (21). The latter has three modifications that allow it to vary from the classic Bethesda assay which entail: use of immune depleted FVIII plasma in place of imidazole buffer as a diluent, buffer normal pool plasma with imidazole at 0.1M and adjust the pH to 7.4 with hydrochloric acid then mixing the normal pool plasma control with plasma deficient in FVIII in place of imidazole buffer (15). These methods only detect inhibitory inhibitors (those decreasing the activity of FVIII) as these are the clinically relevant ones.

Measurement of FVIII inhibitor is done using the Bethesda Assay, a method standardized since 1975. A mix of patients' plasma with pooled normal plasma is incubated for 2 hours then the comparing the remaining FVIII activity with a control mixture. The residual activity in percentage in the patient mix is converted to Bethesda units (BU). One BU is defined as the amount of inhibitor producing a residual activity of 50% (21). The end result is divided either into high titre inhibitors more than 5 BU or low titre inhibitors of less than 5BU.

Patients with inhibitors are stratified as high or low responders, depending on the extent of anamnestic response on repeated exposure to FVIII. High responders develop an increased level of antibodies within a few days of repeated exposure to FVIII and low-responders only have minimum increase after repeated exposures. The International Society of Thrombosis and Hemostasis (ISTH) goes by a cutoff of 5 BU per ml where <5 are low responding and >5 are high responding inhibitors. Many inhibitors develop within 50 exposures where an exposure is an infusion of FVIII product within a 24-hour period.

Transient inhibitors are defined as a positive inhibitor that falls below the definition threshold within 6 months of initial occurrence despite continued antigenic challenge with FVIII (22).

2.6 JUSTIFICATION

The development of inhibitors in hemophilia A patients has been found to have a significant rise in cost of living and a reduction in health-related quality of life. Effective care has been shown to result in satisfactory quality of life (23). A study by Café et al in Portugal found hemophilia A to be responsible for 784 Disability Adjusted Life Years (DALYs) in patients with inhibitors(24). Those with inhibitors develop pain and excessive bleeding episodes that result in life threatening hemorrhage hence increased morbidity and mortality. There are various risk mitigation strategies once the possible triggers are known and better treatment alternatives like immune tolerance induction, bypassing agents or novel therapies with monoclonal antibodies. This care can only be provided once the actual numbers of those with inhibitors and their risk factor profile is known in our population.

The aim of this study was to determine the prevalence of inhibitors in Hemophilia A and selected risk factors at one of the largest hemophilia treatment centres in Kenya. The findings inform on the existing numbers of those with inhibitors and explore the feasibility of establishing routine surveillance for inhibitors in all treatment centres. Knowledge of existing risk factors in our population will enable future mitigation of modifiable risks and early identification and management of those with non-modifiable risks to avert life threatening bleeds.

The results of this study will reflect current hemophilia care practices and may be used to create standardized policies on care of hemophiliacs all over Kenya.

2.7 **RESEARCH** QUESTION

What is prevalence of and selected risk factors associated with development of Factor VIII inhibitors in patients with hemophilia A at Kenyatta National Hospital?

2.8 STUDY OBJECTIVES

2.8.1 Broad objective

To determine the prevalence of and selected risk factors associated with development of Factor VIII inhibitors in patients with hemophilia A at Kenyatta National Hospital.

2.8.2 Specific Objectives

1. To determine the prevalence of FVIII inhibitors in patients with hemophilia A at Kenyatta National Hospital.
2. To determine the prevalence of selected risk factors associated with the development of FVIII inhibitors in patients with hemophilia A seen at Kenyatta National Hospital which include: -

    2.8.2.1 Severity of hemophilia

    2.8.2.2 Family history of inhibitors

    2.8.2.3 Reason for receiving factor

    2.8.2.4 Type of factor product used

    2.8.2.5 Age at first treatment

    2.8.2.6 Current dose of factor

    2.8.2.7 Any history of surgery, trauma, severe bleeds, febrile illness or blood transfusion.

3.0 METHODOLOGY

3.1 Study Design

This was a cross-sectional study to determine the prevalence of and selected risk factors of FVIII inhibitors in patients with hemophilia A.

3.2 Study site and setting

The study was carried out at the KNH Hemophilia Comprehensive Care Clinic, KNH in-patient wards where any hemophiliacs were admitted and the samples processed in the hematology lab at KNH.

Kenyatta National Hospital is the largest public teaching and referral hospital in Kenya located along Hospital road in Upperhill area of Nairobi. It has a specialized hemophilia comprehensive care clinic that provides care for up to two thirds of the registered hemophiliacs in the country, mostly low and middle income earners, where confirmatory diagnosis, treatment and follow-up is done. The Kenya Hemophilia Association (KHA) was established in Nairobi in 1979 and is recognized by the World Federation of Hemophilia (WFH) as the organization in charge of providing clinical support, care and management of those with bleeding disorders in Kenya. It has provided an umbrella covering six treatment centres for hemophilia around the country of which KNH is the largest. All hemophiliacs both in and out patient are attended through this clinic where factor replacement is given to those in need whether as out-patient or during admission. The admitted patients end up in various wards depending on whether male, female, pediatric, adult, surgical or medical cases. The Hemophilia Comprehensive Care Clinic is then notified to facilitate Factor VIII replacement in these patients. Approximately 300 patients with inherited bleeding disorders are seen at this clinic, 75% of which have hemophilia A. The clinic runs 5 days a week attending to Hemophiliacs two thirds of whom are on factor replacement. Routine testing for inhibitors is not done.

3.3 Study duration

January 2021 to January 2022

3.4 Study population

Patients with confirmed hemophilia A seen at the Kenyatta National Hospital.

3.4.1 Selection criteria

The following criteria was used to determine eligibility.

Inclusion criteria: Patients of all age groups with confirmed hemophilia A on factor replacement seen at KNH who gave informed consent, parental or guardian consent and assent where applicable.

Exclusion criteria: Patients with acquired hemophilia as indicated by their history.

3.5 Sample size

To determine the sample size, Cochran's formula was adopted and as the hemophilia population is finite, it was corrected for a finite population (25). A study in Cameroon with demographics similar to ours which had a prevalence of 18.5% was used for estimation of population size (12). As per KNH records, approximately 22 Hemophilia A patients are attended to per month through the Hemophilia Clinic. The total number recorded in the three-month study period was estimated to be 66. This number was used to enable us attain an achievable sample size given the study timeline. Using a 20% non-response rate, a representative sample was calculated.

$$n_0 = Z^2pq/d^2$$

$$n = \frac{n_0}{1+ \frac{(n_0 - 1)}{N}}$$

Where:

n_0 = initial estimated sample size
Z = standard normal deviate at 95% confidence interval (1.96)

p = prevalence of inhibitors in hemophilia A patients in Cameroon 18.5%

q = 1-p

d = precision (0.05)

N = Total population of Hemophilia A patients on factor VIII replacement recorded in three months in KNH (66)

$$n_0 = \frac{1.96^2 * 0.185\ (1-0.185)}{0.05^2} = 232$$

$$n = \frac{232}{1+ \frac{(232 - 1)}{66}} = 52$$

Final sample size anticipating for a 20% non-response rate was calculated.

Final sample size = effective sample size / (1 - non-response rate)

= 52 / (1-0.2)

= 65

3.6 Sampling procedure

Consecutive sampling method was used where all those eligible for the study were recruited until the desired sample size was achieved. Through the Hemophilia Comprehensive Care Clinic, all hemophilia A, both in and out-patient were recruited by the principal investigator or research assistant. They were informed what the study is about, its benefits and risks to them and requested to participate through giving written informed consent, parental consent and assent where applicable. Those who consented were enrolled in the study.

3.7 Variables

The independent variables that we measured included sociodemographic data like age, gender, ethnicity and residence. Clinical features included severity of hemophilia, family history of inhibitors, reason for receiving factor, type of factor product used, age at first treatment, current dose of factor and any history of surgery, trauma, severe bleeds, febrile illness or blood transfusion.

Dependent variables were the presence or absence of inhibitors among the study population. If present, the inhibitors were stratified into low and high titre.

3.8 Data collection

3.8.1 Clinical Procedures

Clinical procedures entailed recruitment of patients with hemophilia A from the Hemophilia Comprehensive Care Clinic where both in and out patients were recruited. Patients were informed what the study entails, its risks and benefits and allowed to freely consent (Appendix 3) or assent (Appendix 4) to participate in the study. A questionnaire (Appendix 2) was administered by the principal investigator or the research assistant collecting both demographic and clinical data and venous samples were drawn after.

3.8.2 Laboratory procedures

Laboratory procedures were carried out at the KNH hematology lab where the collected samples were tested for Factor VIII assays and inhibitor screening. A Bethesda assay was done on those found positive for the screen.

3.9 Specimen management

3.9.1 Specimen collection and transport

Three milliliters of venous blood were collected into a blue capped sample bottle (3.2% buffered sodium citrate) through a clean venipuncture site by the investigator ensuring minimal stasis as per the phlebotomy standard operating procedure (Appendix 4) prior to factor replacement therapy at the hemophilia clinic. It was then gently mixed 5 times by inverting the tube. The sample was transported to the hematology lab within thirty minutes.

3.9.2 Specimen processing

Specimen was immediately separated into platelet poor plasma which was then aliquoted into 2 separate vials of equal volumes then frozen immediately (no longer than 4 hours after collection) at ≤ -80° C awaiting to run tests in batches. During analysis, plasma was thawed at room temperature then mixed before analysis. A one stage APTT based factor assay (Appendix 5) was used to determine FVIII concentration using the Thrombolyzer machine. A second aliquot was used to do a circulating inhibitor screen (Appendix 6) where patients plasma was mixed with normal pooled plasma in a 50:50 ratio, incubated for 2 hours and the APTT ran. The presence of an inhibitor was confirmed by a slow acting (in 2 hours) non correction of this test. Those found positive in the screen proceeded to have a quantitative Bethesda assay (Appendix 7). Here, a mixture of patient's plasma with pooled normal plasma was incubated for 2 hours then compared the remaining FVIII activity with a similarly treated control mixture containing buffer and neutral pooled plasma. The percent residual activity in the patient mix was converted to Bethesda units. Patients results were then classified into those with low and high titre inhibitors using a cut off of 5 Bethesda Units.

3.10 Quality assurance

Quality assurance measures were put in place for accuracy and validity of results.

3.10.1 Pre-analytical

The study investigators ensured correct identification of the study participant and there after gave a unique study number. Venous samples were collected after the patient had relaxed with no tourniquet, or if need be applied for less than one minute. Blood was well mixed with anticoagulant and taken to the lab as soon as possible. Samples were labelled clearly, centrifuged and stored at -80°C awaiting analysis.

3.10.2 Analytical

Daily internal quality controls were performed before the factor assay and circulating inhibitor screen tests are done. A calibrated reference assay was used for each assay. Positive and negative controls were used in the Bethesda assay.

3.10.3 Post-analytical

Test calculation was done using a set calibration standard line and interpreted in % form. Transcription errors were avoided by immediate entry of data and observing care.

3.11 Mitigation of covid-19 transmission risk

Due to the COVID-19 pandemic we took the following measures to protect the researchers and study participants from contracting the disease. Before face to face visits all participants were screened for exposure and symptoms using temperature checks and for defining symptoms like fever, cough and difficulty in breathing. In line with the Ministry of Health guidelines there were hand washing stations with water and soap or sanitizers for all participants. There were also posters on the hospital walls reminding everyone to wear a mask and that no one will be allowed entry into the facility without a mask. The researchers and the participants wore 3-ply face masks correctly at all times while interacting. Physical distancing of a minimum of 1.5 meters was observed. Staff used appropriate personal protective equipment like a pair of gloves per patient when acquiring samples and disposed them accordingly into the hazard waste basket.

3.12 Data management

Data collected was entered in Microsoft Excel and analyzed using SPSS version 21. Patient and clinical characteristics that are categorical were presented as frequencies and proportions, and those that were continuous were presented as means with standard deviation or as median with interquartile range. The prevalence of FVIII inhibitors in patients with Hemophilia A was presented as a percentage of those with the inhibitors over the total sample size. Univariate and multivariate analysis was used to analyze the selected risk factors associated with development of inhibitors using chi square and logistic regression. Odds ratio with 95% confidence intervals were reported where applicable. All statistical tests were considered significant where $p<0.05$.

3.13 Ethical consideration

The study protocol was submitted for approval to the KNH/UON Ethics and Research Committee. Study participation and blood sample collection was carried out only after obtaining a written informed consent, parental/guardian consent and assent where applicable. Study participants had the autonomy to decline participation.

Study participation was on voluntary basis and no form of incentives was given to the participants.

Confidentiality was ensured when conducting interviews and no personal identifying data was be recoded, rather serial labels were used. Total confidentiality was maintained throughout the study results handling process. Raw data was stored in a password protected computer and written records in lockable cabinets then destroyed after 5 years by shredding. Only the study investigators had access to this data.

Phlebotomy in hemophiliacs is standard practice all over the world since they get tested for factor levels frequently. Any risk of bleeding was mitigated by applying pressure on puncture site until the bleeding stopped. Most patients were to receive factor replacement after the procedure so this further averted any risk of bleeding. Patients were observed temporarily before leaving.

Measures were taken during the study to ensure the participants and the researcher and assistants are protected from COVID-19 during this time of pandemic as mentioned in the methodology. The study benefited the participants as each got free factor levels and inhibitor testing. The study findings will contribute to knowledge and improvement of hemophilia care in Kenya.

Any changes in protocol or study site were notified to the committee.

4.0 CHAPTER FOUR: RESULTS

4.1 Study participant characteristics

Seventy patients were eligible to participate in this study as per the selection criteria, 64 were enrolled and 6 were excluded as their parents were not available to give consent. All the 64 had congenital hemophilia A and were being followed up at the Hemophilia Comprehensive Care Clinic at Kenyatta National Hospital. Out of these 64, samples that were viable for assessment for laboratory were 61 as 3 samples were haemolysed. Study participants were enrolled using consecutive sampling.

Table 1: Study Participant Characteristics (N=64)

CHARACHTERISTIC	FREQUENCY (n)
Age distribution (years)	
<10	20.3% (13)
10-19	45.3% (29)
20-29	20.3% (13)
30-39	9.4% (6)
40-49	3.1% (2)
50+	1.6% (1)
County of Residence	
Nairobi	51.6% (33)
Kiambu	18.8% (12)
Kajiado	7.8% (5)
Muranga	7.8% (5)
Machakos	6.3% (4)
Garissa	1.6% (1)
Kisii	1.6% (1)
Marsabit	1.6% (1)
Nyandarua	1.6% (1)
Nyeri	1.6% (1)
Participant knowledge on severity of Hemophilia	
Mild	13 (20.3%)
Moderate	29 (45.3%)
Severe	22 (34.4%)

In this study the participants were all male aged between 4-60 years. The mean age was 17.9 years. The participants originated from ten counties around Kenya, majority being in Nairobi. There was notable under-representation from Western, Coast and Nyanza counties.

4.2 Prevalence of inhibitors in Hemophilia A at KNH

Sixty-one participants had their samples assessed in the laboratory for FVIII levels and Inhibitor screening with further Bethesda assays done for those found positive. Three samples out of initial 64 were not suitable for assessment as the samples were haemolysed. Factor VIII Inhibitor was assessed using inhibitor screening and the tests which did not correct were subjected to the Bethesda assay. Prevalence of participants who had FVIII inhibitors was 19.7% (n=12) and 80.3% (n=49) had no inhibitors. All those found to have inhibitors had low titre inhibitors below 5 Bethesda Units and had severe hemophilia. Half of those who had inhibitors were between 10-19 years.

Table 2: Prevalence of inhibitors (N=61)

Age group (years)	**Number of patients % (n)**	**Inhibitor positive % (n)**
<10	21.3% (13)	25% (3)
10-19	47.5% (29)	50% (6)
20-29	21.3% (13)	25% (3)
30-39	4.9% (3)	0
40-49	3.3% (2)	0
50 +	1.6% (1)	0

4.3 Prevalence of selected risk factors assessed for development of FVIII inhibitors

Figure 1 illustrates the reported knowledge of the participants regarding any family history of inhibitors where 65.6% of the participants had no knowledge of the existence of inhibitors.

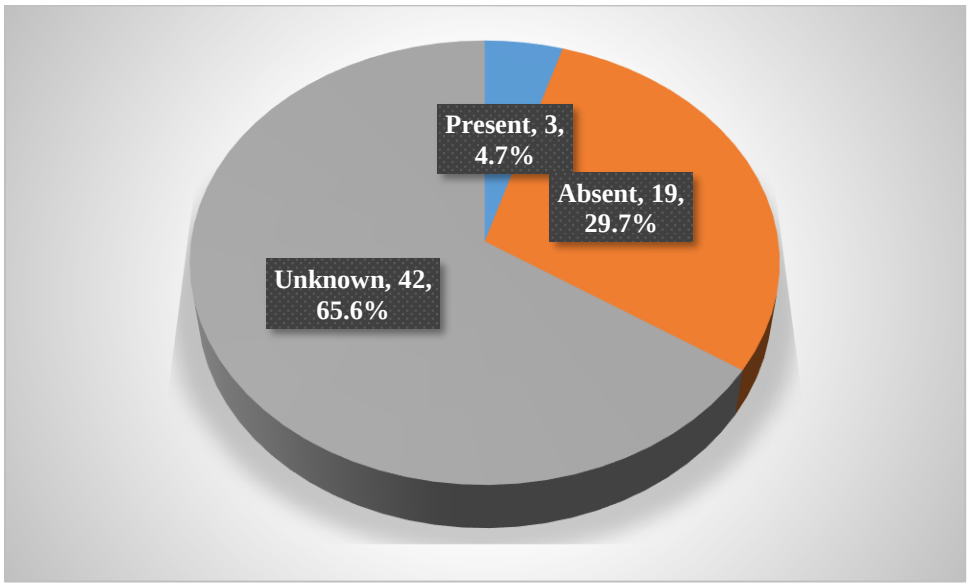

Figure 1: Family history of inhibitors (n=64)

Participants who were receiving factor for prophylactic reasons were 92.2% on demand and 7.8% were on factor for prophylactic reasons.

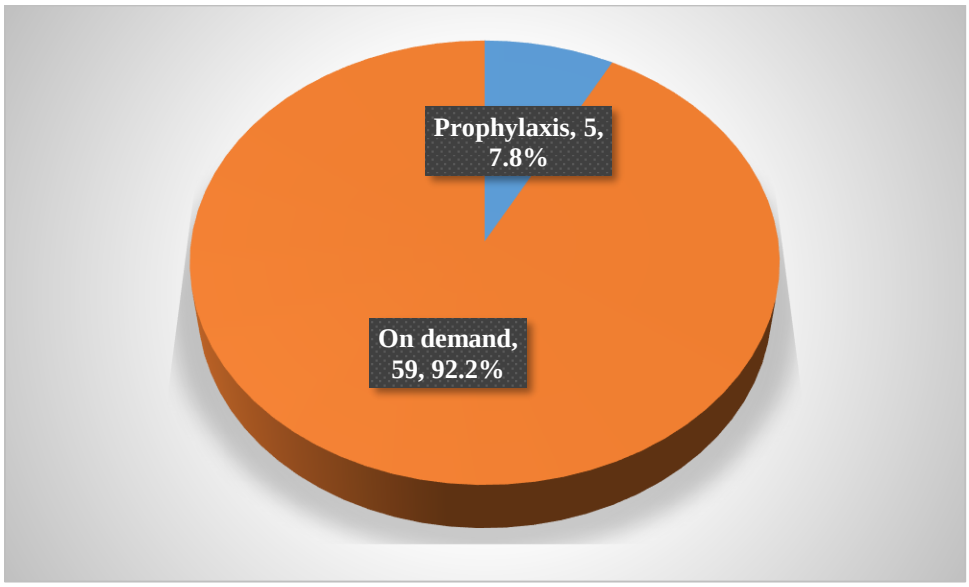

Figure 2: Reason for receiving factor (n=64)

Type of Factor VIII concentrate received was 93.8% recombinant and 6.2% had a history of having received both recombinant and plasma derived factor.

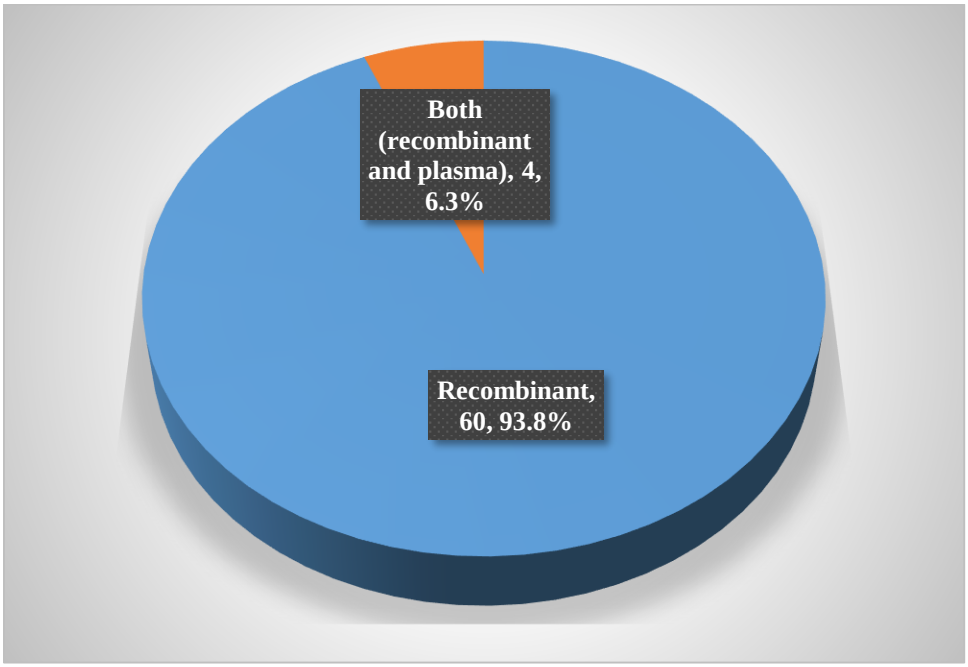

Figure 3: Type of Factor product received (n=64)

Figure 4 shows the dosage of Factor VIII the participants were currently receiving where the dosage ranges from 500-3500IU. Most participants were receiving 1000IU doses.

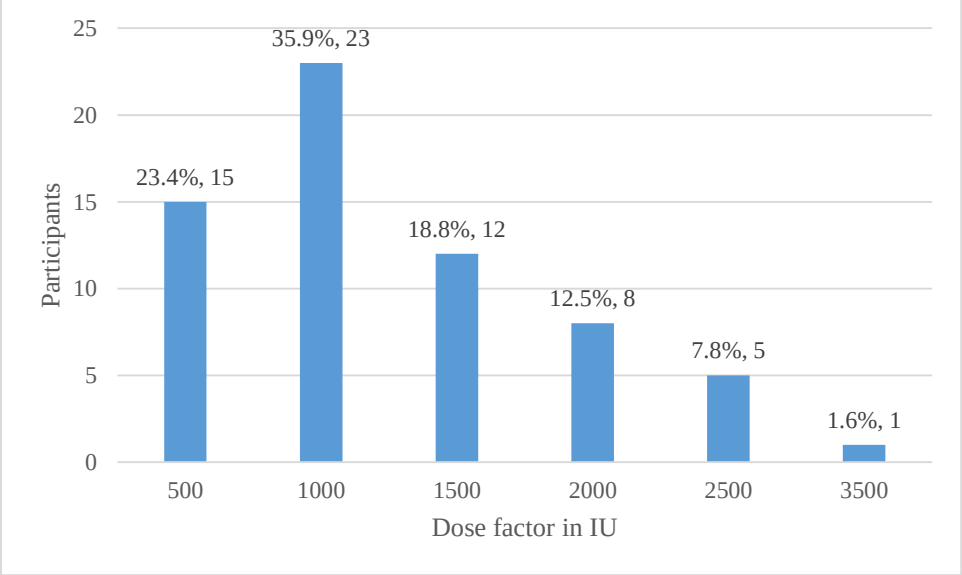

Figure 4: Dosage of FVIII concentrates (n=64)

Participants first received factor at these ages: less than 1year 10.9%, 1-5years 37.5%, 6-10 years 17.2%, 11-15years 7.8%, more than 15 years 17.2% and 6.3% were unsure at what age they first received factor.

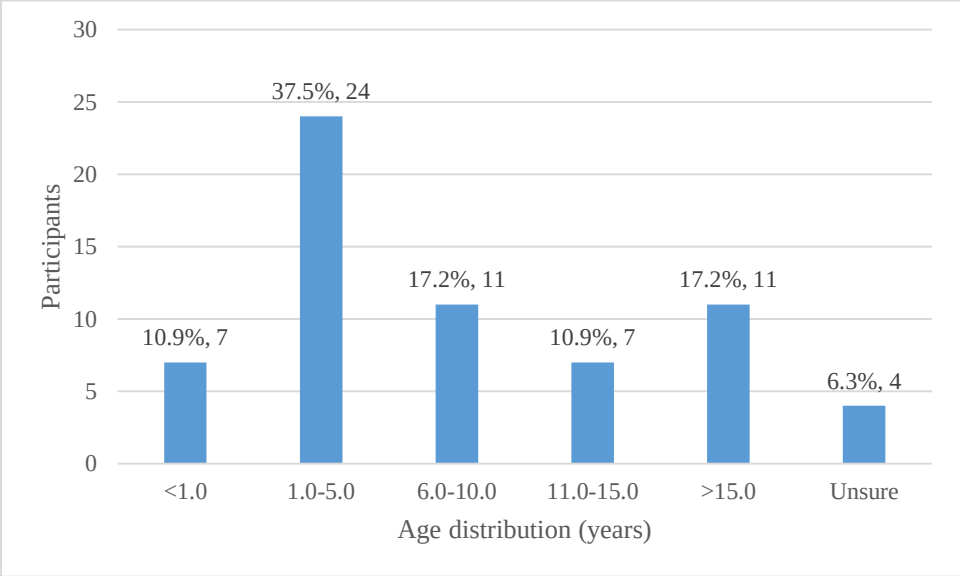

Figure 5: Age at which factor was first received

In past surgical history details, 42.2% had had no surgical procedure done and 57.8% had surgery where 83.8% was minor surgery and 16.2% was major surgery.

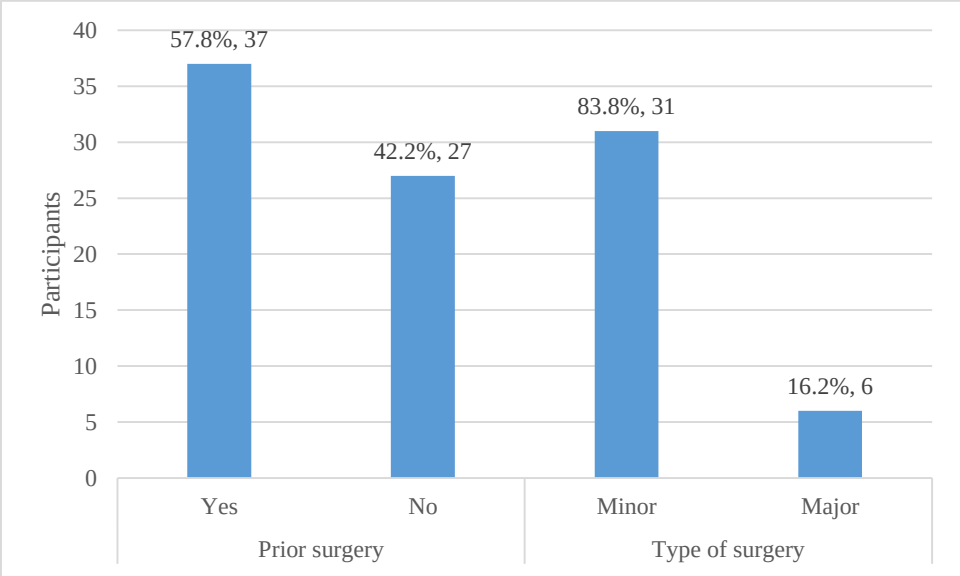

Figure 6: Past surgical history

Medical history of the participants entailed several factors reported like a positive history of trauma in 79.7% of the participants. 37.5% reported having been treated for a febrile illness, 93.8% had major bleeds and 79.7% have received blood transfusion. This is illustrated in the figure below.

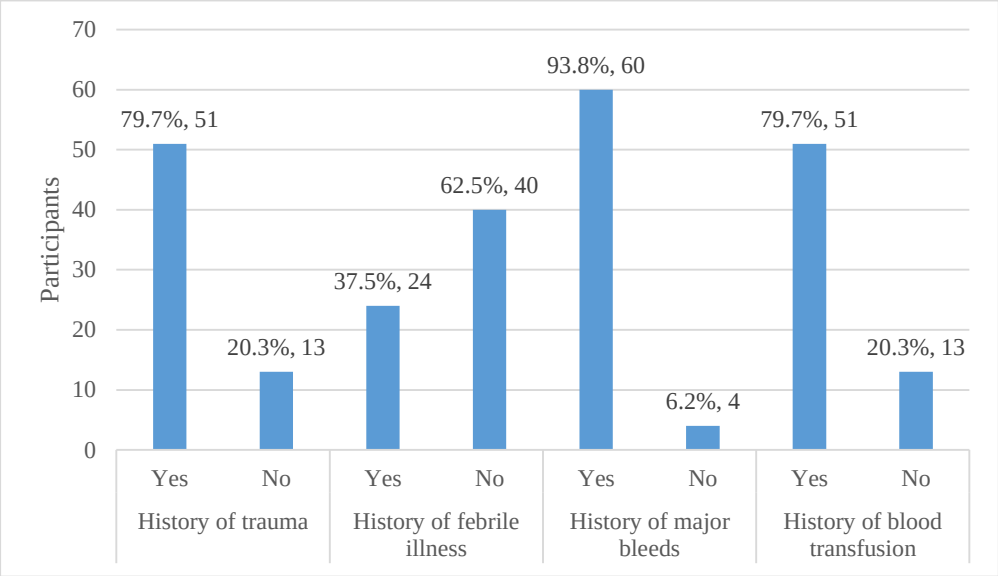

Figure 7: Past medical history

Family history of inhibitors showed that 50% of those with inhibitors had reported the presence of inhibitors in their family as negative. Further associations of odds ratios and significance could not be established as more than half of the participants reported that they did not know about their family history of inhibitors.

Out of those receiving factor for prophylactic reasons, 20% had inhibitors whereas 19.6% of those receiving factor on demand had inhibitors. The odds ratio was 1.0 meaning those on factor for prophylactic reasons were equally as likely to get inhibitors as those on demand. There was however no statistical significance in this parameter.

Participants had either received plasma derived products especially the older patients who then moved to recombinant factor product in the later years or fully received recombinant product. One hundred percent of those on recombinant product were positive for inhibitors. Further associations could also not be carried out here due to lack of any positives in other categories.

Regarding the age at first treatment, participants were stratified into five age groups. Participants positive for inhibitors were found to be mostly less than 1 year of age at 33.3%. These age group was 5 times more likely to get inhibitors compared to those more than 15 years old although no statistical significance was found.

Dose of factor received ranged from 500 to 3500IU and patients were well distributed throughout with 33.3% receiving 1000IU.

Half the participants had had surgery, all of which were minor. The other 50% had had no surgery at all. The odds showed one was 0.7 times less likely to get inhibitors when one had minor surgery than when they hadn't. There was however no statistical significance.

Medical history of the participants looked at their exposure to trauma, where 91.7% of those with positive history of trauma developing inhibitors and the odds showing one who was exposed to trauma is 3.6 times more likely to develop inhibitors. Half of those who had a history of febrile illness had inhibitors with odds of 2.1 times more likely to develop inhibitors if one had febrile illness. All the 58 who had history of major bleeds had inhibitors and 91.7% of those who had history of blood transfusion had inhibitors.

Table 3: Risk factors associated with development of FVIII inhibitors (n=61)

		FVIII inhibitors			
	N	**Yes, *n (%)***	**No, *n (%)***	**OR (95% CI)**	**p-value**
Reason for receiving factor					
Prophylactic	5	1 (8.3)	4 (8.2)	1.0 (0.1 – 10.1)	0.985
On demand	56	11 (91.7)	45 (91.8)	Reference	
Type of factor product used					
Recombinant	57	12 (100.0)	45 (91.8)	-	-
Both	4	0 (0.0)	4 (8.2)		
Age at first treatment					
<1.0	6	2 (18.2)	4 (8.7)	5.0 (0.3 – 71.9)	0.237
1.0 – 5.0	23	5 (45.5)	18 (39.1)	2.8 (0.3 – 27.1)	0.380
6.0 – 10.0	10	3 (27.3)	7 (15.2)	4.3 (0.4 – 50.2)	0.246
11.0 – 15.0	7	0 (0.0)	7 (15.2)	-	
>15.0	11	1 (9.1)	10 (21.7)	Reference	

Dose factor					
500	14	3 (25.0)	11 (22.4)		
1000	23	4 (33.3)	19 (38.8)		
1500	11	2 (16.7)	9 (18.4)		
2000	7	2 (16.7)	5 (10.2)		
2500	5	0 (0.0)	5 (10.2)		
3500	1	1 (8.3)	0 (0.0)		
Surgical history					
Minor	31	6 (50.0)	25 (51.0)	0.7 (0.2 – 2.6)	0.616
Major	6	0 (0.0)	6 (12.2)	-	
None	24	6 (50.0)	18 (36.7)	Reference	
Medical history					
Trauma					
Yes	48	11 (91.7)	37 (75.5)	3.6 (0.4 – 30.6)	0.246
No	13	1 (8.3)	12 (24.5)	Reference	
Febrile illness					
Yes	22	6 (50.0)	16 (32.7)	2.1 (0.6 – 7.4)	0.267
No	39	6 (50.0)	33 (67.3)	Reference	
Major bleeds					
Yes	58	12 (100.0)	46 (93.9)	-	
No	3	0 (0.0)	3 (6.1)	Reference	
Blood transfusion					
Yes	49	11 (91.7)	38 (77.6)	3.2 (0.4 – 27.5)	0.292
No	12	1 (8.3)	11 (22.4)	Reference	

5.0 CHAPTER FIVE: DISCUSSION

This study determined the prevalence of FVIII inhibitors in those with hemophilia A from a study population of 64 participants from January 2021 to January 2022. Sixty-one participants had their samples screened for inhibitors. The prevalence of inhibitors found in this study was 19. 7% (n=12) and all those with inhibitors had low titres ranging from 0.9 to 1.3 Bethesda Units. All our study participants had severe hemophilia with deficient factor levels of between 0.3 to 0.8%. The age distribution of those with inhibitors ranged from 4-29 years with half of those with inhibitors being between 10-19 years.

This study is similar to a study done in Cameroon which found a prevalence of 18.5% (n=38) where 75% of those were low titre inhibitors. If restricted to severe hemophilia cases in their study, where FVIII levels ranged from 0.5 to 0.9%, the prevalence was much higher at 30%. The mean age of participants who had inhibitors was between 2-18 years (12). In Western India, a study by Shah et al found an overall inhibitor prevalence of 20.57% (n=243). Of these 76% had high titre inhibitors and 96% had severe disease. The maximum number of patients who developed inhibitors was between 11 and 30 years (26).

The findings in our study show that there is indeed a high prevalence of inhibitors in our population, unlike that reported by the WFH 2019 at 4% (13). This is probably because inhibitor testing is not routine in our practice. It is also evident that compared to statistics on Caucasians, Africans have higher occurrence of inhibitors (27). This might be attributed to genetics as postulated in the Hemophilia Inhibitor Research Study (HIRS) study where Africans have H3 and H4 haplotypes which are not present in whites. The conventional recombinant FVIII is made from the H1 and H2 haplotypes which are found in all populations hence the presence of additional haplotypes can result in an immune response to administered FVIII molecule (28).

It is evident that a prominent risk factor for development of inhibitors is the severity of hemophilia. The development of inhibitors in patients with severe hemophilia is likely due to repeated exposure to treatment with factor for longer duration hence higher likelihood. It may also be that those who attend the hemophilia clinic frequently are those with severe disease due to increased clinical episodes of bleeding and associated complications hence need for hospital attendance.

All our participants had low titers of <5BU/ml meaning they are more amenable to resolution of the inhibitors using immune tolerance induction which uses higher factor doses. These may

also resolve over time on their own and require repeat testing in 6 months to ascertain they were not transient inhibitors.

Findings on reason for receiving factor product concluded that most of our study participants were on factor only for demand purposes (91%). Eleven out of twelve of those who had inhibitors were in this population. This contrasts the RODIN and CANAL studies that found a decreased incidence of inhibitor development in those treated prophylactically. Prophylactic treatment is said to decrease the formation of inhibitors by inducing immune tolerance. There are said to be less immunologic signals hence no overall inhibitor development occurs in this group. Our local setting has insufficient factor product to adopt this strategy of prophylactic treatment and even the small number said to be on it (n=5) were from a study being recently conducted about prophylactic treatment where factor product was provided via the study funding. Prophylactic treatment is however the WHO recommended standard of care for hemophilia. It may have higher cost implications initially as more factor is required but in the long term has been found effectively cheaper as it improves quality of life and lessens complications (29).

Ninety-three percent (n=57) of the participants in our study were predominantly on recombinant factor products currently and all those who developed inhibitors were from this group. Most studies initially done to compare plasma derived versus recombinant products showed none was more immunogenic than the other until a more recent systematic review depicting one had greater than twice the risk of developing inhibitors on recombinant than on plasma derived products (20). Our study may have been limited on this assessment if we regard the lack of numbers on plasma derived products to compare with.

The age at first treatment in our study reported that the age group that developed inhibitors the most was between 1-5 years where 45.5% (n=5) had inhibitors. These group showed odds of 2.8 more likely to get inhibitors when compared to those who are more than 15 years of age. As a risk factor it is highly debated whether it actually impacts on development of inhibitors or is subject to other variables like intensity of treatment. A study done on a French cohort with severe hemophilia observed a threefold risk of inhibitor development on children treated for the first time before six months as compared to those first treated after twelve months. The CANAL study shows a cumulative risk of 41% when treated first below 18 months and this risk drops to 18% when the treatment is initiated after 18 months (20). In our Kenyan population, the diagnosis of hemophilia is usually delayed and children are usually diagnosed when brought in after bleeding from minor surgical procedures or trauma when at an older age. The severe cases

if occurring in rural areas mostly die before definitive diagnosis is made. This may account for the higher age at which diagnosis is made and hence later initiation of therapy.

Surgical treatment has been shown by Eckhardt et al in a meta-analysis to have an increased risk of development of inhibitors with a pooled OR of 4.1 after first treatment with FVIII (30). In contrast, our study found that half of those who developed inhibitors had had minor surgery and the other half had no surgery at all. Although this finding was not statistically significant, the odds ratio shows a lesser likelihood of developing inhibitors at 0.7 for those who had surgery. Surgery is generally said to induce inhibitor formation by causing tissue damage and releasing endogenous danger signals that escalate to the eventual formation of the antibodies. The probable discord can be due to heterogeneity in type of surgeries done, duration of the procedure and dosing of FVIII prior to the surgery.

The medical history of our participants was tailored to show other events that might induce danger signals. We found that 91.7% of those with inhibitors had had prior exposure to both trauma and blood transfusion products. Compared to those who did not the odds were three times higher that they would get inhibitors. All those who had inhibitors had a positive history of major bleeds. There was an equal number of those who had and those who lacked inhibitors when it came to a history of febrile illness. All these factors are said to induce danger signals within the body leading to upregulation of the immune response to FVIII administration. Further studies regarding these factors are needed to draw conclusions as currently the evidence mostly exists on animal models. (7)

Inhibitor development is a serious complication of treatment. It has several precipitating risk factors that can be avoidable and early testing and recognition of inhibitor presence is recommended.

Conclusion

The prevalence of inhibitors in the 61 patients with Hemophilia A seen at Kenyatta National Hospital is high at 19.7% (n=12). All these patients had low titres (<5BU/ml) and severe hemophilia. The age group found to have most inhibitors was between 10 to 19 years. Although our study was not powered enough to detect statistical significance in the risk factor associations, in absolute numbers, there were significant numbers of those who developed inhibitors being exposed to danger signals through surgery, trauma, major bleeds and blood transfusion. The use of recombinant product and treatment on demand also had significant proportions of developing inhibitors. This concludes that many people with Hemophilia A seen at KNH have inhibitors and need testing to be routine practice in their care. They also have some risk factors that predispose them to develop these inhibitors including surgery, trauma, major bleeds and transfusions which if planned for in the care of hemophiliacs may be avoided and hence reduce the development of inhibitors in this population.

Limitations

There was recall bias when reporting the age at first treatment. This is however a common expectation in studies requiring past history from participants. The study was also conducted in only one hemophilia treatment centre in the country and there are five in total. It however is the biggest one and also doubles as a referral facility for all the other hemophilia centres.

Recommendations

The study findings indicate that the prevalence of inhibitors in the population of Hemophilia A is high and recommend that routine testing of inhibitors be incorporated as standard of care for all Hemophilia comprehensive care centres in Kenya.

Creation of awareness on the existence of this condition is therefore recommended to all clinicians who come into contact with hemophilia patients so as to avoid possible triggers for inhibitors like unplanned surgeries or over judicious use of blood and blood products to reduce risk of inhibitor development. This necessitates the need for a multi-disciplinary approach when it comes to the management of hemophilia patients to reduce modifiable risk factors and detect inhibitor development early.

Health economic assessments like Quality Adjusted Life Years (QALYs) and Disability Adjusted Life Years (DALYs) of those with inhibitors should be done. Information on the cost of care and socioeconomic burden will determine the actual burden of disease and possibly influence policy that avails affordable care and make available essential drugs used in treatment of those with inhibitors.

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APPENDICES

Appendix 1: Data collection tool - Questionnaire

Date:

Participant's code:

A: SOCIODEMOGRAPHIC CHARACTERISTICS

1. Age (years) & Date of birth:

2. Gender

☐ Male ☐ Female

3. Residence: Which county are you from?

4. Ethnicity

B. CLINICAL FEATURES

5. What severity of hemophilia do you have?

☐ Mild ☐ Moderate ☐ Severe

6. Do you have any siblings or family members known to have inhibitors?

☐ Present ☐ Absent ☐ Unknown

7. For what reason do you receive Factor?

☐ Prophylaxis ☐ on demand

8. What type of factor product do you get?

☐ Recombinant ☐ Plasma derived ☐ Both

9. At what age were you first exposed to factor? (treatment with factor)

10. What dose of Factor are you receiving currently?

11. Have you had any surgery before?

☐ Yes ☐ No

If yes which surgery?

☐ Minor ☐ Major

12. Do you have any history of trauma?

☐ Yes ☐ No

13. Do you have any history of febrile illness (infection)?

☐ Yes ☐ No

14. Do you have any history of major bleeds (joint, muscle, visceral)?

☐ Yes ☐ No

15. Do you have any history of blood transfusion?

☐ Yes ☐ No

Appendix 2: Consent

2.1 Informed consent

All consent procedures are sourced from ERC website with adjustment to particular study.

INFORMED CONSENT TO PARTICIPATE IN THE STUDY

SERIAL NUMBER …….

Background

You are being asked to participate in a research study described below. Before you decide, it is important for you to understand why the research is being done and what it will involve. Read the following information carefully and ask if there is anything that is not clear or if you would like more information. Please take time to decide whether you want to volunteer to take part in this study.

TITLE: THE PREVALENCE OF AND RISK FACTORS ASSOCIATED WITH FACTOR VIII INHIBITORS IN PATIENTS WITH HEMOPHILIA A AT THE KENYATTA NATIONAL HOSPITAL.

PRINCIPLE INVESITIGATOR: DR NYACHAE LUCY

Purpose of the study:

The purpose of this study is to determine the prevalence of Factor VIII inhibitors in patients with Hemophilia A and to establish what risk factors are associated with inhibitor development. It is for research purposes and the results will be useful in determining patient management. It aims to establish routine testing for inhibitors as standard of care for hemophiliacs all over Kenya.

Study Procedure:

The study involves filling out a questionnaire capturing your bio data and some clinical details. Your responses will not be linked to you and are completely anonymous and confidential. Blood samples will be drawn for factor VIII assay levels and inhibitor testing.

Risks and benefits

The risks involved in this study is slight pain during sample collection and in case of any prolonged bleeding will be mitigated by applying pressure on the site. Samples will be collected by trained medical personnel. Kindly note that venous sampling for blood tests is a common procedure practiced in healthcare settings all over for Hemophiliacs.

The benefits will be free testing of current Factor VIII levels and inhibitor screening for the patient. The result will be put in the patients file and will be useful in their management. Knowledge of their risk factor profile will enable early planning for future management and how to avert risks. Overall the study will establish the current numbers and potential risk factors for inhibitor development in those with Hemophilia A and aim to make testing of inhibitors routine in all Hemophiliacs.

Alternative Procedures

You may choose not to participate in this study and it will not affect the health care that will be provided to you.

Confidentiality

This research will be conducted in accordance with all the Kenyan laws and regulations that protect rights of human research subjects. All records and other information obtained will be kept strictly confidential and your protected health information will not be used without permission. All data collection tools will be identified by study number or otherwise coded to protect any information that could be used to identify you. Results of this study may be published, but no names or other identifying information will be released.

Voluntary Participation

It is up to you to decide whether you will take part in this study. Refusal to participate or the decision to withdraw from this research will involve no penalty or loss of benefits to which you are otherwise entitled. This will not affect your relationship with the investigators.

Right of investigator to withdraw

The investigator can withdraw you from the research without your approval.

Costs and Compensation to participants

There is no cost to you, and there is no compensation to subjects for participation in this study.

Person to Contact

If you have questions, complaints or concerns about this study, you can contact the principal investigator from University of Nairobi, School of Medicine, Department of Human Pathology, Postgraduate program: Dr. Nyachae Lucy +254723929165 Email: nnyanchama@gmail.com or my Supervisor Dr Kibet Shikuku +254720789843 or you can contact the KNH-UoN ERC at uonknh_erc@uonbi.ac.ke.

Thank you for your participation in this research and we truly appreciate your help.

CONSENT

By signing this consent form, I confirm I have read the information in this consent form and have had the opportunity to ask questions. I voluntarily agree to take part in this study.

Name of participant …….....

Signature/Mark……. …… Date.…….……

Signature of investigator ……. Date…….…….

2.2 Fomu ya Idhini

IDHINI YA KUSHIRIKI KATIKA UCHUNGUZI

NAMBARI YA USAJILI …….

Utangulizi:

Unaulizwa ukubali kushiriki katika utafiti wa kina. Kabla ya uamuzi wako, ni muhimu uelewe kiina na malengo ya uchunguzi huu na yanahusiana na utafiti husika. Soma maelezeo yafuatayo kwa makini na uliza swali ikiwa kuna jambo ambalo haliko wazi.

MADA: MAAMBUKIZO NA SABABU ZA HATARI ZINAZOHUSIANA NA UKUZAJI WA VIZUIZI VYA CHEMBECHEMBE ZA PROTINI NAMBA NANE ZA KUGANDISHA DAMU KWA HIMOFILIA A KATIKA HOSPITALI YA KITAIFA YA KENYATTA.

MDADISI MKUU: DR NYACHAE LUCY

Kusudi la uchunguzi huu:

Madhumuni ya utafiti huu ni kuamua maambukizi ya vizuizi vya chembechembe za protini namba nane za kugandisha damu kwa himofilia a na kuamua ni sababu gani za hatari zinazohusiana na maendeleo ya vizuizi hivi. matokeo ya utafiti huu yatakuwa muhimu katika kuamua usimamizi wa mgonjwa. inakusudia kuanzisha upimaji wa kawaida wa vizuizi hivi kama kiwango cha utunzaji wa walio na himofilia nchini kenya.

Hatua za Zoezi

Utafiti huu unajumuisha kujaza dodoso la kuuliza data yako na maelezo kadhaa ya kliniki. majibu yako hayataunganishwa kwako na ya siri. sampuli za damu zitatolewa kwa upimaji wa viwango vya chembechembe za protini namba nane za kugandisha damu na kuangalia kama kuna vizuizi.

Hatari na manufaa

Hatari zinazohusika katika utafiti huu ni maumivu kidogo wakati wa ukusanyaji wa sampuli na iwapo kuvuja damu kwa muda mrefu kutapunguzwa kwa kuweka shinikizo. sampuli zitakusanywa na wauguzi. kumbuka vipimo vya damu ni utaratibu wa kawaida unaofanywa katika mazingira ya utunzaji wa afya kote kwa walio na himofilia. faida zitakuwa za upimaji bure wa viwango vya chembechembe za protini namba nane za kugandisha damu na uchunguzi wa vizuizi kwa mgonjwa. matokeo yake yatawekwa kwenye faili ya wagonjwa na yatakuwa muhimu katika usimamizi wao. ujuzi wa wasifu wao wa hatari itawezesha upangaji wa mapema kwa usimamizi wa siku zijazo na jinsi ya kuzuia hatari. kwa ujumla utafiti utajulisha nambari za sasa na sababu

za hatari za kukuza kizuizi kwa wale walio na himofilia a na inakusudia kufanya upimaji wa vizuizi utaratibu kwa wote walio na himofilia.

Njia Mbadala:

Unaweza kuamua mwanao asishiriki katika uchunguzi huu ambao hautaathiri huduma ya afya utakayopewa

Uhifadhi siri wa maelezo

Utafiti huu utafanya kwa mujibu wa sharia zote za Kenya zinazolinda utafiti wa vipengele wa haki za binadamu. Rekodi na maelezo mengine yaliyokusanywa yatahifadhiwa kwa njia ya siri na maelezo ya kiafya kuhusu mwanao hayatatumika bila ruhusa rasmi. Vifaa vyote vya ukusanyaji data vitatambuliwa kwa nambari maalum ya uchunguzi huu, ili kulinda habari zozote zile zinaweza kukufichua. Matokeo ya uchunguzi huu yanaweza kuchapiswa lakini hakuna jina au maelezo yoyote ya kukutambulisha yatatumika.

Kushiriki kwa hiari:

Ni wajibu wako kuamua ikiwa utashiriki katika utafiti huu. Kukataa kushiriki au kujiondoa kutoka zoezi hii haitaleta athari mbaya kwako wala haitakuwa na hatia au adhabu yoyote ile. Pia manufaa yoyote kwako hayataathirika kwa vyovyote vile. Uhusiano wako na wadadisi utazidi kuwa mwema.

Haki ya mdadisi kuondoa

Mdadisi mkuu anaweza kukuondoa kutoka kwa zoezi hii bila ruhusa yako.

Malipo kwa washiriki

Hakuna malipo ya aina yeyote ile kwa wanoshiriki katika uchunguzi huu.

Mawasiliano

Ikiwa una maswali, malalamishi au maswala ibuka kuhusu uchunguzi huu, unaweza kuwasiliana na mdadisi mkuu kutoka chou kikuu cha Nairobi, shule ya Udaktari idara ya patholojia.kitengo cha uzamili; Dkt. Nyachae Lucy +254723929165 baruapepe: anwani ya barauapepe ni nnyanchama@gmail.com au msimamizi mkuu Dkt Kibet Shikuku kupitia +254720789843 au unaweza kuwasiliana na kitengo cha KNH-UoN ERC kupitia baruapepe, anwani ambayo ni uonknh_erc@uonbi.ac.ke.

Asanti sana kwa kushiriki katika utafiti huu, tunathamini sana usaidizi wako.

IDHINI

Kwa kujaza fomu hii ya ruhusa, nimekubali kuwa nimesoma maelezo yote yaliyo katika fomu hii na kupata nafasi ya kuuliza maswali. Nimekubali kwa hiari yangu kushiriki katika uchunguzi huu.

Jina la mshiriki: …….

Sahihi/ Alama: …….Tarehe: …….

Sahihi ya mdadisi: ……Tarehe: ……

2.3 Informed Parental Consent

INFORMED PARENTAL CONSENT TO PARTICIPATE IN THE STUDY

SERIAL NUMBER …….

Background

You are being asked to let your child participate in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Read the following information carefully and ask us if there is anything that is not clear or if you would like more information. Please take time to decide whether you want your child to volunteer to take part in this study.

TITLE: THE PREVALENCE OF AND RISK FACTORS ASSOCIATED WITH FACTOR VIII INHIBITORS IN PATIENTS WITH HEMOPHILIA A SEEN AT THE KENYATTA NATIONAL HOSPITAL

PRINCIPLE INVESTIGATOR: DR NYACHAE LUCY

Purpose of the study:

The purpose of this study is to determine the prevalence of Factor VIII inhibitors in patients with Hemophilia A and to establish what risk factors are associated with inhibitor development. It is for research purposes and the results will be useful in determining patient management. It aims to establish routine testing for inhibitors as standard of care for hemophiliacs all over Kenya.

Study Procedure:

The study involves filling out a questionnaire capturing your child's bio data and some clinical details. Your responses will not be linked to your child and are completely anonymous and confidential. Blood samples will then be drawn for factor VIII assay levels and inhibitor testing.

Risks and benefits

The risks involved in this study for your child is slight pain during sample collection and in case of any prolonged bleeding it will be mitigated by applying pressure on the site. Samples will be collected by trained medical personnel. Kindly note that venous sampling for blood tests is a common procedure practiced in healthcare settings all over for Hemophiliacs.

The benefits will be free testing of current Factor VIII levels and inhibitor screening for your child. The result will be put in your child's file and will be useful in their management. Knowledge of their risk factor profile will enable early planning for future management and how to avert risks. Overall the study will establish the current numbers and potential risk factors for inhibitor development in those with Hemophilia A and aim to make testing of inhibitors routine in all Hemophiliacs.

Alternative Procedures

You may choose your child not to participate in this study and it will not affect the health care that will be provided to you.

Confidentiality

This research will be conducted in accordance with all the Kenyan laws and regulations that protect rights of human research subjects. All records and other information obtained will be kept strictly confidential and your child's protected health information will not be used without permission. All data collection tools will be identified by number or otherwise coded to protect any information that could be used to identify your child. Results of this study may be published, but no names or other identifying information will be released.

Voluntary Participation

It is up to you to decide whether your child takes part in this study. Refusal to participate or the decision to withdraw from this research will involve no penalty or loss of benefits to which your child is otherwise entitled. This will not affect your relationship with the investigators.

Right of investigator to withdraw

The investigator can withdraw your child from the research without your approval.

Costs and Compensation to participants

There is no cost to you, and there is no compensation to subjects for participation in this study.

Person to Contact

If you have questions, complaints or concerns about this study, you can contact the principal investigator from University of Nairobi, School of Medicine, Department of Human Pathology, Postgraduate program: Dr. Nyachae Lucy +254723929165 Email: nnyanchama@gmail.com or my Supervisor Dr Kibet Shikuku +254720789843 or you can contact the KNH-UoN ERC at uonknh_erc@uonbi.ac.ke.

Thank you for your child's participation in this research and we truly appreciate your help.

CONSENT

By signing this consent form, I confirm I have read the information in this consent form and have had the opportunity to ask questions. I voluntarily agree to take part in this study.

Name of Child ……

Name of Caregiver / Parent…….....

Signature/Mark……. Date…….

Investigator Signature…… Date…….

2.4 Idhini ya Mzazi

IDHINI YA MZAZI KUSHIRIKISHA MWANAO KATIKA UCHUNGUZI

NAMBARI YA USAJILI …….

Utangulizi:

Unaulizwa umkubalie mwanao ashiriki katika utafiti wa kina. Kabla ya uamuzi wako, ni muhimu uelewe kiina na malengo ya uchunguzi huu na yanahusiana na utafiti husika. Soma maelezeo yafuatayo kwa makini na uliza swali ikiwa kuna jambo ambalo haliko wazi.

MADA: MAAMBUKIZO NA SABABU ZA HATARI ZINAZOHUSIANA NA UKUZAJI WA VIZUIZI VYA CHEMBECHEMBE ZA PROTINI NAMBA NANE ZA KUGANDISHA DAMU KWA HIMOFILIA A KATIKA HOSPITALI YA KITAIFA YA KENYATTA.

MDADISI MKUU: DR NYACHAE LUCY

Kusudi la uchunguzi huu:

Madhumuni ya utafiti huu ni kuamua maambukizi ya vizuizi vya chembechembe za protini namba nane za kugandisha damu kwa himofilia a na kuamua ni sababu gani za hatari zinazohusiana na maendeleo ya vizuizi hivi. Matokeo ya utafiti huu yatakuwa muhimu katika kuamua usimamizi wa mtoto wako. Inakusudia kuanzisha upimaji wa kawaida wa vizuizi hivi kama kiwango cha utunzaji wa walio na himofilia nchini kenya.

Hatua za Zoezi

Utafiti huu unajumuisha kumjazia dodoso la kuuliza data ya mtoto wako na maelezo kadhaa ya kliniki. majibu yake hayataunganishwa kwake na ya siri. Sampuli za damu zitatolewa kwa upimaji wa viwango vya chembechembe za protini namba nane za kugandisha damu na kuangalia kama kuna vizuizi.

Hatari na manufaa

Hatari zinazohusika katika utafiti huu kwa mwanao ni maumivu kidogo wakati wa ukusanyaji wa sampuli na iwapo kuvuja damu kwa muda mrefu kutapunguzwa kwa kuweka shinikizo. Sampuli zitakusanywa na wauguzi. kumbuka vipimo vya damu ni utaratibu wa kawaida unaofanywa katika mazingira ya utunzaji wa afya kote kwa walio na himofilia. Faida zitakuwa za upimaji bure wa viwango vya chembechembe za protini namba nane za kugandisha damu na

uchunguzi wa vizuizi kwa mgonjwa. Matokeo yake yatawekwa kwenye faili ya mtoto wako na yatakuwa muhimu katika usimamizi wake. Ujuzi wa wasifu wao wa hatari itawezesha upangaji wa mapema kwa usimamizi wa siku zijazo na jinsi ya kuzuia hatari. Kwa ujumla utafiti utajulisha nambari za sasa na sababu za hatari za kukuza kizuizi kwa wale walio na himofilia a na inakusudia kufanya upimaji wa vizuizi utaratibu kwa wote walio na himofilia.

Njia Mbadala:

Unaweza kuamu mwanao asishiriki katika uchunguzi huu ambao hautaathiri huduma ya afya utakayopewa. **Uhifadhi siri wa maelezo**

Utafiti huu utafanya kwa mujibu wa sharia zote za Kenya zinazolinda utafiti wa vipengele wa haki za binadamu. Rekodi na maelezo mengine yaliyokusanywa yatahifadhiwa kwa njia ya siri na maelezo ya kiafya kuhusu mwanao hayatatumika bila ruhusa rasmi. Vifaa vyote vya ukusanyaji data vitatambuliwa kwa nambari maalum ya uchunguzi huu, ili kulinda habari zozote zile zinaweza kumfichua mwanao. Matokeo ya uchunguzi huu yanaweza kuchapiswa lakini hakuna jina au maelezo yoyote ya kumtambulisha yatatumika.

Kushiriki kwa hiari:

Ni wajibu wako kuamua ikiwa mwanao atashiriki katika utafiti huu. Kukataa kushiriki au kujiondoa kutoka zoezi hii haitaleta athari mbaya kwa manao wala haitakuwa na hatia au adhabu yoyote ile. Pia manufaa yoyote kwa mwanao hayataathirika kwa vyovyote vile. Uhusiano wako na wadadisi utazidi kuwa mwema.

Haki ya mdadisi kuondoa

Mdadisi mkuu anaweza kuondoa mwanao kutoka kwa zoezi hii bila ruhusa yako.

Malipo kwa washiriki

Hakuna malipo ya aina yeyote ile kwa wanoshiriki katika uchunguzi huu.

Mawasiliano

Ikiwa una maswali, malalamishi au maswala ibuka kuhusu uchunguzi huu, unaweza kuwasiliana na mdadisi mkuu kutoka chou kikuu cha Nairobi, shule ya Udaktari idara ya patholojia.kitengo cha uzamili; Dkt. Nyachae Lucy +254723929165 baruapepe: anwani ya barauapepe ni nnyanchama@gmail.com au msimamizi mkuu Dkt Kibet Shikuku kupitia

+254720789843 au unaweza kuwasiliana na kitengo cha KNH-UoN ERC kupitia baruapepe, anwani ambayo ni uonknh_erc@uonbi.ac.ke.

Asanti sana kwa ushiriki wa mwanao katika utafiti huu, tunathamini sana usaidizi wako.

IDHINI

Kwa kujaza fomu hii ya ruhusa, nimekubali kuwa nimesoma maelezo yote yaliyo katika fomu hii na kupata nafasi ya kuuliza maswali. Nimekubali kwa hiari yangu kushiriki katika uchunguzi huu.

Jina la mtoto: …….

Jina la mzazi: …….....

Sahihi/ Alama: …….Tarehe: …….

Sahihi ya mdadisi: ……Tarehe: ……

Appendix 3: Assent

3.1: Informed Assent Age 8-17

INFORMED ASSENT TO PARTICIPATE IN THE STUDY

SERIAL NUMBER …….

My name is **DR NYACHAE LUCY** (Principle Investigator). I would like you to participate in a research study titled **THE PREVALENCE OF AND RISK FACTORS ASSOCIATED WITH FACTOR VIII INHIBITORS IN PATIENTS WITH HEMOPHILIA A SEEN AT THE KENYATTA NATIONAL HOSPITAL.**

-

Your parent(s) are aware that am talking to you about the study. I will take you through this form, that will inform you about the study to help you decide whether or not you want to take part in it.
What am I being asked to do?

If you decide to be in the study, I will ask you a few questions about the treatment or risk factors you have for your hemophilia like if you have had any operations or if you have had any blood given to you The HCCC sometimes takes your blood samples for testing, this time I will be taking blood for testing factor assay levels and inhibitors. You shall feel a little pain when the blood sample is being withdrawn, we shall ensure that bleeding has stopped before you leave.

What are the benefits to me for taking part in the study?

If you take part in this study, it will include a free test for inhibitors in your file and current factor assay levels which will help in your management. We will also know how many Hemophiliacs here at KNH have inhibitors and what risk factors they have so we can manage them and hemophiliacs all over the country better in future.

Can anything bad happen if I am in this study?

I do not expect anything bad happening to you by agreeing to be part of the study. If there is a bleed during sample collection, we shall ensure we stop it promptly.

Who will know that I am in the study?

If you decide to be in the study, I will not tell anyone else how you respond or act as part of the study.

Do I have to be in the study?

No, you don't. The choice is yours. No one will get angry or upset if you don't want to be in the study.

What if I have questions?

If you have any questions about the study, you can ask me now or anytime during the study. You can also call me at 0723929165 or e-mail me at nnyanchama@gmail.com or contact my supervisor Dr Kibet Shikuku at +254720789843 or you can contact the KNH-UoN ERC at uonknh_erc@uonbi.ac.ke.

Signing below means that you have understood this form and that you are willing to be in this study:

Name of the Participant: _____

Signature of the Participant:_____Date:_____

Investigator Signature_____Date_____

3.2 Idhini ya Mtoto miaka 8-17

IDHINI YA KUSHIRIKI KATIKA UCHUNGUZI

NAMBARI YA USAJILI ……

Jina langu ni Dkt **NYACHAE LUCY** (Mdadisi Mkuu). Ningependa ushiriki katika uchunguzi wa utafiti, mada ikiwa **MAAMBUKIZO NA SABABU ZA HATARI ZINAZOHUSIANA NA UKUZAJI WA VIZUIZI VYA CHEMBECHEMBE ZA PROTINI NAMBA NANE ZA KUGANDISHA DAMU KWA HIMOFILIA A KATIKA HOSPITALI YA KITAIFA YA KENYATTA.**

Mzazi/wazazi wako wana ufahamu wa mazungumuzo yetu kuhusu uchunguzi huu. Nitakupa maelezo kamili kuhusi hii fomu, ambayo itakupa mwelekeo na habari Zaidi ili mwishowe ufanye uamuzi wa kushiriki au kutoshiriki katika zoezi hili.

Je ninaagiziwa nifanya nini?

Ikiwa unaamua kuwa kwenye utafiti, nitakuuliza maswali machache kuhusu matibabu au sababu za hatari ulizo nazo himofilia yako kana kwamba umeshafanyia operesheni yoyote au ikiwa umepewa damu yoyote. Hii kliniki wakati mwingine inachukua damu yako sampuli za upimaji, wakati huu nitakuwa nikichukua damu kwa viwango vya chembechembe ya protini ya uzuizi wa kuganda damu nambari nane na upekuzi wa vizuizi. Utasikia maumivu kidogo wakati sampuli ya damu inatolewa, tutahakikisha kwamba kutokwa na damu kumekoma kabla ya kuondoka.

Je manufaa ya kushiriki katika uchunguzi huu ni yapi?

Ikiwa unashiriki katika utafiti huu, manufaa ni kupata jaribio la bure kwa vizuizi kwenye faili yako na viwango vya chembechembe ya protini ya uzuizi wa kuganda damu nambari nane na kama una vizuizi. Ujuzi huu utasaidia katika usimamizi wako. Pia tutajua ni wangapi wenye Himofilia hapa KNH wana vizuizi na ni hatari gani wanazo ili tuweze kuzisimamia walio na Himofilia kote nchini bora katika siku zijazo.

Kuna uwezekano wa hatari kuzuka ninaposhiriki katika zoezi hili?

Sitaraji uwezekano wa hatari au jambo mbaya kuchipuka kwa kushiriki kwako katika uchunguzi huu. Iwapo kutakuwa na uvujaji wowote katika kukusanya sampuli, tutahakikisha kuwa tumeikomesha mara moja.

Nani atakayejuzwa kuwa ninashiriki katika zoezi hili?

Ukiamua kushiriki katika uchunguzi huu, sitamwambia yeyote kuhusu matokeo yako au sehemu yeyote ya uchunguzi huu.

Je, lazima ni shiriki katika uchunguzi huu?

La, sio lazima. Uamuzi au Chaguo ni lako. Hakuna atakayepandwa na hamaki au hasira au kuudhiwa na uamuzi wako wa kushiriki katika uchunguzi huu.

Je, ikiwa nina maswali?

Ikiwa una maswali yoyote kuhusu uchunguzi huu, unaweza kuniuliza sasa au wakati wowote ule tutakapokuwa katika zoezi hii. Pia unaweza kuwasiliana nami kupitia nambari ya simu ya rununu 0723929165 au anwani ya baruapepe nnyanchama@gmail.com au unaweza kuwasiliana na msimamizi wangu mkuu Dkt. Kibet Shikuku kupitia nambari ya simu tamba +254720789843 au wasiliana na kitengo cha KNH-UoN ERC kupitia anwani ya baruapepe uonknh_erc@uonbi.ac.ke.

Kwa kujaza fomu hii ina maana kuwa umeelewa maelezo yote yaliyo katika fomu hii na kuwa umekubali kwa hiari yako kushiriki katika uchunguzi huu.

Jina la mshiriki: _____ Sahihi ya mshiriki:_____Tarehe: _____

Sahihi ya mdadisi:_____Tarehe: _____

Appendix 4: Standard Operating Procedure on Phlebotomy

Select an ideal vein for procedure through palpation. Select actual site for procedure and ensure all your requirements are present – tubes, needles, gloves, swabs, tourniquet.

Put on gloves

Prepare venipuncture site with alcohol prep cleaning in a circular fashion starting from point of needle insertion outwards. Then allow to dry and do not palpate site after cleaning.

Apply tourniquet 3-4 inches above site selected for < 1 minute and not too tightly.

Remove needle shield and perform venipuncture with patient's arm in downward position and tube stopper uppermost. Push tube into the needle puncturing stopper.

Remove tourniquet once blood appears into tube

Once full to required volume, remove from holder. Invert tube 5 times to mix well, do not shake vigorously.

Place cotton gauze and remove needle.

Apply sufficient pressure to stop bleeding.

Discard needle into biohazard box.

Appendix 5: Laboratory procedures

One stage assay for FVIII:C

Reagents: platelet poor plasma from the patient, FVIII deficient plasma as a substrate, Reagents for APTT, Barbitone buffered saline, plastic tubes and an ice bath.

Method:

Place the APTT reagent and $CaCl_2$ at 37°C, and the patient's, standard, and substrate plasma in the ice bath until used.

Make 1 in 10 dilutions of the test and standard plasma in buffered saline in plastic tubes in the ice bath. Using 0.2 ml volumes, make doubling dilutions in buffered saline to obtain 1 in 20 and 1 in 40 dilutions. Place 0.1 ml of the three dilutions (1 in 10, 1 in 20, and 1 in 40) in glass tubes. If the test plasma is suspected of having a very low factor VIII:C content, make 1 in 5, 10, and 20 dilutions of the test instead.

Add to each dilution 0.1 ml of freshly reconstituted or thawed substrate plasma and warm up at 37°C. Perform APTTs according to the laboratory protocol following a balanced order of duplicates.

The dilutions should be tested at 2 -min intervals on the master watch. The assay must end with a blank consisting of 0.1 ml of buffered saline and 0.1 ml of substrate plasma

Calculation of results:

Plot the clotting times of the test and standard against the concentration of factor VIII:C on semi-log paper. Read the concentration.

The normal range is 50-150iu/dl.

Clinical interpretation: value below 50IU/dl are significant. This will classify the patents into mild (5-30%), moderate (1-5%) and severe (<1%) hemophiliacs.

Once the Factor levels have been determined and severity classified, I will proceed to carry out a circulating inhibitor screen.

Circulating inhibitor screen

Reagents: normal pooled plasma and platelet poor plasma from the patient.

Method:

Prepare 3 plastic tubes as follows: place 0.5 ml of normal plasma in a first tube, 0.5 ml of the patient's plasma in a second tube, and a mixture of 0.25 ml of normal and 0.25 ml of patient's plasma in a third tube. Incubate the tubes for 120 min at 37°C and then place all 3 tubes in an ice bath or on crushed ice. Then, make a 50:50 mixture of the contents of tubes 1 and 2 into a 4th tube, which serves to check for the presence of an immediate inhibitor. Perform APTTs in duplicate on all 4 tubes.

Interpretation of the inhibitor screen based on the activated partial thromboplastin time

Tube	**Content**	**Clotting time**		
1	Normal plasma	Normal	Normal	Normal
2	Patient's plasma	Long	Long	Long
3	50:50 mixture, patient: normal; incubated 2 h	Normal	Long	Long
4	50:50 mixture, patient: normal; no incubation	Normal	Long	Normal
Interpretation		Deficiency	Immediately acting inhibitor	Time-dependent inhibitor

Lewis, S. M., Bain, B. J., Bates, I., Dacie, J. V., & Dacie, J. V. (2006). *Dacie and Lewis practical haematology*. Philadelphia: Churchill Livingstone/Elsevier.

The confirmatory method for tests found to have a time dependent inhibitor is the Bethesda assay which is quantitative.

Bethesda Assay:

In the Bethesda method, 1 Bethesda unit(BU) is defined as the amount of inhibitor that will neutralize 50% of 1 unit of factor VIII:C in normal plasma after 2 hours of incubation at 37°C.

Dilutions of test plasma are incubated with an equal volume of the normal plasma pool at 37°C. The normal plasma pool is taken to represent 1 unit of factor VIII: C. Dilutions of a control normal plasma containing no inhibitor are treated in the same way. An equal volume of normal plasma mixed with buffer is taken to represent the 100% value.

At the end of the incubation period the residual factor VIII:C is assayed and the inhibitor strength is calculated from a standard graph of residual factor VIII:C activity versus inhibitor units.

Reagents: Glyoxaline buffer, kaolin,FVIII, standard plasma

Method:

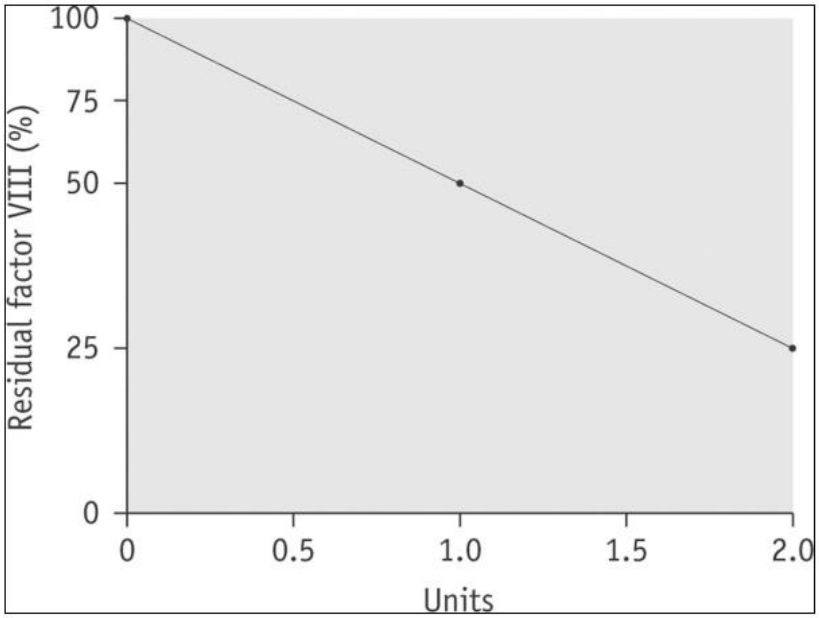
Pipette into each of a series of plastic tubes 0.2 ml of normal pool plasma. Add 0.2 ml of glyoxaline buffer to the first tube (this tube serves as the 100% value); add 0.2 ml of test plasma dilutions in glyoxaline buffer to each of the other tubes. If the patient's inhibitor has been assayed previously, this can be used as a guide to the dilutions that should be used. If the patient has not been tested before, a range of dilutions should be set up ranging from undiluted plasma to a 1 in 50 dilution.

Cap, mix, and incubate all the tubes for 2 hours at 37°C. Then immerse all the tubes in an icebath. Perform factor VIII:C assays on all the incubation mixtures.

Calculation of Results

Record the residual factor VIII:C percentage for each mixture assuming the assay value of the control to be 100%. The dilution of test plasma that gives the residual factor VIII:C percentage nearest to 50% (between 30% and 60%) is chosen for calculating the strength of inhibitor. Results are calculated as shown in Table for three different patients with a mild inhibitor only detected in undiluted plasma, a stronger inhibitor with simple kinetics, and an inhibitor with complex kinetics, respectively.

Interpretation



Lewis, S. M., Bain, B. J., Bates, I., Dacie, J. V., & Dacie, J. V. (2006). *Dacie and Lewis practical haematology*. Philadelphia: Churchill Livingstone/Elsevier.

If the residual factor VIII:C activity is between 80% and 100%, the plasma sample does not contain an inhibitor. If the residual activity is less than 60%, the plasma unequivocally contains an inhibitor. Values between 60% and 80% are borderline, and repeated testing on additional samples is needed before the diagnosis can be established.

Laboratory procedures sourced from Practical Hemostasis.