INHIBITOR TITERS AND LEVELS OF REGULATORY T CELL MARKERS IN PATIENTS WITH HEMOPHILIA AT MOI TEACHING AND REFERRAL HOSPITAL

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DECLARATION

Declaration by the candidate:

This research thesis is my original work and has not been presented for a degree in any other University. No part of this thesis may be reproduced without the prior written permission of the principal investigator.

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DEDICATION

I wish to dedicate this thesis to my beloved mother, Hellen Kiprono, my siblings, Irene, Sharon, Caro, Kibii, Ruto and my lovely daughter Cherotich.

ABSTRACT

Background: Development of inhibitors is one of the most serious complications in treatment of patients with hemophilia (PWH). Inhibitors render replacement therapy ineffective. Inhibitor testing is critical; however this has not been fully implemented at health facilities in resource restricted countries including Moi Teaching and Referral Hospital (MTRH) in Kenya. Several barriers have led to lack of data on the prevalence of inhibitors in these regions. Regulatory T cells (Tregs) have recently been identified as the main cells that control the response of B cells to both FVIII and FIX inhibitors. Typically, the formation of antibodies in response to proteins used as therapeutics is dependent on T-cells whereas active suppression has been suggested to promote tolerance when changing from Tregs activation to Tregs induction.

Objective: To determine the prevalence of FVIII and FIX inhibitors and correlation between inhibitor titers and levels of Tregs (CD4+CD25+FOXP3+) in PWH at MTRH.

Methods: This was a cross-sectional study involving PWH at MTRH. Convenient sampling technique was used to select the study participants between the months of Jan- Oct 2019. Demographic and clinical data were collected using data collection forms. Venous blood in sodium citrate tube (4mL) and EDTA tube (1mL) were collected by a skilled phlebotomist for factor, inhibitor, and flow cytometry assays respectively. The characteristics of the study participants were described using means, standard deviations and relative proportions. Fishers' exact test was used to compare disease severity (%) and inhibitor titers (BU). Spearman's rank correlation was used to determine the correlation between inhibitor titers (BU) and (%) levels of Tregs**.** *P value* < 0.05 was used a cut-off to assess the level of significance of the results.

Results: A total of 88 participants were recruited; out of which hemophilia A (HA) were 71 (81%) and hemophilia B (HB) were 17 (19%). Out of the 71 HA patients 52 (73%) were severe, 10 (14%) moderate and 9 (13%) mild cases. Out of the 17 patients with HB, 14 (82%) were severe, 1 (6%) moderate and 2 (12%) mild cases. The overall prevalence of inhibitors was 14% (12 out of 88). All those who developed inhibitors were HA and their titers ranges were (0.6 BU – 69 BU). Out of the 12 patients who developed inhibitors, 10 of them were severe HA and only 2 were mild HA. The levels of Tregs ranges were between 0.25% and 6%. Inhibitor titers and Tregs were observed to correlate negatively (-0.3803), *p value = 0.003*. Alteration in levels of Treg markers had an effect on inhibitor titers; those with low levels of Treg markers showed high inhibitor titers and vice versa.

Conclusion: The prevalence of inhibitors among PWH at MTRH during the study period was 14%, which was comparable to that reported in other parts of the world. Tregs may have the potential to serve as novel markers and therapeutic target for amelioration or prevention of inhibitor development among PWH.

Recommendations: There is need for adoption of routine inhibitor testing for all PWH throughout the country. Secondly, there is need for further research to explore the potential of Tregs as novel markers and a therapeutic target for the prevention of inhibitor development.

Keywords: Hemophilia, Inhibitors, (CD4+ CD25+ FoxP3+), Regulatory T cells

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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LIST OF ABBREVIATIONS

AMPATH	Academic Model Providing Access to Healthcare
APCs	Antigen Presenting Cells
BU	Bethesda Units
CD	Cluster of Differentiation
CDC	Center for Disease Control
CCCDC	Chandaria Cancer and Chronic Disease Center
ELISA	Enzyme-linked immunoassay
FIX	Factor Nine
FVIII	Factor Eight
HA	Hemophilia A
HLA	Human Leukocyte Antigen
IREC	Institutional Research and Ethics Committee
IHTC	Indiana Hemophilia and Thrombosis Center
ITI	Immune Tolerance Induction
KNH	Kenyatta National Hospital
MU	Moi University
MHC	Major Histocompatibility Complex
MTRH	Moi Teaching and Referral Hospital
PdFVIII	Plasma Derived Factor Eight
PWH	Patients with Hemophilia
RFVIII	Recombinant Factor Eight
SOM	School of Medicine
Tregs	Regulatory T cells
UK	United Kingdom
USA	United States of America
WFH	World Federation of Hemophilia

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Hemophilia A and B are X-linked congenital bleeding disorders that occur at a frequency of 1 in 5000 and 1 in 30000 males worldwide, respectively. The prevalence of hemophilia A varies with the reporting country, with a range of 5.4-14.5 cases per 100,000 males. In the United States hemophilia A affects 1 in 5,000 male births. About 400 babies are born with hemophilia A each year (Alfonso Iorio et al., 2019) . Africa represents less than 3% of patients identified as having hemophilia (Diop et al., 2019). In kenya about 400 patients have been diagnosed with hemophilia (unpublished registry data). Overall, the mortality rate for patients with hemophilia is twice that of the healthy male population. Hemophilia is extremely rare in women; however bleeding symptoms may occur in approximately 10% of female carriers. Various mutations on the genes encoding for factor VIII (FVIII) on the X-chromosome leads to a deficiency or absence of this protein resulting in hemophilia A while a deficiency of factor IX (FIX) causes hemophilia B. Both factors play a key role in the coagulation cascade hence necessary for normal blood clotting to occur (Blanchette et al., 2014). Deficiency or functional abnormality of the factors involved in coagulation causes bleeding disorders such as hemophilia A (HA) and hemophilia B (HB). Hemophilia can be classified into three: mild (5-40% of normal plasma levels of FVIII or FIX), moderate (1-5%) and severe (<1%). These disorders are rare; however they can be life threatening and expensive to treat as they require constant replacement of FVIII or FIX respectively (Rocha, Carvalho, Lopes, & Araújo, 2015). Patients with severe hemophilia experience more frequent bleeding episodes compared to mild or moderate HA (Blanchette, et al., 2014).

The foundation of treatment in hemophilia is replacement therapy of FVIII for HA and FIX for HB patients (Rocha, et al., 2015). Development of inhibitors is one of the most serious complications of factor replacement therapy in patients with hemophilia. It has been established that the inhibitors renders replacement therapy ineffective. As a result, patients who develop inhibitors have increased risk of bleeding and morbidity, decrease in quality of life and increased hospitalization costs (Forsyth et al., 2012). For patients undergoing treatment for hemophilia, introduction of inhibitors is a critical component for consideration when in the process of changing a patient's therapeutic regimen due to the significance of such inhibitors to patient's response to factor infusion. For example, for some of the effective inhibitors with an anamnestic response upon factor infusion, there is need to employ agents that bypass immune responses that may make inhibitors ineffective (Shapiro & Hedner, 2011) and therefore helps in achieving homeostasis in the course of treatment in patients with hemophilia (Wight, Paisley, & Knight, 2003). Homeostatic bypassing agents used in such treatments include NovoSeven which is a recombinant factor VIIIa and an activated prothrombin complex concentrate (aPCC) (Astermark et al., 2006). For other patients with inhibitors, a therapeutic that induces immune tolerance (ITI) by eliminating the production of inhibitors may serve as a reliable alternative therapy.(Franchini & Mannucci, 2011) On the other hand, some inhibitors may lead to anaphylactic reaction which is a serious associated risk that occurs in 25 – 30 % and 1 - 3 % of patients with hemophilia A and hemophilia B respectively.(Mauro, Bonetti, Balter, Poli, & Cesaro, 2016) It is also worth noting that there are some inhibitors which may not cause anamnestic reaction due to their low concentration in the patient's system and therefore allow the administration of the factor products in large treatment doses. Owing to the increased morbidity and mortality in patients with hemophilia following the presence of inhibitors

for a long time, the patients require close monitoring in order to identify the type of inhibitor that is present since this may not be identified with precision at early stages of detection. Accordingly, it is recommended that testing be done frequently in all patients who have been exposed to factors as early diagnosis and monitoring can result in successful therapy in patients who require ITI especially where there is a greater possibility of developing inhibitors.

The development of an inhibitor results from complex interactions between a patient's immune system, genetic and environmental factors. In the process of inhibitor formation, some of the recognizably potential risk factors are genetic factors such as (severity of hemophilia, type of mutation, ethnicity, family history of inhibitors, and HLA genotype) and non-genetic factors (age at first treatment, intensity of treatment, multiple product switches and type of FVIII concentrate) (Fallon, Lavin, & O'donnell, 2018). Studies from different populations have reported varied findings regarding the above factors attributed to development of inhibitors (XF Wang et al., 2010). The major factors which account for the development of inhibitors are *F8* gene mutations and HLA polymorphisms (Garagiola, Palla, & Peyvandi, 2018), (Goodeve, Pavlova, & Oldenburg, 2014), (Elmahmoudi et al., 2011). Some researchers have reported a positive correlation between exposures to different types of factor products and inhibitor development while others found no correlation. Available data on the risk factors attributed to formation of inhibitors are mainly from developed countries where majority of patients have adequate access to factor concentrates and are mostly of Caucasian origin (Soucie et al., 2014).

Black patients with hemophilia have been shown to have a two-fold higher risk of developing inhibitors compared to white patients (Lochan, Macaulay, Chen, Mahlangu, & Krause, 2014). The mechanism that accounts for such variation has not been fully

established, however research suggests that mismatched factor VIII replacement therapy may be a risk factor for the development of FVIII inhibitors (Miller, 2015) . The two full-length recombinant factor VIII products currently approved for use in persons with hemophilia A, Kogenate (Bayer) and Recombinate (Baxter), correspond to the amino acid sequences of H1 and H2, respectively (Howard, 2015). In this regards, it is possible that, one in four blacks with hemophilia A who require replacement therapy with recombinant factor VIII will receive products that differ from their own factor VIII protein at one or two residues, in addition to having amino acid differences attributable to the specific F8 mutation. Plasma-derived factor VIII is also a source of exposure to H1 and H2, because most blood donors are white (Tabriznia-Tabrizi, Gholampour, & Mansouritorghabeh, 2016) (Gunasekera et al., 2015).

In general, adverse-events involving inhibitors are usually captured less accurately despite the fact that inhibitors appear in the list of adverse events in almost all the package inserts of all the treatment products used for treating hemophilia. However, most of the care providers classify inhibitors as contraindications of the treatment as suggested in reported work on inhibitors instead classifying it as an adverse event. This is more especially in the developing countries including Kenya where the frequencies of testing for inhibitors are quite low or even none at all (Kitchen et al., 2009). Possible barriers to testing that have been highlighted include the associated high cost of the inhibitor assays which in addition to that is not listed among the treatments to be covered by the patient's insurance scheme or medical cover as well as the lack of laboratory expertise in inhibitor testing (Favaloro et al., 2010). Another aspect of importance is the necessity for a "washout period" in which routine treatment is

suspended so as to provide space to perform accurate tests especially in pediatric patients (Peerschke et al., 2009).

There are enormous benefits when screening for inhibitors is conducted routinely. These include the possibility of detecting transient inhibitors and also low-level titer inhibitors which can potentially be missed to be detected. In order to gain a good and reliable understanding the clinical significance and the causes of inhibitors, it is crucial to determine the inhibitors whose titer levels are low (Astermark, 2015). Notably, treatment of inhibitors using readily available procedures mainly immune tolerance show high success rate for low-titer inhibitors in comparison to high-titer inhibitors (DiMichele, 2012). There exist protocols involving a combination of frequent high dose factor, through intravenous infusions, with immune suppression and immunoglobulin infusion which are utilized in the treatment of inhibitors (Cao, LODUCA, & HERZOG, 2009).

In an attempt to prevent or reverse the immune response against the therapeutic protein for hemophilia treatment, understanding the basis of immune response and the mechanism of tolerance to these factors is critical. The antibody response to proteins involves an interaction between thymus-derived T helper cells, B cells and antigen-presenting cells (APCs), such as dendritic cells. Dendritic cells take up protein antigens, process and present peptide epitopes that bind to a groove on the major histocompatibility complex (MHC) class II. The MHC may then be recognized by T cell receptors on the T helper cells of an individual, thus eliciting a biochemical signal. This signal is not sufficient to trigger the T cells to divide and produce cytokines for B cells to mature to antibody forming cells. Additional co-stimulatory signals drive T cells into full activation. T-cell help is necessary for antibody formation. There is evidence that shows that immune response to factor VIII is T cell dependent (Aledort,

2007). It is a dynamic process involving mechanisms which limit or delete alloreactive T cell pools and immune regulation through CD 127^-, CD4+ CD25+ and FOXP3+ regulatory T cells (Tregs) (Miao et al., 2009). Recent studies provide evidence that Tregs plays a crucial role in tolerance to coagulation factors delivered by means of gene transfer. Additionally, evidence for involvement of Tregs in limiting inhibitor formation in patients with hemophilia has been provided (Cao, et al., 2009), however the diagnostic and therapeutic relevance of Tregs in patients with inhibitors has not been fully elucidated.

The proportion of patients affected has been reported to range from 3·6% to 25%, but these figures have been derived mainly from retrospective data (B Verbruggen, 2010) (Owaidah et al., 2017). The cause of development of inhibitors in a subset of patients is a pertinent question and remains unknown yet. There is limited data on the pervasiveness of inhibitors in patients with hemophilia especially from low and middle income countries (LMIC) (David et al., 2019). To the best of our knowledge, this will be the first published data on the prevalence of inhibitors in patients with hemophilia in Kenya.

1.2 Statement of the problem

Inhibitors are currently one of the most serious and challenging complications of treatment in hemophillia. The presence of inhibitors renders replacement therapy ineffective, limits patient access to safe and effective standard of care, hence predisposing PWH to increased risk of mortality and morbidity. Despite the significant challenges associated with the development of inhibitors, limited information is available on the prevalence of inhibitors in Kenya, particularly at Moi Teaching and Referral Hospital (MTRH). Barriers to testing include the relatively high cost of testing, lack of laboratory expertise in inhibitor testing and lack of recommended testing for

inhibitors by the national guidelines. These barriers to receiving optimal laboratory services may contribute to suboptimal management of PWH with inhibitors and may result in increased morbidity and mortality.

There has been a slow progress in establishing innovative assay methods for inhibitors in the clinics as illustrated by such advances taking place at an interval of almost 20 years. Additionally, it is evident that Tregs are involved in the process of forming inhibitors in patients with hemophilia, however the potential of Tregs as a diagnostic marker and a therapeutic agent for inhibitors has not been fully elucidated.

1.3 Justification

In the recent past access to clotting factor concentrate has considerably increased in Kenya, largely due to the clotting factor donated through the World Federation of Hemophilia. Increased exposure to clotting factor concentrate is expected to lead to an increased incidence of inhibitors. Therefore, it is extremely important to determine the inhibitor titers for all the patients with hemophilia, to enable clinicians to first identify those at risk, hence enabling them to plan management of the affected patients at an early stage to avoid the likelihood of associated morbidities and mortalities. Since development of inhibitors against the infused factor impairs the effectiveness of treatment, it has become critical to test for inhibitors in all PWH. It was necessary to conduct studies to avail inhibitor prevalence data at MTRH to enable effective planning for management of PWH, particularly those undergoing surgery. In this regard, the inhibitor status of a patient informs the clinicians how to optimize their treatment and whether or not to proceed with surgery based on the availability of by-passing agents which is the recommended treatment for PWH who have developed inhibitors. Secondly, there was need to conduct studies to explore the potential of Tregs as a new diagnostic marker and a therapeutic agent for inhibitors in PWH

1.4 Study significance

Based on the findings from this study, the following major clinical decisions have been made;

i. Availability of data regarding the prevalence of inhibitors at MTRH has shed light on the burden of this complication and has triggered the need to test all patients with hemophilia for existence of inhibitors.
ii. Inhibitor testing is mandatory for any patient with hemophilia prior to undergoing surgery at MTRH. This has played a critical role especially to inform the clinicians whether to proceed with the planned surgery based on the inhibitor results of the patient and the availability of by-passing agents.
iii. The study findings also established the distribution of the hemophilia phenotypes at MTRH. It was evident that majority of the patients are severe hemophilia type.
iv. For patients with hemophilia undergoing circumcision at MTRH, adequate planning has been adopted which includes mandatory testing for inhibitors and ensuring there is adequate supply of by-passing agents for those who have developed inhibitors to ensure optimal management of these patients during circumcision.
v. Currently, treatment regimen for patients with hemophilia who have developed inhibitors has been changed to by-passing agents
vi. Correlation between inhibitor titers and Tregs has provided additional knowledge to the scientific community for future research on new diagnostic markers and therapeutic agents for inhibitors.

1.5 Research questions

i. What is the prevalence of FVIII and FIX inhibitors in PWH at MTRH?

ii. What is the pattern of disease severity in PWH at MTRH?

iii. How do inhibitor titers compare with different phenotypes of PWH at MTRH?

iv. Is there a correlation between inhibitor titers and levels of Tregs (CD4+CD25+FOXP3+) in PWH at MTRH?

1.6 Objectives

1.6.1 General objective

To determine the prevalence of FVIII and FIX inhibitors and correlation between inhibitor titers and levels of Tregs (CD4+CD25+FOXP3+) in PWH at MTRH

1.6.2 Specific objectives

i. To determine the prevalence of FVII and FIX inhibitors in PWH at MTRH

ii. To determine the pattern of disease severity in PWH at MTRH

iii. To compare inhibitor titers in different phenotypes of PWH at MTRH

iv. To correlate inhibitor titers and levels of regulatory T cells (CD4+CD25+FOXP3+) in PWH at MTRH

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Introduction

Hemophilia A (HA) and hemophilia B (HB) are bleeding disorders which arise from abnormality in functions or deficiency of factors. HA is a hereditary disorder which is linked to the X-chromosome and is caused by dysfunction or absence of blood coagulation factor VIII while deficiency of coagulation factor IX gives rise to HB (Blanchette, et al., 2014). Based on severity level, HA or HB can be categorized into three classes, namely: Mild (when containing 5-40% of FVIII or FIX relative to normal plasma levels), moderate (when having 1-5% of FVIII or FIX with respect to normal plasma levels) and severe (when having <1% of FVIII or FIX with respect to normal plasma levels). Patients with severe hemophilia experience more frequent bleeding episodes compared to mild or moderate hemophilia (Rosendaal, 2001).

It is notable that clot formation is the main product when multiple plasma proteins interact following an injury. The formed clot is stabilized when fibrinogen is converted to fibrin and then the formed fibrin undergoes cross-linking upon activation by factor XIII.

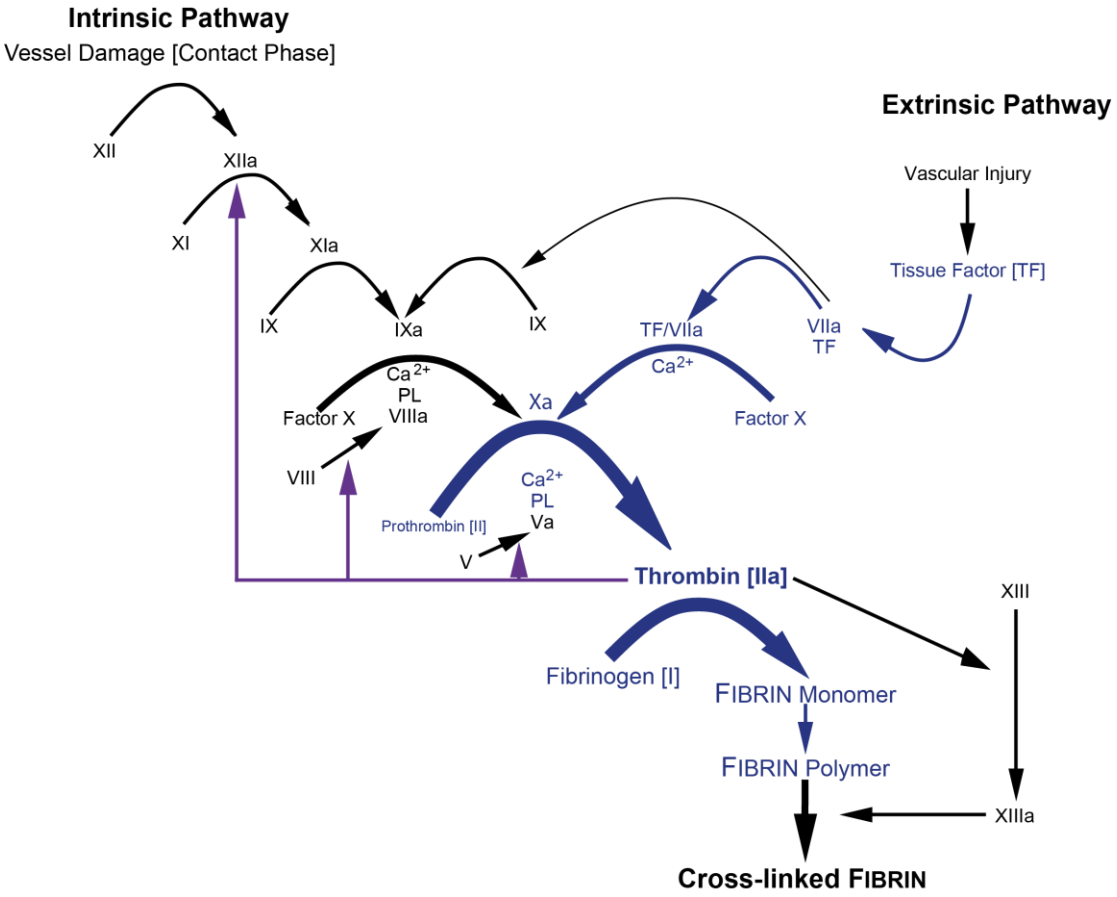


Figure 1: Summary of the coagulation cascade (Perry, 2020)

Intrinsic & extrinsic pathway leads to formation of prothrombin activator (Xa).Xa is responsible for the conversion of prothrombin to thrombin. Thrombin in turn leads to activation of fibrinogen to fibrin monomers. Fibrin monomers are not stable, so XIIIa acts on them and lead to fibrin mesh formation through covalent bonding. Fibrin mesh attracts phospholipids and platelets, and leads to the formation of a stable clot.

In hemophilia therapeutics, factor replacement therapy is a fundamental and effective treatment option except in cases where alloantibodies (which are basically, inhibitors) are expressed in patients as a response to counter exogenous factor products (Forsyth, et al., 2012). As mentioned above, the inhibitors may form in patients with hemophilia A or hemophilia B. These inhibitors are antibodies which are produced when such patients receive or are administered with external factor VIII (or IX) products, a common approach applied

in treatment of patients showing deficiency in factor VIII (or IX) (Mauro, et al., 2016). These antibodies are produced naturally when the clotting factor employed in treatment fails immune system's recognition either because the patient does not produce clotting factors or the patient produces abnormal proteins. Therefore, the interaction of a factor and an inhibitor leads to a response which may eliminate it from circulation or render it ineffective in the clotting process. Whereas inhibitors mainly deactivate or restrain the activity of the clotting factor, some of the factor VIII or factor IX inhibitors which do not deactivate the clotting factor, and therefore referred to as non-inhibitory antibodies, may also be present. It has also been shown that such inhibitors of factor VIII or factor IX being part of the autoimmune process may be present in persons who do not have hemophilia.

Research has shown that inhibitors formation in patients with hemophilia presents one of the most serious complications which can arise from factor replacement therapy. It has been established that the inhibitors renders replacement therapy ineffective. As a result, patients who develop inhibitors have increased risk of bleeding and morbidity, decrease in quality of life and increased hospitalization costs (B Verbruggen, 2010). The proportion of patients affected has been reported to range from 3·6% to 25%, but these figures have been derived mainly from retrospective data(Owaidah, et al., 2017).

The various differential diagnoses in patients with bleeding disorders may inclusively range from antibodies involved in inhibition to proteins involved in blood coagulation. In most cases, the immune system commonly targets factor VIII as a coagulation protein. It is noticeable that factor VIII inhibitors occur in form of alloantibodies in transfused patients with hemophilia whereas they arise as autoantibodies in healthy populations (Ma & Carrizosa, 2006). For example, these antibodies are produced, in about 20-30% of patients with hemophilia A, as a response to factor VIII infusions.

Also, it is important to note that factor VIII inhibitors are polyclonal IgG populations which are used in directing against multiple epitopes (Astermark, 2015). Formation of inhibitors is therefore one of challenging problems in treatment of hemophilia, and thus leads to substantial increase in morbidities, mortalities and the treatment cost. (Forsyth, et al., 2012).

2.1.1 Prevalence of inhibitors in patients with hemophilia

The development of inhibitors against FVIII or FIX is the most serious complication of haemophilia occurring in close to 30% of patients with severe haemophilia A, and about 10% of patients with mild-to-moderate haemophilia A, and fewer than 5% of patients with severe haemophilia B(David, et al., 2019). These complication render replacement therapy ineffective, increases the risk of serious bleeding and an earlier onset of progressive arthropathy and also higher treatment-related costs. While inhibitors usually develop within the first 20 exposure days and thus are an issue in young patients who receive prophylaxis, inhibitors are also a concern for older patients(Astermark, 2015). A total of 950 patients cared for at 16 U.S. hemophilia treatment centers were enrolled on The Hemophilia Inhibitor Study (HIS). Of these subjects, 283 hemophilia A had inhibitors(Alfonso Iorio, et al., 2019). In the United Kingdom (UK), a study investigating the effect of recombinant factor VIII (rFVIII) brand on inhibitor development enrolled 407 severe hemophilia A previously untreated patients born between 1 January 2000 and 31 December 2011(Garagiola, et al., 2018). An inhibitor developed in 118 (29%) patients, 60 high and 58 low titer, after a median (interquartile range) of 7.8 (3.3-13.5) months from first exposure and 16 (9-30) EDs. The introduction of viral inactivation steps to produce new plasmaderived products improved the safety by minimizing the potential of bloodborne pathogen transmission. However, these additional steps in the manufacturing process probably made plasma-derived products

more immunogenic with a higher risk of inhibitors which was estimated up to 20%-25%.23–27 For instance(Wali, 2018), the introduction of a pasteurized version of a previously dry-heated FVIII concentrate in order to obtain a higher purity concentrate (CPS-P) was associated with an outbreak of inhibitors in multi-transfused patients in the Netherlands and Belgium(Margaglione & Intrieri, 2018).

Inhibitors in hemophilia B are much less prevalent than hemophilia A, especially in patients with mild disease. Similar factors associated with inhibitors in hemophilia A also seem to be present for hemophilia B. the prevalence of inhibitors in patients with hemophilia B has generally been estimated using data from small, single institution studies, or from clinical trials of new factor IX products. A large survey of North American Hemophilia Treatment Centers (HTC) found a prevalence of inhibitors in hemophilia B patients of 1.5%.Of the 3785 hemophilia B subjects enrolled into the UDC surveillance project, 75 (2%) had an inhibitor at some point during the study period(Puetz, Soucie, Kempton, Monahan, & Investigators, 2014). The majority of subjects (59) had evidence of an inhibitor (either elevated inhibitor titer, or receipt of ITI or bypass agent) at time of enrollment into the UDC. Among those with an inhibitor, 24 (0.6%) had low titer inhibitors, while the remaining 51(1.3%) had high titer inhibitors. Of those with a low titer inhibitor, 10 (42%) received treatment with ITI or a bypassing agent during the study period (Soucie, et al., 2014).

92 patients diagnosed with Hemophilia A or B from North Eastern India were included in a study to determine the prevalence of inhibitors. The age of patients ranged from 2.5 month to 53 years. Out of 92, seventy nine (85.87%) were Haemophilia A and thirteen were (14.13%) Hemophilia B patients. 3.50% (2/55) cases of treated Hemophilia A patient developed an inhibitor.

2.2 Features of factor VIII and factor IX

Factor VIII is structurally a large 300-kDa glycoprotein with 2332 amino acids and produced in the sinusoidal cells of the liver as well as in the endothelial cells from the other parts of the body. It is established to circulate in plasma as a complex with von Willebrand factor (VWF) formed without the contributions of covalent interactions. VWF plays a role in protecting factor VIII from deactivating or getting cleared rapidly. In this complex, factor VIII protein binds as a dimer containing a heavy chain and a light chain, consisting of a number of domains which include domains A, B and C. Domains A1, A2 and B are part of the heavy chain (90 – 250 kDa) whereas domains A3, C1 and C2 form part of the light chain of 80kDa of the factor VIII glycoprotein. The heavy chain is quite heterogeneous owing to the variations in the formation of the B-domain (Jacquemin et al., 2003). Activation of factor VIII to factor VIIIa occurs upon its release from the complex along with the removal of B-domain. Factor VIII is a cofactor that plays a role in coagulation of blood as well as a cofactor for factor IXa which undergoes complexation in the presence of phospholipids and Ca^{2+} to form a complex which transforms factor X to active form factor Xa. Hemophilia factor products in conventional therapy include plasma-based factor VIII, plasma-based factor VIII with VWF, recombinant factor VIII without the B-domain and recombinant complete factor VIII. Among the recent developments include the use of pegylation to deliver the factor products with elongated half-life ($t_{1/2}$), glycopegylation, fusion of factor VIII to albumin or fusion of factor VIII to the Fc domain of immunoglobulins (Ig) and creation of sites for site-directed pegylation (Ekladious, Colson, & Grinstaff, 2019; Konkle et al., 2015). It is notable that a common pegylation procedure involves modification of the factor products so as to improve their $t_{1/2}$ and circulation of the proteins with therapeutic activity (Konkle, et al., 2015). For instance, emicizumab is a

bivalent antibody that has shown activity in the production of factor Xa from factor VIIIa and therefore used clinically to substitute for factor VIII. Also, emicizumab may be utilized as a therapy for individuals with inhibitors since it is a non-factor VIII-based product (Lenting, Denis, & Christophe, 2017). Recombinant factor VIIIa and aPCC represent two other categories of bypassing agents for patients with inhibitors.

On the other hand, factor IX is protease product dependent on vitamin K having a protein length containing 415 amino acids. It has a number of domains which include two-epidermal growth factor-like domains, a catalytic domain mainly serine protease and a gamma-glutamic acid domain. Factor IX undergoes rapid activation through regulated proteolysis by factor VIIa in the presence of calcium ions and cell-surface tissue factor (Komiyama, Pedersen, & Kisiel, 1990; Samelson-Jones, Finn, George, Camire, & Arruda, 2019).

2.3 Characteristics of inhibitors

Inhibitors are commonly polyclonal and mostly of immunoglobulin G (IgG) class while some are monoclonal with characteristic IgG subclass and oligoclonal with IgG subclass 1 and subclass 4 (Giddings, Bloom, Kelly, & Spratt, 1983). IgG1 and IgG4 are common subclasses for factor VIII inhibitors. However, IgG1 have been reported to be present in some patients who do not have functional inhibitors. On the hand, factor IX inhibitors show characteristic IgG4 subclass while the other subclasses are rarely observed. It is worth mentioning that functional inhibitors in both hemophilia A & B are associated mostly with the detection of IgG4 subclass of antibodies. Activation of factor VIII inhibitors occurs slowly, time bound and dependent on temperature as well with optimal activity at 37 °C. Such a reaction profile of factor VIII inhibitors is useful in differentiating between factor VIII inhibitors and other non-specific inhibitors as well

as lupus anticoagulants which are not time bound. Unlike Factor VIII inhibitors, anti-factor IX antibodies are not sensitive to exposure time and therefore not time-dependent in their action. These differences in activity profiles are believed to be driven by the large size of factor VIII-VWF complex which leads to steric hindrance. Like other IgG4 antibodies, factors VIII and factors IX inhibitors do not form precipitating complexes in gels. In vitro experiments have demonstrated that these inhibitors form immune complexes which circulate with their respective antigens in patients with hemophilia. Separation of such factor VIII and factor IX immune complexes involve acidification or heating. Although less effective, heating may also be applied in separating factor IX from factor IX inhibitors. These factors have been proposed to work preferably by clearing circulating immune complexes instead of blocking of factor VIII or factor IX activities. As a result, this leads to removal of antibodies accompanied by no inhibitory detectors in the presence of excess antigen.

It has been observed that factor VIII inhibitors show selective interaction with the factor VIII molecule with A2, C1 and C2 being the common domains. Also, different factor VIII inhibitors have varied affinities and interaction kinetics for factor VIII. There are two common kinetics of interaction for these inhibitors, Type 1 kinetics and Type 2 kinetics. Type 1 kinetics occurs when inhibitors are completely neutralized on addition of factor VIII while the interaction is classified as Type 2 kinetics when the activity of the added factor VIII remains even in the presence of excess inhibitor. Therefore, Type 1 and Type 2 inhibitors display different reaction profiles including affinity with the added factor VIII. Although these differences have been observed in immunologic assays, their effect in vivo on functional inhibitors assays and kinetic is still unclear. On the contrary, kinetics studies on factor IX inhibitors are however limited and more

detailed studies are required. For factor IX inhibitors, their target domains are mainly the protease domains.

2.4 Molecular mechanisms of inhibitor formation

Anti-thrombin III, proteins C & S are natural inhibitors in the coagulation cascade. These proteins play a crucial role in the regulatory mechanisms that serve to limit the extension of the clot through inactivation of specific clotting factors following their activation (Blanchette, et al., 2014). In contrast, inhibitors to coagulation factors (also known as circulating anticoagulants) refer to antibodies which interfere with, and thus neutralize, the normal functioning of clotting proteins. These antibodies may work against against factor VIII or factor IX inhibitors in the case of Hemophilia A and B respectively. Being a coagulation protein, it has been observed that the immune system commonly targets factor VIII (Astermark, 2015). Some of the patients with hemophilia A express these neutralizing antibodies (inhibitors) in response to factor VIII infusions during therapy. Even though the underlying causes of the immunogenicity of factor VIII protein is not well established, recent studies have significantly provided more data on antigen-presenting cells (APCs) and factor VIII, with some insights into the way these cells take up and process factor VIII (Fallon, et al., 2018). Following these recent studies, it has been observed that factor VIII protein uses its C1 domain to gain entry into APCs. Nonetheless, the mechanism of endocytosis is still not clear yet. In the APCs, factor VIII protein is subsequently processed in the endolysosomes producing a heterogeneous mixture of peptides derived from itself as factor VIII-derived peptides mainly on major histocompatibility complex (MHC) class II. Therefore, anti-factor VIII antibody are then produced when cognate effector $CD4^+$ T cells recognize the complex of peptide-MHC class II (Astermark, 2015).

2.5 Risk factors for the development of inhibitors

Complex interactions involving environmental and/or genetic factors and the immune system of the patients lead to the development of an inhibitor. Some of the recognizably potential risk factors in the process of inhibitor development include genetic factors such as the type of mutation, HLA genotype, ethnicity, family history of inhibitors and severity of hemophilia. Other risk factors include non-genetic factors such as the kind of concentrate used in treatment, multiple switches in the products used, the intensity of the treatment and age of the patient at first treatment (Johannes Oldenburg et al., 2004).

2.5.1 Genetic factors

Multivariate analysis by Goudemand et al identified independent risk factors including the ethnic origin; Nonwhites were found to be at 3.5- to 6.7-fold higher risk than whites. Additionally, patients with family history of inhibitors were at a 5.8- and 6.3-fold higher risk than patients with a family history of hemophilia without inhibitors, a link that has also previously been reported (Goudemand et al., 2006).

Factor VIII genotype is a critical risk factor for the development of inhibitors. It is notable that there is a good correlation between severity of HA and development of inhibitors with about 20 % of patients in the reported data showing a positive correlation between development of inhibitors and the severity of Hemophilia A (XF Wang, et al., 2010). Researchers have gone ahead to conduct genetic analysis of these patients and strikingly 90% had large insertions/deletions of >1 exon, inversions in intron 1 or intron 22, or nonsense mutations on factor VIII gene's light chain (J Oldenburg & Pavlova, 2006). The major deletions results in absence of circulating factor VIII antigen causing the immune system to recognize the exogenous factor VIII as a "foreign protein" thus formation of inhibitors against it (Saint-Remy, 2002). On the contrary, there is less risk

of formation of inhibitors in patients with moderate or mild HA. Nonetheless recent studies though limited shows that inhibitors also arise in patients with mild or moderate HA (Lenk & Kertzscher, 2002). The widespread presence of inhibitors in patients with moderate or mild HA ranges between 3 to 13 %. Based on this prevalence data, it is believed that the development of inhibitors in patients with mild hemophilia may occur through an alternative mechanism. Alterations in the immunogenicity of factor VIII or IX may be as a result of missense mutations especially in its C1/C2 domains thus may generate a response from inhibitors against the epitope that underwent mutations (Eckhardt et al., 2012). For example, a study conducted by Hay et al., 1998, found that missense mutations were common in the nine patients who had mild HA and this may have contributed to the abnormal but relatively stable conformation because of the introduction of new cysteine residues which could regulate the formation of disulphide bridges (C. Hay et al., 1998). In another retrospective study involving 16 patients with hemophilia containing a T295A missense mutation, two of the three patients who developed inhibitors experienced low-titer inhibitors while the titer level for one of the three patients was found to be high. Therefore, patients with hemophilia are predisposed to inhibitor development as a result of mutations causing infused factor VIII or IX to be perceived as either a completely novel antigen or an immunologically altered antigen (Ivaskevicius et al., 2014)

2.5.2 Non-genetic factors

Environmental factors have also been shown to contribute significantly to the development of inhibitors. For instance, a study on the incidence of inhibitor development in patients with hemophilia whose factor VIII or factor IX activity was 5% or less, and who had received replacement therapy at least once found that inhibitors developed only in hemophilia A patients who had previously been treated with factor

VIII products. All inhibitors were first detected when patients were aged 0·08-5·2 years. The cumulative risk was 33% at age 6 years.

Young age at first factor treatment has been reported to be a potential risk factor for inhibitor development (Chalmers et al., 2007). Goudemand and colleagues found a 0.3 fold fewer inhibitors in children treated for the first time after 12 months of age compared with those treated before 6 months of age. They also compared the incidence of inhibitors in previously untreated patients with severe HA treated with either rFVIII (Recombinate or Kogenate) or PdFVIII (FVIII-LFB). They found that rFVIII (Recombinate or Kogenate) carries about a 2.5- to 3-fold higher risk than PdFVIII (FVIII-LFB) for inhibitor development (Goudemand, et al., 2006). Researchers have put forward two possible explanations for a lower risk of inhibitor development with the PdFVIII namely; the presence of immunomodulatory activity co-purified along with factor VIII and the presence of von Willebrand factor (VWF). Immunomodulatory activity has been associated with transforming growth factor (TGF) in some, but not all, plasma-derived products. For instance, TGF was found to be in relatively high quantities in factor VIII LFB even though it is not the only cytokine with immunomodulating properties that is available in the concentrates derived from plasma. Through studies involving animal models, the VWF in PdFVIII has been found to offer protection against the development of inhibitors. To facilitate this protective action, the VWF attaches mainly to the C2 domain of factor VIII which is a common domain targeted by antibodies working against factor VIII. On the contrary, rFVIII lacks both immunomodulatory activity and von Willebrand factor hence a higher risk for inhibitor formation (Gouw et al., 2007).

As a result of the recent work on inhibitors, exposure of multi-transfused patients to specific factor products revealed another set of different inhibitors specific to multi-

transfused patients. This variation is evident in the recent study where a change of therapeutics to a concentrate containing factor VIII with an inactivated double-virus derived from plasma led to development of inhibitors in 8 of the 140 multi-transfused patients with severe HA. In the above study, the titer level of the inhibitors of the 8 patients were in the range of 2.2 to 60 Bethesda Units whereas the transfused factor VIII recovery was between 0.21 and 0.68 (expressed as i.u./dl factor VIII rise per i.u./kg administered). The unique thing about this category of inhibitors is that they showed complex kinetics of inhibition which were observed to be specific to the light chain of factor VIII protein. In spite of the elongated treatment using concentrates containing factor VIII, the level of these inhibitors was observed to interestingly decrease gradually upon withdrawal of pasteurization products. According to the above findings, there is need for clinical studies which are monitored prior to the introduction of a modified or a new concentrate containing a clotting factor in both untreated patients and previously treated patients (Coppola, Di Minno, & Santagostino, 2010).

2.6 Correlation of inhibitors and tregs

The immune process in inhibitor development is a complex process. When exogenous factor VIII or FIX is administered to a patient with hemophilia, some of the factor is processed by antigen presenting cells to present antigen in the context of Major Histocompatibility Complex (MHC). CD4+ cells, recognize this antigen presentation, mediated by their T cell receptors, and become activated to secrete cytokines that will drive B cell differentiation and antibody production targeted against the antigen. CD4+ CD25+ FoxP3+ Tregs are associated with inhibiting these ‘effector T cells,’ mediated in part by cell-cell contact, thereby invoking tolerance and limiting B cell antibody

production (Ettinger et al., 2016). Tregs expressing CD25 and the transcription factor FOXP3 account for 5-10% of normal human peripheral blood CD4+ T cells (Sakaguchi, 2005).

Tregs can inhibit effective T cell proliferation and cytokine production to also prevent CD8+ cells from differentiating into fully functional cytotoxic cells. When Tregs are lacking, inhibitory function returns (Tang and Bluestone, 2008). Findings from *in vitro* experiments showed that CD25+ cells immunosuppressive effects and the number and function of Tregs were related to pathogenesis in a variety of immune diseases (Kamate, Lenting, Van Den Berg, & Mutis, 2007). A study by Ding, 2014 found that CD4+ CD25+ $CD127^-$ were higher in patients with inhibitors compared to those without inhibitors. However the percentage of CD4+ CD25+ $CD127^-$ in non-inhibitor was not significantly different from that of healthy controls. They argued that the increase in the number of Tregs may be due to feedback to the produced antibodies thereby reducing the body's immune response to factor VIII. They added that the high proportion of CD4+ CD25+ $CD127^-$ T cells in inhibitor positive may be as a result of CD4+ T cells releasing immunosuppressive factors to inhibit production of factor VIII antibodies (Ding, Ji, Wu, Li, & Sheng, 2014).

In contrast to the above, El-Asrar et al. 2016 found that the frequency of Tregs was significantly decreased among patients with hemophilia A in the groups which had developed inhibitors as well as those without inhibitors relative to the healthy control group. From their study, it was found that the lowest levels of Tregs occurred in the group which had developed inhibitors of factor VIII. In addition, it was also observed that Tregs levels were more frequent in the group of patients with severe HA in comparison to the patients with moderate or mild HA. By using the receiver operating curve (ROC) to analyze patients' samples, patients who have developed inhibitors

could be distinguished from those patients who haven't developed inhibitors, with Tregs cut-off value at 1.91%, 91.3% specificity and sensitivity of 100%. However, they recommended that more studies must be conducted so as to verify the above Tregs threshold values (El-Asrar, Hamed, Darwish, Ismail, & Ismail, 2016).

2.7 Laboratory diagnosis of inhibitors

The differential diagnosis in patients with hemophilia includes inhibitory antibodies to blood coagulation proteins. In order to reduce the possibility of anamnesis, there is need for diagnosis of inhibitors in the early stages of the diagnosis process as this will provide an opportunity for the commencement of immune toleration induction (ITI) in the event level of the inhibitor does not go above the threshold 10 BU/mL. This in turn will reduce exposure to the undesirable sub-optimal therapy. Additionally, some of the circumstances under which the testing for inhibitors is prompted in the diagnostic process include; (i) prior to elective procedures which are invasive in nature, (ii) when the concentrate used in infusion leads to sub-optimal laboratory or clinical response, (iii) prior to a change of concentrate and in subsequent change of concentrate, and (iv) 2 – 3 weeks following a surgery or a treatment which is intensive in patients with mild or moderate hemophilia. The UK guidelines states that the screening for inhibitors should be carried out in patients with severe hemophilia an inhibitor screen should be performed in patients with severe hemophilia at least on every 3rd Exposure Days (ED) or every 3 months in the event the exposure to the concentrates has happened continuously until 20 EDs (C. R. Hay, Brown, Collins, Keeling, & Liesner, 2006).

A measurement on the clearance rate of the factor in the infusions offers the most sensitive method to detect as well as determine the quantity of inhibitors in the sample. Such tests for inhibitors have been found to give the most sensitive results after a

washout period especially when the level of the factor has gone back to the baseline in a period of 24 h. This will help to minimize errors arising from the masking or quenching of a low-titer inhibitor in the residual concentrate used in infusions (B Verbruggen, 2010). Testing for inhibitors is often carried out using Bethesda assay, though Nijmegen-Bethesda test method is highly recommended since it is more sensitive than Bethesda assay (Bert Verbruggen, van Heerde, & Laros-van Gorkom, 2009). There is also another screening approach that is used to determine the activity of inhibitors against a patient's factor concentrate described in 2006 (C. R. Hay, et al., 2006). It is a useful screening test and appears to be more sensitive than a Bethesda assay; however, it may be less specific and associated with false-positive results. In patients using standard prophylaxis (20–50 iu/kg alternate days), a measureable factor trough level at 48 h can pragmatically be interpreted as a negative inhibitor screen because this is likely to be associated with a half-life >7 h. In patients with mild or moderate hemophilia A the sensitivity of an inhibitor test may be improved by heating the plasma at 58°C for 90 min to inactivate residual FVIII (Kitchen et al, 2009 and Miller et al, 2012).

2.8 Treatment and management of inhibitors

Treatment strategies for inhibitors depend on the patients' inhibitor titer levels. The frequency of the dosage of factor FVIII or FIX can be increased or even higher doses can be used in the treatment of patients who have low-level of titer inhibitors (<5 BU/mL). This regimen will not only give rise to pre-existing inhibitor saturation but also give sufficient factor that will be enough to bring back homeostasis as well normal coagulation (Collins et al., 2013). In contrast, a simple infusion treatment has been shown to be ineffective for patients whose level of titer inhibitors is high (>5 BU/mL).

Such situations therefore have been effectively overcome using bypassing agents which include factor VII that has been activated or a concentrate containing prothrombin-complex which has also been activated. However, the mainstay of treatment for patients with high level of titer inhibitors is through immune tolerance induction (ITI). ITI consists of regular injections of factors for a period varying from several weeks up to two years. ITI remains the only strategy that proved to both eradicate FVIII inhibitors as well as lead to induction of factor VIII-specific immune tolerance (Coppola, et al., 2010). There exist two protocols which are mostly in common use, namely; the Bonn and the Malmö protocols. Interestingly, these two protocols have resulted in remarkable and comparable rates of success of up to 87 % in spite of their substantial differences. Such an occurrence may be explained by the activation of regulatory T cells following excessive exposure to high concentration of antigen and thus could possibly suppress effector T cells which are specific to antigens with subsequent tolerance induction. Therefore, B-cells which are specific to factor VIII would likely be eliminated because of their inability to differentiate into durable plasma cells which can produce antibodies as a consequence of the absence of support from T cells (Saint-Remy, 2002).

It is noteworthy that ITI is considered as a long-term investment in the treatment of inhibitors because eradication of inhibitors require effective strategies since it encompass prevention and control of bleeds in the course of inhibitor treatment (Coppola, et al., 2010). For instance, it is advisable for patients who have factor VIII inhibitors determined on a number of occasions to receive ITI in order to get rid of the inhibitors and bring back their normal effectiveness in clinical response to factor VIII. This is because such regular presence of factor VIII inhibitors causes interference in treatment or prophylaxis of bleeds in the course of standard treatment doses. Even so, a number of aspects that could likely influence the results of ITI have been described,

mainly good risk patients (defined as having an inhibitor titer 500 BU/mL upon introduction of ITI) on several occasions give poor results (DiMichele, 2012). Therefore, after a period of 6 – 9 months, ITI can be withdrawn from this category of patients. Subsequently, a second alternative line of treatment should be sought as long as there is evidently no significant continuous reduction in the level of titer inhibitor; that is, of more than 20 % reduction in the level of titer inhibitor after a period of 6 months. Additionally, the choice of factor products has been shown to also influence the results of ITI. It has been observed that by using factors based on plasma which is of low-purity, tolerance is easily achieved compared to when using recombinant factor products (Kurth et al., 2011). However, these findings still remain controversial as evidenced by a number of other studies which have shown that the type of factor product does not seem to influence the success rate of ITI. Notably, the most powerful predictor of ITI success is the patients' starting titer (Goudemand, et al., 2006). It has been established that regimens which elongate the treatment to such a time that the level of titer inhibitor is below 10 BU/mL have registered high rates of success in treatment (Mauser-Bunschoten, Nieuwenhuis, Roosendaal, & van den Berg, 1995). During this time, a recombinant factor VII (rFVIIa) which has been activated ought to be used in the treatment of bleeds so as to eliminate an anamnestic response (O'connell et al., 2002). Some of the research data further suggest that efficacy in induction of tolerance can be achieved by using high dose regimens (200 and 100 iu/kg/d) in good risk patients, however the influence of these high dose regimens on bleeding is yet to be determined. On the other hand, use of low-doses (50 iu/kg three times a week or on alternate days) to induce tolerance has been associated with elongated time to reach a negative Bethesda titer and further linked with substantially a lot inter-current bleeding prior to the negative Bethesda titer (DiMichele, 2012).

It is recommended that patients with low-titer inhibitors (historic peak titre 5 BU/mL requiring ITI should be started with high-dose to minimize inter-current bleeding. However, in good risk patients, dose reduction in stages can reduce costs as long as there is no subsequent increase in breakthrough bleeds that require by-passing therapy. Hay and Dimichele, 2012 concluded that the above approach will not expectedly elongate the time that will be taken to reach tolerance.

2.8.1 Novel techniques to modulate inhibitor formation

Several novel techniques being developed to modulate inhibitor formation are currently at various stages of translation to the clinic. The limitations of ITI such as; inability to resolve high titer inhibitors, also prohibitively expensive for many patients has inspired further search for novel techniques currently undergoing clinical trials (Mariani, Siragusa, & Kroner, 2003). Some of these approaches include gene therapy, use of immunosuppressive drugs, blockade of co-stimulation, oral tolerance, nanoparticles and generation of Tregs. Gene therapy is ideal for monogenic hereditary diseases like hemophilia; however the underlying problem in this approach is the patients' immune response to both the therapeutic protein and its transmission vector (Liu, Ye, Lin, Djukovic, & Miao, 2014). Recent advances include use of AAV virus and manipulation of responses with microRNA. With regards to engineered Tregs, they have been proposed to treat undesirable immune responses. Researchers agree that Tregs have the potential to be useful but are generally not antigen specific which may lead to global immunosuppression. Therefore activated Tregs generated in response to an antigen are more desirable, however obtaining large numbers from a patient is technically challenging. Fortunately, scientists have now created antigen specific Tregs by transduction of T-cell receptor (TCR) variable regions into expanded human FOXP3+ Tregs (James et al., 2011). In so doing they were able to suppress the factor VIII-

specific effector cells thus validating their potential to treat inhibitor antibody formation in hemophilia.

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study site

The study was carried out at Academic Model Providing Access To Healthcare - Moi Teaching and Referral Hospital (MTRH) situated in the Western parts of Kenya. AMPATH is a partnership between Moi University School of Medicine, Moi Teaching and Referral Hospital (Kenya's second largest national referral hospital), and a consortium of U.S. medical schools led by Indiana University. AMPATH promotes and fosters a comprehensive approach to HIV/AIDS and other diseases including hemophilia. AMPATH hemophilia program was started in 2012, supported by Indiana Hemophilia and Thrombosis Center (IHTC). Since then a number of patients have been diagnosed and continue to receive care through the follow up clinics at AMPATH-MTRH, Chandaria Cancer and Chronic Disease Center (CCCDC).

Figure 2: Aerial view of AMPATH-MTRH, CCCDC (Photo courtesy of Pfizer pharma)

3.2 Study design

This was a cross-sectional study involving patients with confirmed diagnosis of hemophilia attending follow up clinics at AMPATH- MTRH in Western Kenya. The study participants, parents/guardians were briefed on the nature and purpose of the study and participation was by voluntary informed consent or assent where applicable. Participants were recruited and their blood samples collected between the months of January and October, 2019.

3.3 Study population

The target population consisted of patients with confirmed diagnosis of hemophilia attending hemophilia follow up clinic at Chandaria Cancer and Chronic Disease Center in AMPATH-MTRH. Announcements inviting participants to the study was placed at the notice board near the hemophilia clinic at CCCDC, MTRH from Jan- Oct, 2019

3.4 Target population

3.4.1 Sample size determination

The objective of the study is to determine the prevalence of inhibitors and levels of Tregs markers in patients with hemophilia at MTRH. Previous studies show that the overall prevalence of inhibitors in patients with hemophilia is approximately 3-30%. In Kenya, there are about 400 persons diagnosed with hemophilia. Those diagnosed and treated at MTRH are about 120 (WFH data). Thus in order to determine a sufficient sample size that will give us 95% level of confidence, we used the following formula (Cochran, 1963).

$$n_o = \frac{Z^2 P(1-P)}{e^2}$$

$$n_o = \frac{1.96^2 \times 0.3 \times (1-0.3)}{0.05^2} = 322 \text{……..Equation 1}$$

Where;

Z = is the standard normal variate at 5% type 1 error (P<0.05) which is 1.96. In majority of studies p values are considered significant below 0.05.

P = is the expected proportion in the population based on previous studies or pilot studies. In this case we take a P of 30% (0.3).

e = is the absolute error or precision decided by the researcher. In this case we use 5% (0.05).

Since the population of persons diagnosed with hemophilia at MTRH is small i.e 120, a sample size of 322 may not be feasible. Therefore the sample size was adjusted as follows;

$$n = \frac{N_o}{1+\frac{(n_{o-1})}{N}}$$

Where N is the total number of patients with hemophilia at MTRH
Hence;

$$n = \frac{322}{1+\frac{(322-1)}{120}}$$

n = 88……..Equation 2

3.4.2 Inclusion criteria:

i. All PWH who are currently on factor replacement therapy during the study period.

3.4.3 Exclusion criteria:

i. PWH and a known diagnosis of HIV/AIDS.

ii. PWH and a known diagnosis of Systemic Lupus Disease.

3.5 Study procedure

Every participant was assigned a unique number for purposes of identification on the recruitment day. We did not include their names and phones numbers to ensure confidentiality. Their demographic and clinical data were collected using a predesigned data collection form. Two samples of venous blood in sodium citrate tubes (4mL) and EDTA tube (1mL) were collected by a skilled phlebotomist. The sample in sodium citrate tubes was centrifuged at 1500g for 15 minutes, plasma aliquoted into cryo vials for factor and inhibitor assays. The EDTA sample was used for flow cytometry assays of CD4+ CD25+ FoxP3+ Tregs. The characteristics of the study participants were described using means, standard deviations and relative proportions. Fishers' exact test was used to compare disease severities (%) and inhibitor titers (BU). Spearman's rank correlation coefficient was used to determine the correlation between inhibitor titers (BU) and percentage (%) levels of (CD4+ CD25+ FoxP3+) Tregs. A P value of 0.05 or less was considered significant. Data analysis was conducted using R-studio software in the month of October, 2019. Thesis writing took place between the months of November, 2019 and March, 2020. The study timeline and the budget are attached on the appendix 1 and 2 respectively. The study budget mainly covers the reagents for factor and inhibitor assays which were purchased from Stago Diagnostica as well as the reagents for flow cytometry which were purchased from Elab Sciences.

3.6 Sampling techniques

Hemophilia is classified as a rare bleeding disorder; in this regard the number of patients enrolled in this program is few, therefore convenient sampling where subjects meeting the inclusion criteria were recruited consecutively until the desired sample size (88) was achieved. All patients with hemophilia who were diagnosed from 2012 when the AMPATH-MTRH Hemophillia program was started to a month prior to the study (Dec 2018) were considered in order to give all the patients attending follow up clinics at MTRH the opportunity to participate in the study if they met the inclusion criteria.

3.7 Sample collection and measurements

Patient demographics and clinical data were collected using a predesigned data collection form. Venous blood was collected into a 3.2% BD sodium citrate tubes (4mL) in ratio 1:9 and EDTA (1mL) by a skilled phlebotomist. The sample collected in sodium citrate tubes were centrifuged at 1500g for 15 minutes. Diagnosis of hemophilia and disease severity was determined using factor assay while inhibitor titers were measured using Bethesda assay described below. The sample collected in EDTA tubes (1mL) was used to measure the percentage levels of (CD4+ CD25+ FoxP3+) Tregs using flow cytometry. Both the Bethesda assay and flow cytometry assay were performed following the manufacturers' instructions.

3.8 Laboratory measurements

3.8.1 Factor VIII assay

Factor VIII assay was used to confirm the diagnosis and severity of hemophilia A.

Principle:

The assay is based on a comparison of the ability of dilutions of standard and test plasmas to correct the APTT of plasma known to be totally deficient in factor VIII but containing all other factors required for normal clotting.

Procedure:

Cuvettes strips were placed in the incubation area for pre-warming at 37 degrees for at least 3minutes. To the cuvettes 50µL of the calibrator was added, factor VIII deficient plasma, APTT reagent then the timer was started. The same steps were followed for the patient sample. When the instrument started to beep the cuvettes were transferred to the test column area. The finn tip pipette was primed once with the start reagent calcium chloride. The pipette key was pressed to activate the finn tip pipette. 50µL of calcium chloride pre-warmed at 37ºc was dispensed into the cuvette. A percentage check of calibrator standard was done. The patient results were plotted against the factor VIII calibrator curve to determine the factor VIII levels.

3.8.2 Factor IX assay

Factor IX assay was used to confirm the diagnosis and severity of hemophilia B.

Principle:

The assay is based on a comparison of the ability of dilutions of standard and test plasmas to correct the APTT of plasma known to be totally deficient in factor IX but containing all other factors required for normal clotting.

Procedure:

Cuvettes strips were placed in the incubation area for pre-warming at 37 degrees for at least 3minutes. To the cuvettes 50µL of the calibrator was added, factor IX deficient plasma, APTT reagent then the timer was started. The same steps were followed for the patient sample. When the instrument started to beep the cuvettes were transferred to the

test column area. The finn tip pipette was primed once with the start reagent calcium chloride. The pipette key was pressed to activate the finn tip pipette. 50µL of calcium chloride pre-warmed at 37ºc was dispensed into the cuvette. A percentage check of calibrator standard was done. The patient results were plotted against the factor IX calibrator curve to determine factor IX levels.

3.8.3 Factor VIII inhibitor assay

Factor VIII inhibitor assay is the Quantitative determination of inhibitors in hemophilia A.

Principle:

Factor VIII inhibitors were quantified by mixing the test plasma with a known amount of factor VIII present in normal pooled plasma. After a 2 hour incubation period, the residual factor VIII activity was measured. By comparing the difference in factor VIII activity of the patient incubation mixture and a control mixture, the amount of inhibitor present was calculated in Bethesda Units. One Bethesda unit of inhibitor is defined as the amount that will inactivate 50% of the factor VIII activity present (Giles et al., 1998).

Procedure:

Plastic tubes were labelled as per dilutions ranging from 1:2 to 1:1024. 200 µl of Imidazole buffer was transferred into the labelled tubes 200 µL of test plasma was added into tube no.1 which is labelled as 1:2, mixed and serial dilution done till last tube. (1:1024). 12 glass tubes were labelled as follows:

a. Tube 1: 200 µL of buffer (Imidazole)+ 200 µL control (Pooled normal plasma)

b. Tube 2: 200 µL of test plasma + 200 µL of control plasma (Pooled normal plasma)

c. Tube 3 -12: 200 µL of respective diluted test plasma from 1:2 to 1:1024 + 200 µL of Control plasma (Pooled normal plasma) in all the tubes. (All tubes were pluged with non-absorbent cotton.)

All the tubes were then incubated in a Water bath at 37°C for 2 hours. The standard curve was generated for factor VIII assay. At the end of two hours, factor VIII assay (refer to 3.8.1) was performed on each incubation mixture by using 1/5 dilution.

Note:

Factor activity of control + buffer should always be more than 80%, otherwise the test is invalid.

Result interpretation:

The factor VIII activity of the control and the patient incubation mixtures were determined from the factor VIII assay curve. The residual factor VIII activity was determined using the Factor VIII activity of the control and the dilution of the patient plasma having a factor VIII activity that yields a residual factor VIII activity greater than 25% lesser than 50%. Residual factor VIII activity (%) = factor VIII activity (Patient)/ factor VIII activity (Control) x 100. The Residual Factor VIII activity was converted to Bethesda Unit using the provided Bethesda chart.The Bethesda unit obtained was multiplied by the dilution factor.

3.8.4 Factor IX inhibitor assay

Quantitative determination of inhibitors in hemophilia B was performed using factor IX inhibitor assay.

Principle:

Factor IX inhibitors were quantified by mixing the test plasma with a known amount of factor IX present in normal pooled plasma. After a 2 hour incubation period, the residual factor IX activity was measured. By comparing the difference in factor IX activity of the patient incubation mixture and a control mixture, the amount of inhibitor present was calculated in Bethesda Units. One Bethesda unit of inhibitor is defined as the amount that will inactivate 50% of the factor IX activity present (Giles, et al., 1998).

Procedure:

Plastic tubes were labelled as per dilutions ranging from 1:2 to 1:1024. 200 µl of Imidazole buffer was transferred into the labelled tubes. 200 µL of test plasma was added into tube no.1 which is labelled as 1:2, mixed and serial dilution done till last tube. (1:1024). 12 glass tubes were labelled as follows:

Tube 1: 200 µL of buffer (Imidazole)+ 200 µL control (Pooled normal plasma)

Tube 2: 200 µL of test plasma + 200 µL of control plasma (Pooled normal plasma)

Tube 3 -12: 200 µL of respective diluted test plasma from 1:2 to 1:1024 + 200 µL of Control plasma (Pooled normal plasma) in all the tubes. (All tubes were plugged with non-absorbent cotton.)

All the tubes were then incubated in a Water bath at 37°C for 2 hours.

The standard curve was generated for factor IX assay.

At the end of two hours, factor IX assay (refer to 3.8.2) was performed on each incubation mixture by using 1/5 dilution.

Note:

Factor activity of control + buffer should always be more than 80%, otherwise the test is invalid.

Result interpretation:

The factor IX activity of the control and the patient incubation mixtures were determined from the factor IX assay curve. The residual factor IX activity was determined using the Factor IX activity of the control and the dilution of the patient plasma having a factor IX activity that yields a residual factor IX activity greater than 25% lesser than 50%. Residual factor IX activity (%) = factor IX activity (Patient)/ factor IX activity (Control) x 100. The Residual factor IX activity was converted to Bethesda Unit using the provided Bethesda chart. The Bethesda unit obtained was multiplied by the dilution factor.

Definition of inhibitor unit: One Bethesda Unit of inhibitor is defined as the amount of antibody that will inactivate 50% of added normal plasma FVIII or IX activity after 2 hours incubation at 37^{o}C. For practical purposes, the level of inhibitor detected is that corresponding to the inverse of the dilution leading to an apparent assayed FVIII or IX residual value of 50% of that in tube.

3.8.5 Flow cytometry assays

Levels of Tregs (CD4+ CD25+ FoxP3+) in patients with and without inhibitors, was determined using flow cytometry.

Immunophenotyping by flow cytometry

Venous blood collected in EDTA tube was stained for surface markers using anti-CD25 allophycocyanin (APC) and anti-CD4 (FITC) for 30 minutes at room temperature (Elab Sciences, U.S.A). Subsequently, the cells were fixed, permeabilized with 1X permeabilisation buffer (eBioscience, CA, USA), after which intracellular staining with anti FoxP3 antibodies (Elab sciences) is performed for 30 minutes at 4ºC. Data was

then acquired and analyzed using Flow Jo software. Results were presented as mean percentage of FOXP3 positive cells gated on (CD4+CD25+) cells.

3.9 Data management plan

3.9.1 Data collection, entry and storage

- All the generated data were entered into Ms Excel for cleaning then exported to R-studio for analysis.
- Data was presented using graphs and pie charts, flow cytometry scatter dot plots and frequency distribution tables.
- All soft copy data was kept in a password protected computer while the hard copy data was kept in a lockable cabinet located in a restricted-access room.

3.9.2 Statistical analysis

Demographic data and laboratory results were entered into a Microsoft Excel spread sheet (2010) to enable data review and cleaning. Cleaned data was then exported to R-studio for coding and analysis. The characteristics of the study participants were described using means, standard deviations and proportions. Fishers' exact test was used to compare disease severities (%) and inhibitor titers (BU). Spearman's rank correlation coefficient was used to determine the correlation between inhibitor titers (BU) and percentage (%) levels of Tregs (CD4+ CD25+ FoxP3+). For all tests, a *P* value of 0.05 or less was considered statistically significant.

3.10 Ethical considerations

- The study proposal was approved by the Institutional Research Ethics Committee (IREC) of MTRH/Moi University.

- Participants were recruited into the study only after giving an informed consent, and assent where applicable.
- Participants' identities were concealed throughout the study period.
- All soft copy data was kept in a password protected computer while the hard copy data was kept in a lockable cabinet located in a restricted-access room.

3.10.1 Informed consent

The study purpose and procedures were explained to each participant in Swahili and English. Participants were recruited into the study only after giving an informed consent, and they were at liberty to withdraw from the study at any time without question.

3.10.2 Confidentiality

All the information obtained from the participants was kept private and confidential. The samples collected as well as the laboratory generated data were recorded using unique identification numbers. To further ensure confidentiality, the lab generated reports were kept in secure cabinets and password protected computers, only accessible to authorized personnel.

CHAPTER FOUR

4.0 RESULTS

4.1 Participants' characteristics

The analysis of this data was done using R-studio software. A total of 88 participants were recruited in the study. The mean age in years was 16.90 (SD=12.22) with a median of 14 years (IQR: 7.5, 24.5). Majority of the participants were HA 71 (81%) while the remaining 17 (19%) were HB. All of the participants were Male and of the ages between 1.75 years (approx. 21 months) to 61.75 years old (approx. 741 months).

Table 1: General characteristics of the participants based on their severity and age

Severity	**Age (years)**	**Hemophilia A** (Factor VIII, n=71)	**Hemophilia B** (Factor IX, n=17)	Total n=88
Mild (>5%)	<5	1	-	1
	5-9	1	-	1
	10-14	3	-	3
	15-19	-	1	1
	>20	4	1	5
		9	2	11
Moderate (1-5%)	<5	1	-	1
	5-9	1	-	1
	10-14	1	1	2
	15-19	1	-	1
	>20	6	-	6
		10	1	11
Severe (<1%)	<5	8	2	10
	5-9	12	4	16
	10-14	11	1	12
	15-19	5	2	7
	>20	16	5	21
		52	14	66

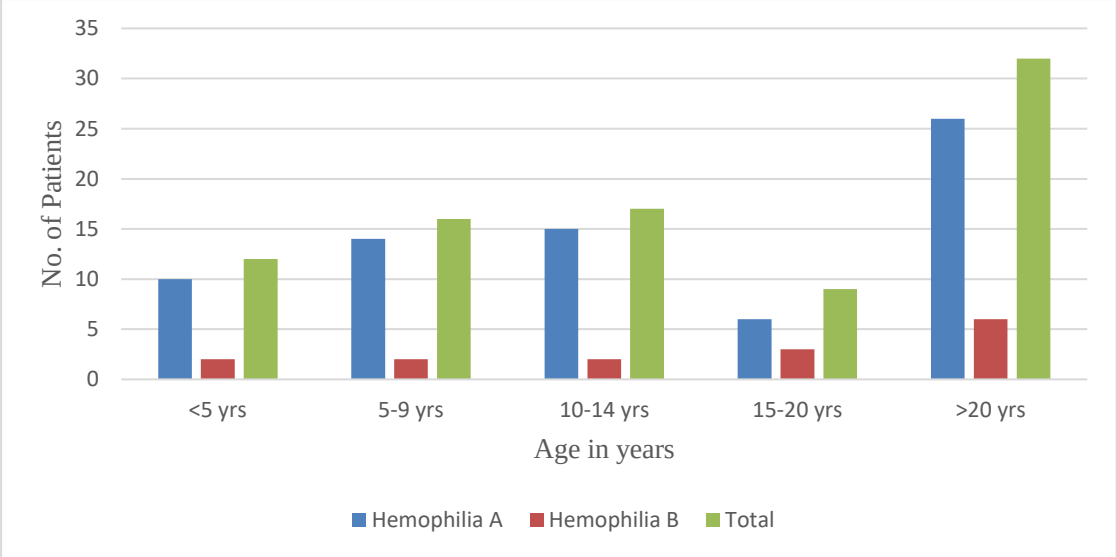
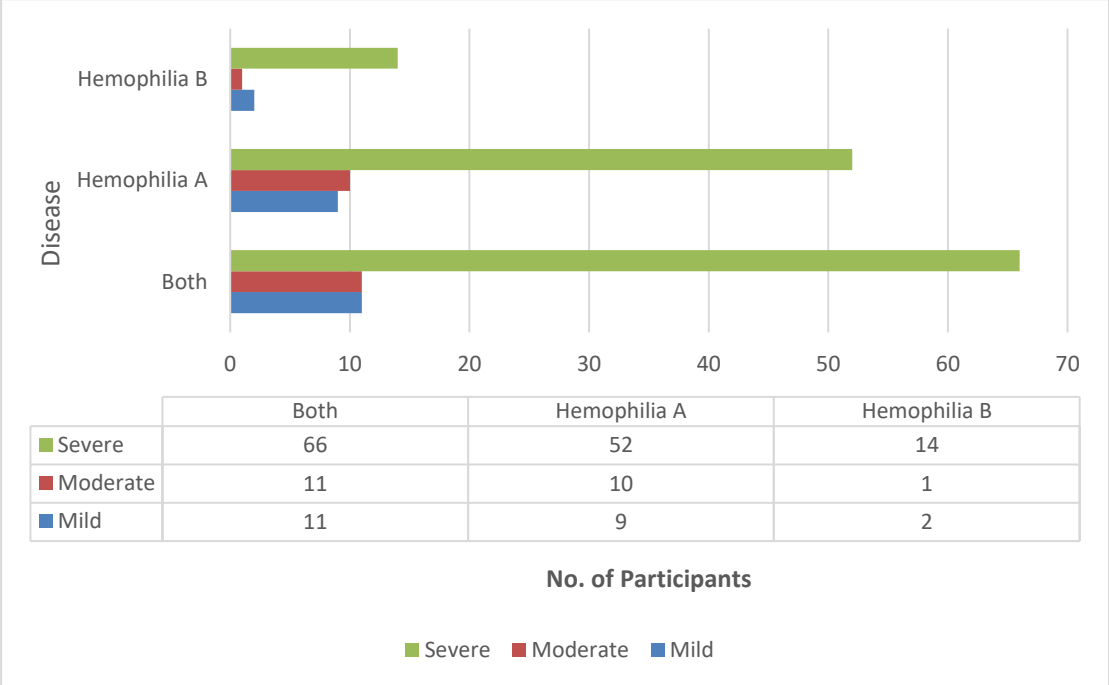


Figure 3: Age distribution of Hemophilia cases



	Both	Hemophilia A	Hemophilia B
Severe	66	52	14
Moderate	11	10	1
Mild	11	9	2

Figure 4: Distribution based on disease severities

As seen in figure 5 above, 75% (64) of the participants had severe hemophilia whereas 12.5 % (11) had moderate hemophilia and so was the number equal for those who had

mild hemophilia. Out of the 88 patients, 71 (81%) had HA and 17 (19%) had hemophilia B (HB). Out of the 71 HA patients 52 (73%) had severe factor VIII activity, 10 (14%) moderate and 9 (13%) mild cases. Out of the 17 patients with HB, 14 (82%) had severe factor IX activity, 1 (6%) moderate and 2 (12%) had mild HB.

Prevalence of inhibitors in PWH

Table 2: Prevalence of inhibitors

	Inhibitors		
Diagnosis	No	Yes	Total
Hemophilia A	59 (83)	12 (17)	71(100)
Hemophilia B	17 (100)	0 (0)	17(100)

N.B: Values in brackets represent the percentages

Of all the participants 12 had inhibitor titers greater than the cutoff 0.6. Thus the overall prevalence of inhibitors was 14% (95%CI: 7, 22.61). We observed that all those who developed inhibitors were HA and their titer ranges were (0.6BU – 69BU). Factor VIII inhibitors were observed in 12 (17%) of the 71 patients. Among those who developed inhibitors 6 (50%) had high titers (>5BU/mL) and 6 (50%) had low titers (<5BU/mL). Most patients who developed inhibitors had severe hemophilia A (83%) 10/12, the remaining 2 had mild HA. None of the HB patients developed factor IX inhibitors.

Inhibitors titers in different phenotypes of hemophilia

Table 3: Inhibitors titers and disease severity

	Inhibitors		Fishers' exact
Severity	No	Yes	P-value
Mild	9 (81.82)	2 (18.18)	0.458
Moderate	11 (100)	0 (0)	
Severe	56 (84.85)	10 (15.15)	

We observed that there was no statistically significant association between disease severity and presence of inhibitors.

Inhibitor titers and levels of regulatory T cells in PWH

Table 4: Inhibitor titers and Tregs

				t-test
Inhibitors	n	Mean	Std	p-value
No	12	4.578	2.022	0.003
Yes	12	2.102	1.535	

The table above shows the results comparing the mean % of Tregs by inhibitors. We observed that there was a statistically significant difference in the mean % of Tregs

between those with inhibitors and those without. The mean was higher among those without inhibitors.

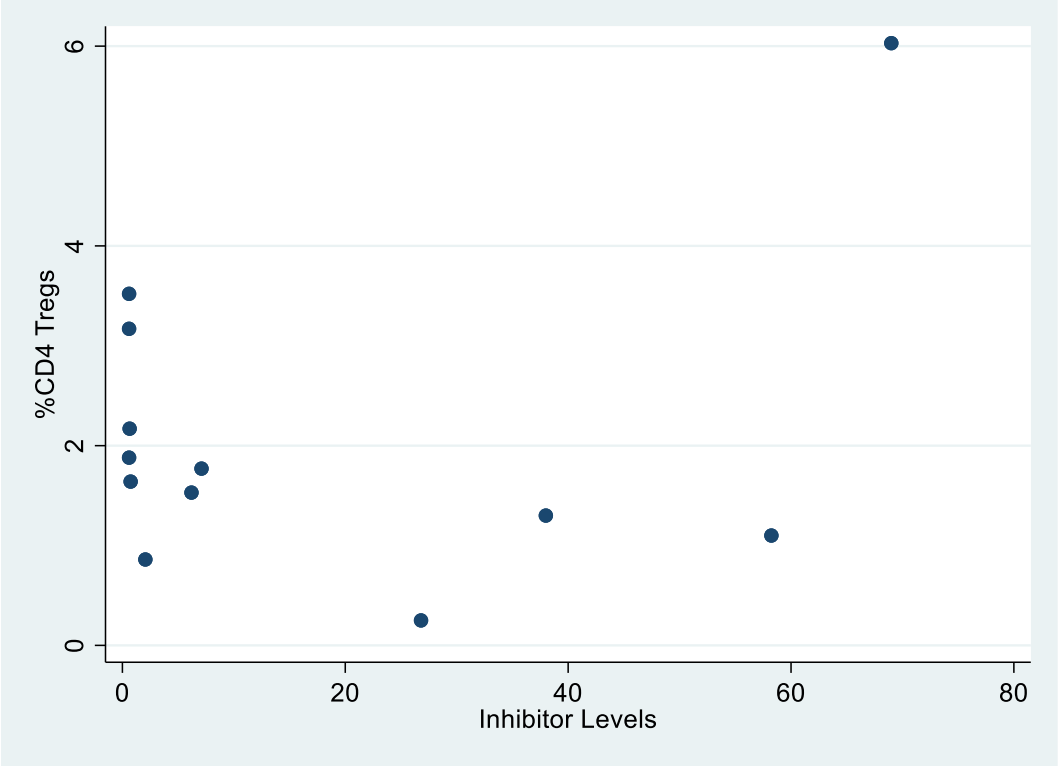


Figure 5: Scatter dot plots of levels of Tregs (%) and the inhibitor titers (B.U)

The correlation coefficient was -0.3803 (p-value=0.003).

CHAPTER FIVE

5.0 DISCUSSION

An estimated 4,000 people have hemophilia in Kenya, yet only 400 have been identified. While there is significant progress in the management of hemophilia in developed world allowing patients to lead a normal life, that is not the case for the more than half a million people in low and middle income countries (Okoye, Nwogoh, Adediran, & Nwagha, 2019). This debilitating disease remains largely unknown in Kenya where hemophilia is still considered a “curse” a cause for stigma, financial burdens and sometimes a death sentence. Some of the gaps in the management of people living with hemophilia include, access to diagnosis, care and genetic counseling. Furthermore, factor replacement therapy is very expensive and many resource limited countries depend solely from donations from WFHs humanitarian aid programme. Currently, the Kenyan government is not buying any factor concentrates for treatment of patients with hemophilia, thus most factor is mainly from WFH donations. As a result, the amount of factor that is consumed is quite low compared to the quantity of factor consumed in the developed nations. The development of inhibitors against the infused FVIII or FIX impairs the effectiveness of treatment and is the most serious and challenging complication in the management of patients with hemophilia. However, there is a scarcity of data regarding the occurrence and the widespread presence of the inhibitors in the population of developing nations as well as a perception these data are likely to be lower than the estimates from the developed nations (Taresh & Hassan, 2019). It is possible that inhibitors continue to be under reported due to various reasons such as lack of laboratory expertise to screen for inhibitors, lack of awareness that this event should be reported as an adverse reaction, absence of recommended testing to detect inhibitor development and physician apathy (Soucie, et al., 2014).

The current study determined the phenotypes and the prevalence of inhibitors in patients with hemophilia at the second largest HTC in Kenya. Our study evaluated 71 patients with HA and 17 patients with HB. In our study, none of the patients with hemophilia B had inhibitors. This finding is consistent with the reports from Centers for Disease Control (CDC) which reported low incidence of inhibitors of 2% among 3785 patients with hemophilia B (Puetz, et al., 2014). A Canadian study also reported a low prevalence of inhibitors of 0.6% in patients with hemophilia B and 2.1% in those with severe forms (Webert et al., 2012). The overall prevalence of inhibitors in our study was 14% (12/88), a figure comparable with that reported in other countries. The highest prevalence of inhibitors ever reported in hemophilia patients is 29.3% in a recent study conducted among Saudi patients (Owaidah, et al., 2017). In a meta-analysis conducted by Messori et. al. the pooled incidence of low-titer inhibitors was 10% (95% confidence interval [CI]: 8.3-12%) for studies including previously untreated persons (Messori, Peyvandi, Mengato, & Mannucci, 2017). In our study, half of the inhibitor positive patients had low titers 50% (6/12). This finding is comparable to results from an Indian study which reported a prevalence rate of 64.3% (56/87) of patients with low titer inhibitors (David, et al., 2019). Such figures may suggest the need for a prospective follow up of those patients who have transient inhibitors considering that treatment regimen ought to change to by-passing agent yet they may be wrongly labeled as having inhibitors. Also, none of our participants had undergone immune tolerance induction (ITI).

Research has reported race and ethnicity has a predisposing factor for the development of inhibitors. A study in the U.S found that black patients were more likely to develop inhibitors as compared to white patients while in another study patients of Hispanic origin reported a high prevalence of 24.5% (Carpenter, Michael Soucie, Sterner, &

Presley, 2012). Among the Chinese population, the overall prevalence of HA patients was 3.9% and the severe HA was 4..3% (XF Wang, et al., 2010). In Irag, 18.6% (22/143) of patients with hemophilia from a single center study developed inhibitors (Taresh & Hassan, 2019). A recent study from India involving 447 patients with HA found 87 (19.5%) of them positive for inhibitors (David, et al., 2019). A prevalence of 29.3% have been reported among Saudi patients with hemophilia (Owaidah, et al., 2017). Among the few studies that have been conducted in Africa, inhibitors were detected in 18.2% in HA and 9.1% in HB in Egypt (Abdelrazik, Rashad, Selim, & Tharwat, 2007) and a much lower prevalence of 5% in Tunisia (Kraiem et al., 2012). In West Africa, a study from Senegal reported 20% prevalence among 50 patients with HA, a figure comparable with our findings of 16.90% prevalence of inhibitors among 71 patients with HA. A review of 24 studies on the rate of inhibitor development among 2094 pups with hemophilia A found that 14.3% of the patients using plasma derived factor VIII concentrates developed inhibitors and a higher rate of 27.4% in patients using recombinant factor VIII concentrates (A Iorio et al., 2010).

The mechanism involved in the development of inhibitors in patients with hemophilia still remains unclear. However, it has been described to be a T helper dependent process involving antigen uptake and presentation of the antigen presenting cells (APC) (Scott, 2014). Despite very little information on the immune interactions preceding inhibitor development, some studies have demonstrated the role of regulatory T cells (Tregs) in modulating tolerance to FVIII protein (Xiaomei Wang, Terhorst, & Herzog, 2016). Treg are a subset of lymphocytes that regulate humoral and cell mediated responses by inducing tolerance, and dampening inflammation thus plays a pivotal role in maintaining the homeostasis of the immune system (Sakaguchi, Yamaguchi, Nomura, & Ono, 2008). Tregs (CD4+CD25+Foxp3+) can be divided into naturally occurring

Treg (nTreg) derived from the thymus of naïve animals and peripherally induced Treg (iTreg) induced in the periphery by stimulation of naive T cells with antigen. Multiple studies have demonstrated that perturbation of Treg may lead to autoimmune diseases or other immunopathology. The Transcription factor (Foxp3) is a master regulator for CD4+CD25+ Treg differentiation, maintenance, and function. Mutation of Foxp3 gene in human causes severe autoimmune disease – IPEX syndrome (Zheng et al., 2010). In such conditions, the ratio of Tregs to immune effector T cells is perturbed, and an immune response is mounted against self-proteins (Uchida & Okazaki, 2016).

The association between Tregs and inhibitors in hemophilia still remains controversial. However, rapidly growing evidence shows that Tregs may be crucial in tolerance to coagulation factors and thus may be involved in the pathogenesis of inhibitors in PWH (Xiaomei Wang, et al., 2016). The normal range of Tregs (%) in human is 5 – 10% of peripheral CD4+ T cells. In the current study, the levels of Tregs ranges were between 0.25% and 6%. We compared the levels of Tregs (%) and inhibitor titers (BU) in PWH (n=12). The results from this study showed a negative correlation (-0.3803) between inhibitor titers and levels of Tregs. Alteration in levels of Treg markers had an effect on inhibitor titers; those with low levels of Treg markers showed high inhibitor titers and vice versa. Additionally, we compared the mean % of Tregs in PWH with and without inhibitors. Upon comparing their means, we found a lower mean % of Tregs in those with inhibitors (2.1) compared to those without inhibitors (4.6).

In the case of genetic disorders like hemophilia, significant mutations in either factor 8 or factor 9 gene do not allow expression of protein of interest thus hindering establishment of central tolerance which results in very few antigen specific Tregs (Margaglione & Intrieri, 2018). This is mostly true for severe hemophilia cases whereby the underlying mutation is as a result of large deletions of the factor 8 or factor 9 gene

(Miao, 2010). Since Tregs have been shown to suppress B and T cell responses to coagulation factors, their depletion may result in formation of antibodies against the infused factors during replacement therapy for PWH. Similar results were reported by El-llasra *et.al.* (2016), who found that the frequency of Tregs were significantly lower among all patients with hemophilia A compared to healthy controls (2.59 versus 3.73%; P=0.002). Further, Tregs were significantly decreased among patients with FVIII inhibitors compared with the inhibitor-negative group (P<0.001). A recent in- vivo study revealed that adoptive transfer of reprogrammed CD4+ Treg cells that expresses FOXP3+ prevents inhibitor response to FVIII protein therapy in hemophilia A mice. They concluded that reprogramed FoxP3+ expressing cells can induce *in vivo* conversion of endogenous FVIII peripheral Tregs, resulting in sustained suppression of FVIII inhibitors (Biswas & Herzog, 2019).

In contrast to the results from the present study, previous work by Ding *et al.,* (2014) noted that CD4+CD25high had a higher frequency in patients who tested positive for inhibitor relative to the patients who tested negative for the inhibitor. However, the level of CD4+CD25high Tregs in the group that tested negative for inhibitor did not show any significant difference relative to the healthy controls. They argued that the increased number of Treg cells in the inhibitor positive group may be due to a probable self-response to the antibodies that were produced which in turn leads to a reduction in the immune response to factor FVIII (Ding, et al., 2014). Another study from India found no qualitative and quantitative differences in the total number of T cell subsets CD4+ CD25+ (P = 0.593) among the inhibitor negative and inhibitor positive PWH (David, et al., 2019).

CHAPTER SIX

6.0 CONCLUSION

This study provides evidence that, though previously ignored, inhibitors still exists in our population; the overall prevalence of inhibitors in PWH at MTRH (14%) is comparable to previous findings from other countries. Even though, disease severity is a known risk factor for development of inhibitors in PWH, we found no statistical significance between disease severity and inhibitor titers in our study population (both the inhibitor negative and inhibitor positive group). However, considering only the inhibitor positive group, it appears that patients with hemophilia A (12/12) and severe hemophilia are more prone to develop inhibitors (10/12) compared to the mild and the moderate groups. Additionally, we found a negative correlation between Tregs and inhibitor titers (-0.3804); low frequency of Tregs (CD4+CD25+ and FOXP3+) may contribute to development of inhibitors in PWH. Tregs therefore have the potential to serve as novel diagnostic markers and therapeutic agents for inhibitors. This study was limited because it was conducted in a single site (MTRH), therefore the findings cannot be generalized in other populations. However it provides preliminary findings that can serve as a basis for design of larger multicenter studies to further investigate this phenomenon.

6.1 Recommendations

Based on our findings, there is need to fully adopt inhibitor testing in all PWH nationwide. Also, further research is warranted to explore the potential of Tregs as a novel marker and a therapeutic agent for inhibitors.

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APPENDICES

Appendix 1: Consent form-English version

Inhibitor Titers and Levels of Regulatory T cell Markers in Patients with Hemophilia at Moi Teaching and Referral Hospital

Participants' ID_____

Dear Parent/Guardian

Thank you for allowing me the opportunity to screen you/your child for inhibitors and levels of regulatory T cells (CD4+ CD25+ FoxP3+) because you/he has hemophilia. Hemophilia is an inherited bleeding disorder that causes continuous bleeding whenever a person with factor VIII or IX deficiency is injured. Inhibitors may develop in some of these patients as a complication to treatment. If detected in time there are medical interventions that can be given to improve outcome of the disease and reduce complications.

The procedure involves cleaning of the site of sample collection and drawing blood using a needle which may cause minimal pain.

The procedure is not known to predispose a person to any complications but in a rare situation that the site of prick is contaminated then an infection may arise.

The results obtained from the test will only be communicated to you and your clinician. Otherwise the information will be de-identified for research purposes. The personal information that will be collected will help me to communicate the results to you.

If you/your child is found to have inhibitors, he will be referred to a hematologist for further management when he comes for his routine hemophilia clinics.

There is no penalty for refusing to participate in this study, also there are no monetary benefits for accepting to participate.

Participants/Parents:

Name_____Sign_____Date_____

Interpreter:

Name _____Sign_____ Date_____

Principal Investigator:

Name _____Sign _____Date_____

Appendix 2: Fomu ya idhini- Swahili version

Inhibitors and Regulatory T cell Markers in Patients with Hemophilia at Moi Teaching and Referral Hospital

NAMBARI YA MHUSIKA _____

Kwa Mhusika/Mzazi/Msimamizi,

Nashukuru kwa kunipa idhini kupima damu yako/ ya mwanao kuhusu ugonjwa wa hemofilia. Hemofilia ni ugonjwa wa kurithi ambao unasababisha kuvuja damu isiyoganda wakati mtu aliye na ukosefu wa protini inayoitwa factor VIII ama IX anapojeruliwa. Matibabu ya hemofilia yaweza pia kusababisha kutokea kwa tatizo la kufanyika kwa "inhibitors" damuni. "Inhibitors" husababisha dawa ya hemofilia kutofanya kazi ipasavyo. Upimaji huu utawezesha kujulikana kwa mapema kwa tatizo hili iwapo lipo na wewe/ mwanao ataweza kusaidika kwa haraka kwa kupewa madawa mengine ili kuzuia madhara zaidi ya ugonjwa.

Utafiti huu haiwezi kuleta madhara yoyote kwako/mtoto wako. Tutakapopata majibu, tutakujulisha wewe pamoja na daktari wako/ wa mwanao. Usiri utahakikiswa kwa majibu yako nakama utapatikana/mwanao atapatikana kuwa na "Inhibitors" tutakutuma/ tutamtuma moto kwa daktari ili apate matibabu zaidi ya manufaa.

Ni haki yako/ya mwanao kukataa kushiriki kwa utafiti huu ama kujiondoa baadaye wakati wowote. Hakuna ubaguzi ama adhabu ya anina yoyote ikiwa hautakubali kushiriki na pia hakuna faida ya kifedha utakayo lipwa kwa kushiriki katika utafiti huu.

Mgonjwa/mzazi_____Sahihi_____Tarehe_____

Mtafsiri_____Sahihi_____Tarehe_____

Mtafiti

mkuu_____Sahihi_____Tarehe_____

Appendix 3: Assent form

Inhibitor Titers and Levels of Regulatory T cell Markers in Patients with Hemophilia at Moi Teaching and Referral Hospital

I voluntarily agree to participate in this research study.

I_____ have been explained about this study and do understand its purpose and do voluntarily agree for an examination and blood samples to be taken from me. I understand that I will not suffer any extra discomfort and that I will not pay any extra cost or be paid for accepting to participate in this study.

The records and results relating to my participation in this study will remain confidential and will be communicated to me, my parent and my doctor. I may withdraw from this study at any time and this will not result in denial of any health benefit that I am entitled to.

Participants Name _____Sign _____Date _____

Parents Name _____ Sign _____ Date _____

PIs Name_____ Sign_____ Date _____

Appendix 4: Fomu ya idhini ya mtoto kati ya miaka (10-17) “Assent form”

Inhibitor Titers and Levels of Regulatory T cell Markers in Patients with Hemophilia at Moi Teaching and Referral Hospital

Mimi_____nimeelezwa juu ya utafiti na naelewa madhumuni ya utafiti na nimekubali kujitolea kukaguliwa na kutolewa vipimo vya damu. Naelewa sitaadhirika na maumivu na sitaharamika kwa kitu chochote. Matokeo na nakala zitakazo nihusu kwa kuhusika kwenye utafiti huu zitabaki siri na nitaelezwa mimi, mzazi na daktari wangu.

Naweza kujiondoa katika utafiti huu wakati wowote nikiamua na uamuzi huu hautanizuilia kupata faida za afya yangu zilizo dhamiriwa.

Mgonjwa_____Sahihi_____Tarehe_____

Mzazi_____ Sahihi_____Tarehe_____

Mtafiti mkuu_____Sahihi_____Tarehe_____

Appendix 5: Data collection form

Inhibitor Titers and Levels of Regulatory T cell Markers in Patients with Hemophilia at Moi Teaching and Referral Hospital

Participants No: _____

Part A (to be completed by the PI) **Date:** …../……/…….

Name of child: _____ Date of Birth: …../…./…..

Mother	**Father**
Name: _____	_____
Age: _____	_____
Telephone numbers: _____	_____

Family's residence (county): _____

Parent willing to participate in this study: **O** Yes **O** No

Part B

Sample Collection Date: _____ Time: _____

Test Date: _____

Name of person performing the test: _____

Results:

Inhibitor titers: _____

Levels of $CD4^+$ $CD25^+$ $FoxP3^+$: _____

Results Communicated? **O** Yes; to who: _____ **O** No

If positive for inhibitors, refer to a hematologist for further management

Date of referral: _____

Name of the PI: _____ Sign _____

Date: _____