

https://dspace.org/

Diagnostic and clinical evaluation of in hibitors in a cohort of haemophilia patients in a quaternary care centre

Wan, Yuen On

2016-10-12
http://hdl.handle.net/10539/21170

Downloaded from a DSpace repository. https://dspace.org/

DIAGNOSTIC AND CLINICAL EVALUATION OF INHIBITORS IN A COHORT OF HAEMOPHILIA PATIENTS IN A QUATERNARY CARE CENTRE

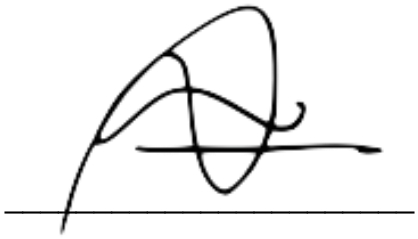
Yuen On Wan

A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Medicine in Haematology

Johannesburg, 2016

DECLARATION

I, Yuen On Wan declare that this research report is my own unaided work. It is being submitted for the degree of Master of Medicine in Haematology in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



Yuen On Wan

On 6th day of June, 2016

DEDICATION

To my parents, Hinda and Stanley, for their wisdom and making me be who I am today.

To my husband, Graham, for his constant reassurance, love and support.

To my mentor and supervisor, Professor JN Mahlangu, for his ongoing encouragement and support throughout my educational journey and his passion for the field of haemophilia care.

PRESENTATION ARISING FROM THIS RESEARCH

Wan YO, Mahlangu JN.

Analytical performance of the Nijmegen assay using different buffered reagents.

Accepted for presentation at the World Federation of Hemophilia Congress, USA in 2016.

ABSTRACT

Background: Haemophilia A is a congenital bleeding disorder due to deficiency of clotting Factor VIII (FVIII) and is treated by replacement therapy using FVIII clotting factor concentrates. The most serious complication is the development of a neutralizing inhibitor to exogenous FVIII requiring treatment with a bypassing agent. Routine measurement of inhibitors is important in the diagnosis, therapeutic monitoring and surveillance of inhibitor patients. The testing of inhibitor is historically performed with the Bethesda assay (BA) which lacks specificity and reliability when it comes to the low-titre inhibitor ranges. The Nijmegen modification of the Bethesda assay (NA) was developed and proven superior to the BA. With routine inhibitor monitoring, the phenomenon of transient low-titre inhibitor is noted, they disappear spontaneously and patients do not require a change in treatment. The aim of this study was to validate NA in the routine National Health Laboratory Service (NHLS) haematology laboratory at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) and the haemophilia clinic at CMJAH using different buffered reagents and to determine the significance of the transient inhibitors using a cohort of inhibitor patients attending our haemophilia clinic. **Methods:** A total of 100 samples from both low- and high-titre inhibitor patients were collected in our centre over the period of 2007 to 2013 when patients had their routine clinic visits. Validation of NA, including the set-up and optimization of the NA, was done as described by Verbruggen (2014). The normal pooled plasma (NPP) and Dade® Citrol 1® Coagulation Control Level 1 (Citrol 1) were buffered with imidazole. Using either the NPP or Citrol 1, the NA was performed on 3 control samples (provided by External quality Control of diagnostic Assays and Tests (ECAT) foundation) and on all 100 patient samples. Precision

analysis was performed on a control sample to determine standard deviation (SD) and coefficient of variation (CV). Accuracy analysis was performed on patient-samples against BA and results expressed as bias and 95% confidence interval. Linearity analysis was performed in the low-titre range between 0 to 5 BU/mL. Clinical data of the corresponding patients were collected and analyzed to determine the clinical significance of transient inhibitor. **Results:** Of the 100 patient-samples,100 were analyzed with NPP and 43 with Citrol 1. In the NA using NPP, the control plasma CV was 8.44% (95%CI of 0.77 ± 0.05) with a SD of 0.06. In the NA using Citrol 1, the control plasma CV was 10% (95%CI of 0.93 ± 0.02) and SD was 0.09. In the Bland-Altman plot of NA against BA using the NPP, the bias was 0.49 (95%CI of -8.1 to 9.1). The bias between NA and BA using Citrol 1 was 0.8 (95%CI of -6.8 to 8.5). The correlation coefficient of NA vs BA using the NPP was 0.93 and that of the NA vs BA analysis using the Citrol 1 was 0.76. All control plasma analyses using NPP buffered by 4M imidazole solution were within reference values whilst only 2 of 3 values were within the assigned values using Citrol 1. Only 3 of 20 high-titre inhibitor patients have a history of transient inhibitor. The inhibitors in the residual 44 patients with low titre inhibitors were of transient nature and these patients have not developed clinically significant inhibitors to date. **Conclusion:** In this analysis of patient and control samples with inhibitors, the analytical performance of the NA was comparable to BA. The performance of the Nijmegen assay with buffered pool plasma was better than that of the same assay using buffered Citrol 1. In this study, the presence of transient inhibitor did not seem to predict future inhibitor development in our haemophilia cohort.

ACKNOWLEDGEMENTS

I would like to express my great appreciation to the patients with haemophilia who were involved in this study as well as the following people and institutions who assisted me with this research report:

- My supervisor Professor Johnny Mahlangu for ongoing advice, encouragement and support throughout this project.

- Dr Bert Verbruggen and Dr Piet Meijer for the technical advice on the Nijmegen assay.

- Mr Jonas Shibambo for his guidance on the initial set-up of the Stago® instrument and inhibitor testing menu setup.

- Sr Bongi Mbele and Mr Kabelo Modisenyane from Charlotte Maxeke Johannesburg Academic Hospital Haemophilia Comprehensive Care Centre for the ongoing help with the patient aspect of this project.

- The National Health Laboratory Service and Faculty of Health Sciences Research Committee individual research grant for their financial contribution in the execution of this project.

I would also like to extend my thanks to the scientists, technologists and technicians of the cytogenetics and coagulation laboratory benches of the Department of Molecular Medicine & Haematology for their help in offering me the resources during the conduct of this study.

TABLE OF CONTENTS

DECLARATION ii

DEDICATION iii

PRESENTATION ARISING FROM THIS RESEARCH iv

ABSTRACT v

ACKNOWLEDGEMENTS vii

TABLE OF CONTENTS viii

LIST OF FIGURES xiii

LIST OF TABLES xiv

ABBREVIATIONS xv

1 INTRODUCTION 1

1.1 Overview of haemophilia 1

1.1.1 Genetics of haemophilia 1

1.1.2 Clinical presentation 2

1.1.3 Diagnosis 3

1.1.4 Treatment 6

1.2 Complications of haemophilia treatment 6

1.3 Inhibitors 7

1.3.1 Types of inhibitor 7

1.3.1.1 Transient inhibitors 9

1.3.2 Definition of clinically relevant inhibitor development 10

1.3.3 Risk factors 10

1.3.3.1 Patient-related factors 11

1.3.3.1.1 Age 11

1.3.3.1.2 Severity of disease 11

1.3.3.1.3 Genetics 12

1.3.3.1.4 HIV status 13

1.3.3.2 Treatment-related factors 14

1.3.3.2.1 Prophylaxis 14

1.3.3.2.2 Age at first exposure to treatment 14

1.3.3.2.3 Intensity of treatment 14

1.3.3.2.4 Exposure days 15

1.3.3.2.5 Types of replacement products 15

1.3.3.3 Risk factors for acquired inhibitor development 17

1.3.4 Clinical significance 18

1.3.4.1 Morbidity and quality of life 18

1.3.4.2 Therapeutic management and cost of inhibitor patients 18

1.3.4.2.1 Management of haemorrhagic episodes 18

1.3.4.2.2 Long term management 19

1.4. Measurement of inhibitors 21

1.4.1 Bethesda assay (BA) and its limitations 21

1.4.2 Nijmegen modification of the Bethesda assay (NA) 23

1.5 Rationale for the study 25

2 MATERIALS AND METHODS 27

2.1 Study design and site 27

2.2 Study population 28

2.3. Blood samples handling 28

2.3.1 Blood samples collection 28

2.3.2 Samples Preparation and Storage 29

2.4 The Classic Bethesda Assay 29

2.5 Establishment of the Nijmegen Modification of the Bethesda Assay 33

2.5.1 pH measurement 33

2.5.2 Preparation of Imidazole buffer 33

2.5.3 FVIII inhibitor testing 33

2.5.4 Reagents storage and preparation 34

2.5.5 The Normal Pooled Plasma 35

2.5.6 Preparing a 4M imidazole solution at a pH of 7.4 35

2.5.7 Preparation of Buffered Normal Pooled Plasma / Citrol 1 35

2.5.7.1 Direct buffering of NPP/Citrol 1 with solid Imidazole 35

2.5.7.2 Buffering of NPP/Citrol 1 using 4M Imidazole solution 36

2.5.8 The Nijmegen Modification of the Bethesda Assay 36

2.5.9 Control samples 41

2.5.10 Precision Analysis 42

2.5.11 Accuracy Analysis 42

2.5.12 Linearity Analysis 43

2.5.13 Clinical data collection 43

2.5.14 Establishing the clinical significant cut-off value 44

2.5.15 Statistical Analysis 45

3 RESULTS 46

3.1. Laboratory validation: 46

3.1.1 NA (Direct buffering of NPP with solid Imidazole) 46

3.1.1.1 Precision analysis 46

3.1.1.2 Accuracy analysis 46

3.1.2 NA (Direct buffering of Citrol 1 with solid Imidazole) 47

3.1.2.1 Precision analysis 47

3.1.2.2 Accuracy analysis 48

3.1.3 NA (Buffering of NPP using 4M Imidazole solution) 48

3.1.3.1 Precision analysis 48

3.1.3.2 Accuracy analysis 49

3.1.3.3 Linearity analysis 52

3.1.4 NA (Buffering of Citrol 1 using 4M Imidazole solution) 53

3.1.4.1 Precision analysis 53

3.1.4.2 Accuracy analysis 53

3.1.3.3 Linearity analysis 57

3.2. Clinical correlation studies 57

4 DISCUSSION 61

4.1 Validation of NA using different buffered reagents 62

4.1.1 NA (Direct buffering with solid Imidazole) 62

4.1.2 NA (using 4M Imidazole solution) 62

4.1.3 Other observations 64

4.2 Clinical correlation and low-titre transient inhibitors 64

4.3 Limitation of the study 65

4.4 Recommendation 65

5 Conclusion 67

APPENDICES 68

Appendix A Permission letter from CMJAH 68

Appendix B Ethics clearance certificate 69

Appendix C Turnitin result 70

REFERENCES 71

LIST OF FIGURES

Figure 1.1. Inheritance pattern of Haemophilia..... 4

Figure 1.2. Kinetics of type 1 and type 2 inhibitors against Factor VIII. 8

Figure 1.3. Flow diagram of Bethesda Assay..... 22

Figure 1.4. Flow diagram of Nijmegen Assay. 24

Figure 2.1. Flow diagram demonstrating the Bethesda Assay set up process. 31

Figure 2.2. Flow chart demonstrating the setup of doubling dilutions. 32

Figure 2.3. Flow diagram demonstrating the Nijmegen set up process..... 38

Figure 3.1. Linear regression graph demonstrating NA using BNPP against BA. 50

Figure 3.2. Bland-Altman plot demonstrating accuracy between BA and NA with BNPP. 51

Figure 3.3. Linearity analysis of NA with BNPP. 52

Figure 3.4. Linear regression graph demonstrating NA using BC against BA. 55

Figure 3.5. Bland-Altman plot demonstrating accuracy between BA and NA with BC. 56

Figure 3.6. Linearity analysis of NA with BC..... 57

Figure 3.7. Flow diagram indicating the number of patient files reviewed, exclusion criteria and total number analyzed. 58

LIST OF TABLES

Table 1.1. Differences between allo- and autoantibodies 9

Table 1.2. Risk of inhibitor development in PUPs and PTPs. 11

Table 1.3. Incidence of inhibitor development with severity of haemophilia A 12

Table 1.4. FVIII mutations and inhibitor prevalence of Haemophilia A 13

Table 1.5. Prospective observational studies (RODIN, EUHASS, GTH PUP and SIPPET) results for inhibitor development 16

Table 1.6. The advantages and disadvantages of aPCC (FEIBA®) and rFVIIa (NovoSeven®) 19

Table 1.7. Cost of care in haemophilia patients with inhibitor 21

Table 2.1. Scheme of Nijmegen Assay sample preparation. 39

Table 2.2. Bethesda Chart 41

Table 2.3. ECAT control samples and expected values with 2SD 42

Table 3.1. Precision analysis of NA using BNPP 46

Table 3.2. Accuracy analysis of NA using BNPP (control samples) 47

Table 3.3. Precision analysis of NA using BC 47

Table 3.4. Analysis of NA using BC (control samples) 48

Table 3.5. Precision analysis of NA using BNPP 48

Table 3.6. Analysis of NA using BNPP (control sample) 49

Table 3.7. Precision analysis of NA using BC 53

Table 3.8. Analysis of NA using BC (control sample) 53

Table 3.9. Characteristics of patients analyzed. 59

ABBREVIATIONS

aPCC	Activated prothrombin complex concentrate
aPTT	Activated partial thromboplastin time
BA	Bethesda Assay
BC	Buffered Citrol 1® Coagulation Control Level 1
BDDRFVIII	B-domain-deleted recombinant factor VIII
BNPP	Buffered Normal Pooled Plasma
BU/mL	Bethesda unit per millilitre
CFC	Clotting factor concentrate
Citrol 1	Dade® Citrol 1® Coagulation Control Level 1
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
CI	Confidence interval
CV	Coefficient of variation
ECAT	External quality Control of diagnostic Assays and Tests Foundation (Dobbeweg 1, 2254 AG Voorschoten, The Netherlands)
ER	Emergency room
EQAP	External Quality Assessment Programme
FEIBA	Factor Eight Inhibitor Bypassing Activity
FLRFVIII	Full-length recombinant factor VIII
FVIII	Factor VIII
FIX	Factor IX
HCCC	Haemophilia Comprehensive Care Centre
HCl	Hydrochloric acid
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus

ITI	Immune tolerance induction
LTA	Low-titre FVIII inhibitor assay
mcL	Microlitre
NA	Nijmegen modification of the Bethesda Assay
NBU/mL	Nijmegen-Bethesda unit per millilitre
NHLS	National Health Laboratory Service
NPP	Normal Pooled Plasma
pdFVIII	Plasma-derived factor VIII
PPP	Platelet poor plasma
PTP	Previously treated patients
PUP	Previously untreated patients
QC	Quality control
QOL	Quality of life
RCF	Relative centrifugal force
rFVIIa	Recombinant activated Factor VII
rFVIII	Recombinant factor VIII
SD	Standard deviation
SOP	Standard operation procedure

1 INTRODUCTION

1.1 Overview of haemophilia

Haemophilia is a congenital bleeding disorder due to deficiency of a coagulation clotting factor. Deficiency of factor VIII (FVIII) is called Haemophilia A and occurs in 1 in 5000 live male births. Factor IX (FIX) deficiency is called Haemophilia B and occurs in 1 in 25 000 live male births[1]. Treatment of haemophilia is by replacement of the missing protein, which may be plasma derived or recombinant factor. Haemophilia patients are categorized into three groups depending on their baseline factor levels: mild (>5% - <40%) , moderate (1% - 5%) and severe (<1%)[2].

1.1.1 Genetics of haemophilia

Haemophilia is a X-linked recessive inherited disorder (see Figure 1.1) where the mutated gene occurs on the X chromosome causing the expression of phenotype in males (only one X chromosome) and homozygous females. Mutation in the F8 gene leads to abnormal FVIII clotting factor production resulting in Haemophilia A and F9 gene mutations similarly result in Haemophilia B[3].

The F8 gene is located cytogenetically on the long arm of the X chromosome at band Xq28. Two additional genes F8A and F8B are nested within intron 22 which is the largest intron within the F8 gene. The most common mutation in Haemophilia A involves intron 22 inversion due to homologous recombination[4]. F9 gene is also located on the long arm of the X chromosome but on band Xq27. The most common mutation in Haemophilia B result from single-base substitution[5] .

Acquired haemophilia is however not an inherited disorder but relates to the production of autoantibodies targeting specific coagulation factors such as FVIII and FIX. Causes include drugs, pregnancy, postpartum state, underlying autoimmune diseases and dermatological conditions or it can be idiopathic. The management of acquired haemophilia involves bypassing agents for acute bleeds and inhibitor eradication long term[6].

1.1.2 Clinical presentation

The deficiency of clotting factor (FVIII or FIX) leads to bleeding due to impaired haemostasis. Clinical presentation depends on the severity of the disease. In mild haemophilia, patients are generally asymptomatic and bleeds often occur after trauma or surgery (delayed bleeding may be experienced after dental procedure). Individuals with mild haemophilia may present later in life if they have not had a haemostatic challenge[7]. In moderate haemophilia, individuals usually bleed after minor injury or invasive procedure. In the presence of a target joint, bleeding may be more frequent and spontaneous. In severe haemophilia, bleeds are usually disproportionate to the degree of trauma, spontaneously bleeding into joints is common. These individuals usually present early in life (within first year of life) with haemarthrosis, spontaneous haematoma in muscles or after invasive procedures (such as venepunctures and circumcision)[8, 9]. Urogenital bleeds (kidney or bladder) are frequent in severe haemophilia and if managed appropriately, does not result in loss of renal function[10]. Although it is rare, spontaneous or post-traumatic intracranial haemorrhage is one of the most serious, life-threatening bleeding events in severe haemophilia which can occur at any age[11-13]. Occasionally, intracranial haemorrhage can occur at birth[14].

1.1.3 Diagnosis

The diagnosis of haemophilia is made by a combination of patient's bleeding history, family history and laboratory testing.

Pertinent bleeding history in haemophilia includes bleeding symptoms, severity of bleeding and response to haemostatic challenges[15]. Family history may be negative in up to one third of haemophilia patients due to spontaneous mutation in F8 or F9 gene[16].

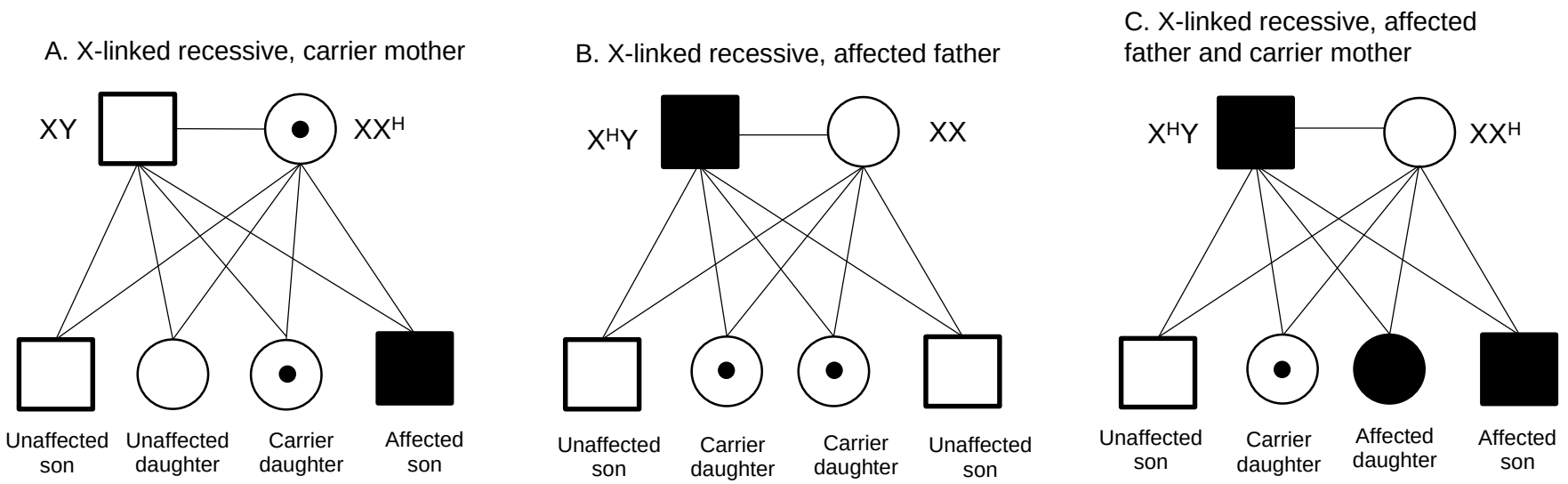


Figure 1.1. Inheritance pattern of Haemophilia.

A. Carrier mother with unaffected father and possible offspring. B. Affected father with unaffected mother and possible offspring. C. Affected father with carrier mother and possible offspring. Symbols: ☐, unaffected male; ○, unaffected female; ⊙, carrier female; ■, affected male. Abbreviations: XY, father; XX^H, carrier mother; X^HY, father with haemophilia; XX, mother.

An approach to laboratory diagnosis includes a tiered approach starting with screening tests moving to definitive tests:

- Screening tests using:
    - prothrombin time (PT) for extrinsic coagulation pathway;
    - activated partial thromboplastin time (aPTT) for intrinsic coagulation pathway;
    - bleeding time;
    - platelet count and functions for platelet quantity and quality testing[15].
- An isolated prolonged aPTT which corrects on mixing studies (by mixing 50% test plasma with 50% normal pooled plasma) indicate a factor deficiency. Individual factors such as FVIII and FIX should then be assayed. A normal aPTT does not exclude the diagnosis of mild haemophilia, therefore definitive testing such as factor activity should be performed regardless of aPTT results if clinically indicated. In the presence of FVIII deficiency, von Willebrand disease should be excluded[15].
- If screening tests yield an isolated prolonged aPTT which does not correct, the presence of an inhibitor should be suspected. Inhibitor testing by Bethesda assay (BA) or Nijmegen modification of the Bethesda assay (NA) should be performed[15].
- Genetic testing for specific mutations is indicated in most haemophilia patients once the diagnosis is made. It aids to predict the risk of inhibitor formation and identification of carrier status in female family members[17].

1.1.4 Treatment

The principle to achieve haemostasis in haemophilia is to replace the missing clotting factor. Clotting factor concentrates (CFC) are the treatment of choice (i.e. FVIII concentrates for haemophilia A and FIX concentrates for haemophilia B). The CFC are available in plasma-derived and recombinant formulations. Other sources for clotting factors include activated prothrombin complex concentrate (aPCC), cryoprecipitate and fresh frozen plasma (FFP), but these are generally utilized when CFC are not available. In addition to CFC, other pharmacological agents, which include antifibrinolytics and desmopressin, showed variable success[15].

1.2 Complications of haemophilia treatment

Clotting factor concentrate and many plasma-derived sources of clotting factor may give rise to transfusion-transmitted viral infections such as human immunodeficiency virus (HIV), Hepatitis B virus (HBV) and hepatitis C virus (HCV). Bacterial infections are possible because venous access is necessary[15, 18].

Intra-articular joint and intramuscular bleeds are common and frequent in severe haemophilia. Inadequate and/or delayed CFC treatment and recurrent joint bleeds lead to progressive deterioration of joints and permanent joint deformities and dysfunction[18].

The most serious and challenging complication of CFC replacement therapy in haemophilia is the development of antibodies (inhibitors) against replacement coagulation protein[19] rendering the substitution factor therapy ineffective. A recent

systematic review demonstrated that the overall prevalence of inhibitors in the haemophilia population was 5% - 7%[20]. In the case of congenital haemophilia these are alloantibodies characterized by an anamnestic response and in acquired haemophilia these are autoantibodies[21, 22].

In up to 50% of haemophilia B patients with inhibitors, severe allergic reaction may occur to FIX concentrates administration[15].

1.3 Inhibitors

Inhibitors are polyclonal high-affinity immunoglobulin G (IgG) antibodies against FVIII[23, 24] or FIX[25]. Inhibitors develop in ± 25-30% of severe haemophilia A and 3-5% in haemophilia B[18].

1.3.1 Types of inhibitor

There are two types of inhibitors: Type 1 and Type 2. Type 1 inhibitors are alloantibodies that neutralize factor VIII in direct proportion to their concentration[21] (see Figure 1.2). It is subdivided further into 2 types: high responding and low responding. High responding inhibitors are defined as those with high antibody titre of > 5 BU/mL which appear rapidly after factor exposure. Low responding inhibitors show little to no response upon exposure to factor and titres remain less than 5 BU/mL[2, 26]. Type 2 inhibitors are usually autoantibodies that neutralize factor VIII rapidly initially, followed by a slow phase of inactivation where residual factor VIII can be measured [21] (see Figure 1.2). The *in vivo* autoantibody potency is poorly correlated with the residual FVIII measured *in vitro*[22]. The differences between auto- and allo-antibodies are summarized in Table 1.1.

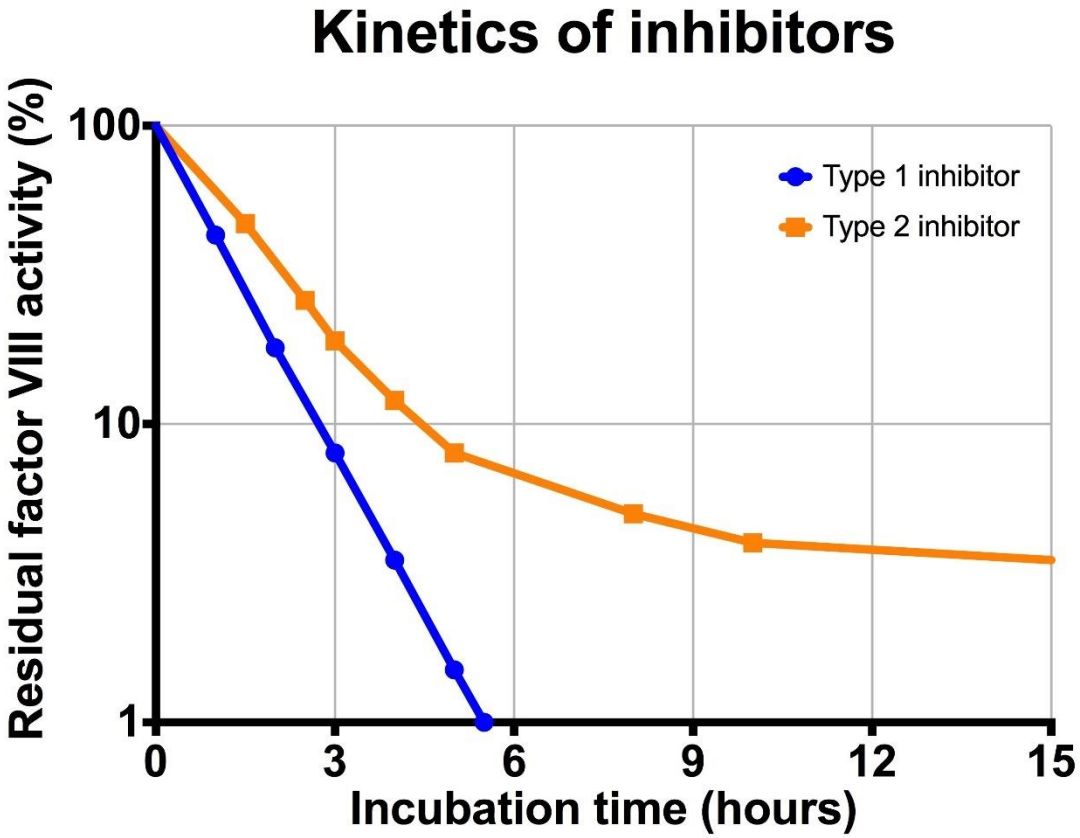


Figure 1.2. Kinetics of type 1 and type 2 inhibitors against Factor VIII.

Type 1 inhibitor shows a linear relationship between time incubated and neutralisation of Factor VIII activity with eventual complete neutralisation of Factor VIII. Type 2 inhibitor shows an initial neutralisation phase then a plateau with some residual Factor VIII (which can be detected *in vitro*). Adapted from Ma and Carrizosa[21]

Table 1.1. Differences between allo- and autoantibodies. Adapted from Green[22]

	Alloantibody	**Autoantibody**
Patients type	Haemophilia	Non-haemophilia
Origin	Therapy-related	Spontaneous
Bleeding site	Predominantly Joints	Predominantly skin & soft tissue
Effect on haemostasis	Disrupt FVIII-FIX complex formation	Alter binding of VWF and phospholipids to FVIII
Kinetics	Type 1	Type 2
Acute Management	<5 BU/mL: FVIII concentrates; >5 BU/mL: Bypassing agents	Bypassing agents
Long Term Management	Immune tolerance induction	Inhibitor Eradication with immunomodulatory therapies

Abbreviations: FVIII, factor VIII; FIX, factor IX, VWF, von Willebrand factor; BU/mL, Bethesda unit per millilitre.

1.3.1.1 Transient inhibitors

"Transient" inhibitors are low-titre antibodies that appear and disappear spontaneously over a non-defined amount of time without any specific intervention[23, 27]. These transient inhibitors represent 20-55% of all inhibitors reported in recombinant FVIII studies[28, 29]. Some literature defined transient inhibitors as inhibitors that spontaneously disappear within 6 months[28]. However, a recent prospective study suggests some of these low-titre inhibitors only eventually disappear over 3-6 years[30]. The presence of transient inhibitors in haemophilia patients suggests that possible natural tolerance occurred to the

given exogenous factor[23, 27]. There is not much literature available regarding the long term relevance of these inhibitors. It is also not clear if the transient inhibitors can predict future inhibitor development.

1.3.2 Definition of clinically relevant inhibitor development

Clinically relevant inhibitor development was defined by at least two positive inhibitor titre measurements with reduced *in vivo* factor recovery[31]. A positive inhibitor titre is defined as $\geq$0.6 BU/mL. The predicted factor recovery 30 minutes after FVIII infusion is based on the assumption that 1IU of FVIII per kilogram of body weight will raise the FVIII level to 2%. A reduction of factor recovery is defined as less than 66% of the expected rise in factor level[32].

1.3.3 Risk factors

Risk factors for inhibitor development in congenital haemophilia are multifactorial and include both genetic and acquired causes (see Table 1.2). Research conducted in PUP (previously untreated patients) for the development of inhibitors are important because these patients are FVIII naïve and therefore give a better indication of immunogenicity of CFC. In PUP research, both patient- and treatment-related factors contributing to the patients' immune response to the exogenous FVIII are considered. In PTP (previously treated patients), the development of inhibitors is considered less likely as they are already tolerized to replacement CFC[33, 34].

Table 1.2. Risk of inhibitor development in PUPs and PTPs[33, 34].

Previously untreated patients	Previously treated patients
Age of first exposure	Product-related immunogenicity (including neo-epitopes in novel products)
Genetics - Mutation type - Polymorphisms - Immune regulatory genes	
Race	
Family history	
Environmental: - Intense FVIII exposure - Surgery - Early introduction to prophylaxis	

Abbreviations: PUP, previously untreated patients; PTP, previously treated patients.

1.3.3.1 Patient-related factors

1.3.3.1.1 Age

Inhibitor development is highest in children ≤ 5 years of age with a cumulative risk of 16%. A second peak is seen at an older age of ≥ 60 years with a cumulative risk of 36% by 75 years[35, 36]. The reason for inhibitor development at older age may be due to loss of tolerance and exposure to danger signals (such as surgery and intensive therapy) and immune dysregulation associated with advanced age[35, 37].

1.3.3.1.2 Severity of disease

Haemophilia patients with severe disease have a significant higher incidence of inhibitor development than patients with mild to moderate disease. The prevalence

for the severe haemophilia population to develop inhibitors is 12% - 13%[20, 35] and incidence of up to 30%[20, 38] in severe haemophilia A and 2% - 5% in severe haemophilia B[39]. Incidence of inhibitor development as a function of severity of haemophilia is demonstrated in Table 1.3.

Table 1.3. Incidence of inhibitor development with severity of haemophilia A[20, 39].

Haemophilia Severity	Incidence of inhibitor development
Mild/moderate haemophilia	2 – 8%
Severe haemophilia	Up to 30%

1.3.3.1.3 Genetics

The type of FVIII mutation is strongly correlated with the risk for inhibitor development. High risk mutations include large deletion, null mutation, nonsense mutation and introns 22 inversion (see Table 1.4). Large multidomain deletions are particularly associated with increased prevalence of high-titre inhibitor development. In addition, immune response gene polymorphism (e.g. involvement of interleukin 10 and tumor necrosis factor alpha) shows a contribution to inhibitor development[40, 41].

Table 1.4. FVIII mutations and inhibitor prevalence of Haemophilia A[41].

Mutation	Inhibitor prevalence (%)
Large deletions	41
Multidomain	88
Single domain	25
Non sense mutations	31
Light chain	40
Heavy chain	17
Intron- 22 inversion	21
Small deletions	16
Missense	5
C1/C2 domain	10
Non C1/C2 domain	3
Splice site	17

In addition to the F8 gene mutations, a positive family history with inhibitor development as well as ethnicity (in particularly black and subgroup of Hispanic, Asian and Indian families) were associated with a higher incidence of inhibitor development[42-45].

A recent study conducted in South Africa showed that black patients with haemophilia had a higher frequency of the intron 22 mutation and inhibitor development than white patients in South Africa. The findings support a significant association between inhibitor development, ethnicity and F8 gene mutation type[46].

1.3.3.1.4 HIV status

Infection with the HIV, in the majority of haemophilia patients, is a consequence of transfusion transmission which predates the general screening methods used to

date. The impact of HIV on haemophilia has unprecedented high morbidity, however, some patients are long term non-progressors. There is no documented association between the HIV and incidence for inhibitor development[35, 36].

1.3.3.2 Treatment-related factors

1.3.3.2.1 Prophylaxis

Factor replacement therapy can be given by two different strategies, namely: on-demand and prophylactic therapy. On-demand treatment is defined as factor replacement therapy given when bleeding has already occurred. Prophylactic therapy is factor replacement therapy given to prevent bleeding. On-demand treatment is associated with higher risk of developing inhibitor whilst prophylactic treatment seems to have a protective effective against inhibitor development[38, 47]. Prophylaxis, particularly in children appears to have a tolerizing effect with resultant low incident of inhibitors

1.3.3.2.2 Age at first exposure to treatment

Younger age (especially <1 year of age) of the first exposure to replacement therapy is strongly associated with higher risk of inhibitor development[45, 48, 49]. This is in part because the immune system is not yet mature to differentiate self from non-self antigens.

1.3.3.2.3 Intensity of treatment

Inhibitor development in mild to moderate haemophilia patients are usually rare (risk of ~5%)[24]. However, intensive factor therapy (in particular, via continuous infusion) is associated with increased incidence of inhibitor formation in children

with mild or moderate disease[50]. Intensive factor therapy is defined as high dose replacement therapy with CFC over a short period of time.

In adult patients, intensive treatment is commonly associated with surgery and the post-operative period. The intensive CFC treatment contributes to inhibitor formation in the non-severe adult haemophilia population[51].

Trauma, ischemia and tissue damage can cause inflammatory responses. These cell-damage associated molecule patterns (DAMPs) present danger signals that can stimulate inflammatory responses of the innate immune system that ultimately, but indirectly, lead to upregulation of antibody responses[52]. It was proposed that avoiding these immunological danger signals decreased the development of inhibitors[53].

1.3.3.2.4 Exposure days

Most inhibitors tend to develop within 10-15 exposure days (but generally within 50 exposure days) to CFC in the severe haemophilia population. After 50-75 exposure days, the cumulative rate for inhibitor development reaches a plateau[54, 55] and the formation of inhibitor after 150 exposure days is very rare[41, 56].

1.3.3.2.5 Types of replacement products

Multiple prospective observational studies (RODIN, EUHASS, GTH PUP study and PEDNET) and randomized controlled trials (SIPPET) have been conducted to study the relationship between inhibitor development and the type of replacement products (recombinant factor and plasma-derived factor products). See Table 1.5

for results. The SIPPET study showed rFVIII is associated with a 1.87-fold higher incidence for inhibitor development than pdFVIII products. In addition, switching between products does not make a difference to the development of inhibitors[56-58].

Table 1.5. Prospective observational studies (RODIN, EUHASS, GTH PUP and SIPPET) results for inhibitor development[29, 58-60].

Study	**pdFVIII** [n/N, cumulative incidence (%)]	**rFVIII** [n/N, cumulative incidence (%)]
EUHASS 2015	11/51 (21.6%)	97/366 (26.5%)
RODIN 2013	29/88 (33.1%)	148/486 (32.3%)
GTH 2003	11/49 (22%)	20/63 (32%)
SIPPET 2015	29/125 (26.7%)	47/126 (44.5%)

Abbreviations: pdFVIII, plasma derived factor VIII; rFVIII, recombinant factor VIII; n, number of positive patients; N, total number of patients in the study.

There are two types of recombinant CFC, namely full-length recombinant factor VIII (FLRFVIII) and B-domain-deleted/-truncated recombinant factor VIII (BDDRFVIII). The BDDRFVIII is a recombinant factor VIII product made with the B-domain (which is 908 amino-acid) removed except for a 14 amino-acid linker sequence (which is known as the SQ-peptide). Some authors suggest the function of the large and highly glycosylated B-domain is important for the intra-cellular processing and secretion of FVIII but unnecessary for coagulant activity[61, 62]. Both efficacy and immunogenicity (i.e. inhibitor development) of BDDRFVIII have been shown to be similar to that of FLRVIII in a few prospective studies and meta-analysis[33, 63, 64].

1.3.3.3 Risk factors for acquired inhibitor development

Occasionally autoantibodies against factor VIII develop in non-haemophilia patients (acquired factor VIII inhibitors). Some of the reasons for development of autoantibodies include the following:

- HIV infection: very rare, only a few cases reported internationally and appears to be independent of treatment or CD4 count/degree of immunodeficiency[65-67],
- pregnancy and postpartum state (8%) usually at term or early postpartum period, alteration of immune state of third trimester or parturition may be contributing to the development of inhibitors[68-70],
- underlying autoimmune disorder (17-18%) such as rheumatoid arthritis (8%), systemic lupus erythematosus (6%) and Sjögren's syndrome[68],
- malignancy (12%) including both solid tumours (squamous cell carcinoma of lung, prostate, pancreas, colon and hypernephroma) and haematological malignancy (such as plasma cell dyscrasias and lymphoproliferative disorders) in both cancer and precancerous state[68, 69],
- drugs (5-10%) including interferon for the treatment of HCV, penicillin, sulfa drugs, phenytoin and chloramphenicol[6, 67, 69],
- dermatological conditions (5%) such as psoriasis, pemphigus vulgaris, erythema multiforme and non-specific dermatosis which may be related to drug reaction or underlying autoimmune state[68],
- idiopathic which comprises approximately 50% of the cases[69, 70].

1.3.4 Clinical significance

1.3.4.1 Morbidity and quality of life

Patient with inhibitors experience more difficulty in achieving haemostasis which leads to more frequent bleeding phenotype. Acute and chronic synovitis also contribute to the increased bleeding episodes. Musculoskeletal bleeds and orthopaedic procedures in inhibitor patients are significantly increased in comparison to patients without inhibitors[39]. Hospitalization, outpatient routine, emergency room (ER) visits and subsequently absence from work/school are noted to be higher in inhibitor patients. Significant amount of inhibitor patients reported chronic joint pain and reduced mobility which require walking aids or wheelchair usage. These complications resulted in general decreased QOL[39, 71, 72].

1.3.4.2 Therapeutic management and cost of inhibitor patients

Management of inhibitor patients generally requires a highly trained team as well as resources that are not readily available. Overall, the therapeutic options for haemostasis, as well as inhibitor eradication/immune tolerance induction, are not optimal with unpredictable effectiveness[24].

1.3.4.2.1 Management of haemorrhagic episodes

Treatment of acute bleeds should be provided as soon as possible. For patients with low-responding inhibitors, the treatment usually involved replacement therapy with CFC (at normal or higher dosage). Patient with high-titre inhibitors will require bypassing agents (such as aPCC/factor eight inhibitor bypassing activity (FEIBA®) or rFVIIa/NovoSeven® or both, see Table 1.6). Patients may respond better to one

than the other. Owing to the unpredictable response, general recommendation is not possible[24, 41].

Table 1.6. The advantages and disadvantages of aPCC (FEIBA®) and rFVIIa (NovoSeven®). Adapted from Kulkarni *et al*.[73]

Type of product	Advantages	Disadvantages
rFVIIa (NovoSeven®)	No known risk of viral transmission Devoid of human serum or proteins Absent anamnestic response	Short half-life Risk of thrombosis
aPCC (FEIBA®)	Long half-life Virally inactivated Less expensive than rFVIIa	Plasma-derived product Risk of thrombosis Possible anamnestic response Allergic reaction

Abbreviation: rFVIIa, activated recombinant factor 7; aPCC, activated prothrombin complex concentrate.

1.3.4.2.2 Long term management

Long term management of high-titre patients are generally aimed at immune tolerance induction (ITI) in patients (mostly children) with alloantibodies and inhibitor eradication in adults with autoantibodies[6, 24].

Eradication of autoantibodies involves the use of prednisone (1 mg/kg/day) alone or in combination with oral cyclophosphamide (1-2 mg/kg/day) as first line treatment[74, 75]. Rituximab is an anti-CD20 monoclonal antibody used alone or in combination with other immunosuppressant as first- or second-line therapy for autoantibodies eradication. In addition, calcineurin inhibitors and mycophenolate mofetil are used as alternatives to rituximab in patients who did not respond to the

first line treatment[74]. There is no recommendation for optimal immunosuppressive regimen and duration of treatment for autoantibodies eradication[76].

Immune tolerance induction can be high (FVIII, 200 U kg^{-1} day^{-1}) or low (50 U kg^{-1} thrice weekly) dose regimen and involves repeated doses of FVIII product and bypassing agent infusion with or without immunomodulation until the disappearance of alloantibodies[24, 41]. Dosage of the ITI, time between inhibitor diagnosis and initiation of ITI, maximum titre and age at treatment all affect the outcome. It was noted that high dose ITI has less bleeding complication than low-dose ITI[77]. If patients do not achieve tolerance by 2 years (i.e. inhibitor persisted), it is deemed as failure of ITI[24, 78].

Short and long term management of inhibitor patients remain extremely expensive and estimated in the region of 18000 Euros per month[79] (see Table 1.7).

Table 1.7. Cost of care in haemophilia patients with inhibitor. Adapted from Di Minno *et al*.[79]

Average annual concentrate costs 1.5- to 3-fold higher in inhibitor patients than in non-inhibitor patients
The highly expensive care provides a satisfactory quality of life in haemophilia with inhibitors
Monthly cost/patient ~18 000 €; ~50% of patients require <5000 € per month, only 2% needing >100 000 €
Costs of ITI: ~8-fold higher FVIII consumption and costs in patients with unfavourable prognosis
Mean annual costs of on-demand treatment in an inhibitor patient are 3-fold higher than in non-inhibitor patients
Mean ITI costs are 3-fold higher in high-responder than in low-responder children. Higher differences in adults

Abbreviation: ITI, Immune tolerance induction.

1.4. Measurement of inhibitors

1.4.1 Bethesda assay (BA) and its limitations

The presence of inhibitor is most commonly diagnosed using the Bethesda Assay (BA) which was developed in 1975[19, 80]. The BA involves serial dilution of patient's plasma (containing the inhibitor) with normal pooled plasma (source of FVIII). The mixtures are then incubated at 37˚C for 2 hours (allowing FVIII from NPP to be inactivated by inhibitor from test plasma). Residual clotting factor activity in the mixture is determined by standard one stage clotting assay.

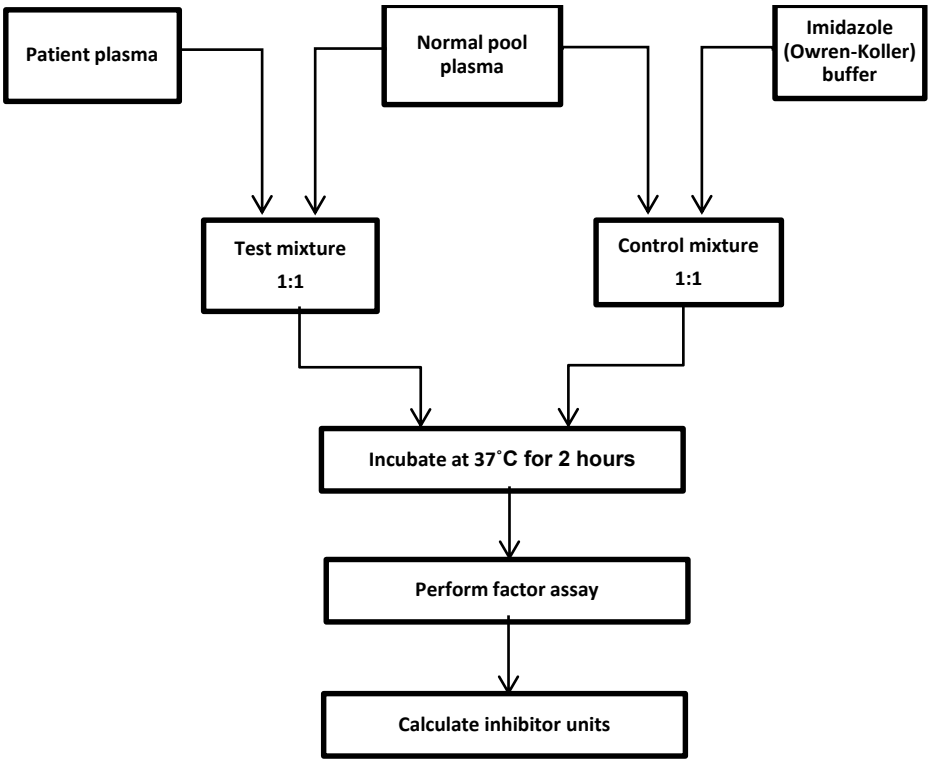


Figure 1.3. Flow diagram of Bethesda Assay[81].

Clinically relevant inhibitor development was defined by two positive-titre measurements (>0.6 BU/mL) with reduced factor recovery of <66% of expected rise. Bethesda Assay is sensitive, however lacks specificity and reliability in the low titre inhibitor range (defined as 0.4 – 5 BU/mL, but in particularly 0.4 – 1.0 BU/mL) owing to the following:

- pH of the test mixture increased to 8.4 – 8.6 during the 2-hour incubation in 37°C waterbath denaturing the FVIII;
- protein denaturation during the incubation period further decreasing FVIII activity.
- it was also noted that during serial dilution, the inhibitor activity increased proportionally despite no clinical evidence of an inhibitor.

Overall, the non-specific low-titre inhibitor measured is due to the presence of experimental artefact[82, 83].

The Nijmegen modification (NA) of the Bethesda assay was therefore developed to improve the specificity and reliability for low titre inhibitor testing in 1995[82].

1.4.2 Nijmegen modification of the Bethesda assay (NA)

The Nijmegen modification proposed two changes to the current Bethesda assay: (a) normal pooled plasma is buffered to pH7.4 with 0.1M imidazole buffer and (b) FVIII-deficient inhibitor-free plasma is used in the place of imidazole buffer (in our centre - Owren-Koller buffer)[82]. This is subsequently followed by serial dilution, incubation, residual clotting factor activity measurement and calculation of inhibitor level as per original BA.

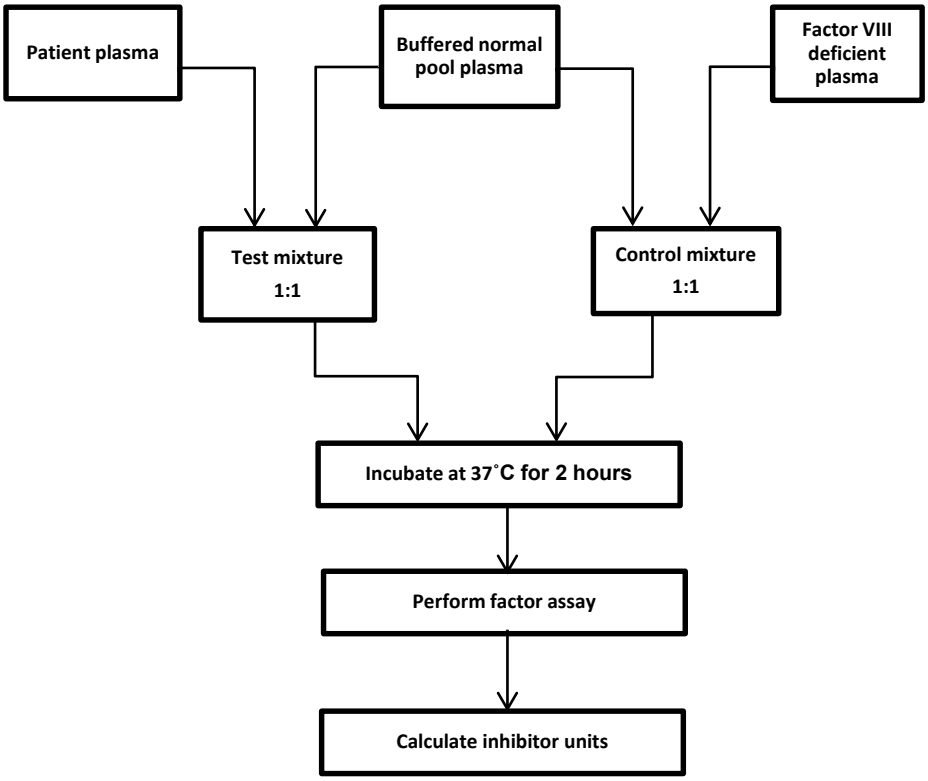


Figure 1.4. Flow diagram of Nijmegen Assay[81].

These modifications improve the intrinsic buffering effect after serial dilution thereby improving the specificity and reliability of the test for low-titre inhibitor without altering the sensitivity. With the regards to high-titre inhibitors, NA offers the similar specificity to BA but the quantification is erroneous owing to the complex non-linear kinetics between FVIII and its inhibitors[82, 84].

The inter-laboratory CV for NA is approximately 50% and had remained high over the years due to the differences in reagents used, normal pooled plasma as well as buffering methods. Centralized testing was recommended[85, 86].

Additional recommendation of using 4M imidazole solution instead of solid imidazole for the buffering of incubation mixture was received in 2014 to further

standardize the NA to increase test specificity (Verbruggen, personal communication).

The frequently used cut-off value for both BA and NA is 0.6 BU/mL, however, owing to the increased specificity of the NA, the cut off value may be lower[87].

Despite all attempts to improve the sensitivity and specificity of the NA, low-titre inhibitors are still being missed (in particularly late ITI and early post ITI periods). A low-titre FVIII inhibitor assay (LTA) was developed in 2012 which was based on the principle of NA with the following changes:

a) concentration of test plasma by selective protein filtration;
b) the ratio for incubation mixture was 3:1 (concentrated test plasma: BNPP);
c) residual FVIII activity was measured by the use of chromogenic substrates.

The LTA offers the lower limit of detection of 0.03 BU/mL which cannot be detected by BA or NA, however clinical significance of these inhibitor <0.6 BU requires further studies[87, 88].

1.5 Rationale for the study

In our centre, the currently used method for inhibitor measurement is the BA. Using this method, the estimated inhibitor frequency is 10-15% in the population attending the haemophilia treatment centre at the CMJAH. In view of the high treatment costs associated with management of inhibitors, it is important that the measurement is accurate and reliable. Using the BA, a large number of patients appear to have low levels of inhibitors with no clinical inhibitor effect. It is therefore

important to establish the clinical significance of these low level inhibitors using the more reliable NA.

The following objectives were set in order to achieve the above aim:

- To validate the NA against the BA in inhibitor measurement in a cohort of known inhibitor patients at the CMJAH Haemophilia Clinic;
- To establish the clinically significant inhibitor cut-off value using the Nijmegen assay for our laboratory;
- To retrospectively correlate borderline inhibitor levels obtained with BA and NA to the risk of development of low responding and high responding inhibitors.

2 MATERIALS AND METHODS

2.1 Study design and site

This study comprised of two parts:

The laboratory validation of the NA was a cross-sectional study whilst the clinical correlation was done retrospectively.

a) Laboratory: The method validation of the NA and establishing a clinically significant cut-off value as a cross-sectional study. This part of the study was conducted at the Department of Molecular Medicine and Haematology, National Health Laboratory Service, CMJAH. The validation of the NA also comprised of 2 parts where: 1) NA performed with buffered normal pooled plasma and 2) NA performed with buffered Dade® Citrol 1® Coagulation Control Level 1 (Siemens Healthcare Diagnostics, Newark, DE, USA).

b) Clinical correlation: Retrospective data analysis was performed to determine the correlation between borderline inhibitor level and bleeding phenotype, as well as the risk of high responding inhibitor development. This part of the study was carried out at the Haemophilia Comprehensive Care Centre, Area 294, CMJAH.

Permission to conduct this study, to use stored samples and to review patient's files was given by the Chief Executive Officer of CMJAH and the Human Research Ethics Committee of the University of Witwatersrand, Johannesburg (see Appendix A for the permission letter and Appendix B for the ethics clearance certificate number M130539).

2.2 Study population

a) Laboratory: 100 stored samples collected between January 2007 and 2013 from patients previously tested with a positive inhibitor level, who attended HCCC, were utilized. The samples were collected during routine visits in diagnostic, treatment and/or monitoring follow-up of patients. Only the remaining samples after performing routine analyses were stored (refer to section 2.3 for samples handling).

b) Clinical correlation: Data from the patients of whom specimens were used in the validation study and known patients with high responding inhibitor, which were followed up at the CMJAH HCCC, were collected. Patients were of all ages and all visits in which inhibitor testing was performed were included.

2.3. Blood samples handling

2.3.1 Blood samples collection

5 or 10mL of blood were routinely collected in BD Vacutainer® Citrate Tubes with 3.2% buffered sodium citrate solution (BD Biosciences, San Jose, CA, USA) for inhibitor testing and/or monitoring as part of their full assessment while patients attended the clinic. The blood samples were taken by a trained haemophilia nurse or a haematology registrar using BD Vacutainer® Safety-Lok™ Blood Collection Sets with Luer Adaptors (BD Biosciences, San Jose, CA, USA) and BD Vacutainer® Holders (BD Biosciences, San Jose, CA, USA) to ensure that the samples were collected correctly.

2.3.2 Samples Preparation and Storage

The samples were centrifuged in a Hettich® Rotofix 32A (Hettich, Tuttlingen Germany) centrifuge at 3500rpm (relative centrifugal force (RCF) of 1900 x g) for 15 minutes in order to obtain platelet poor plasma (PPP). Plasma was removed and aliquoted into separate plastic tubes and/or Nunc CryoTube® Vials (ThermoFisher Scientific, Massachusetts, USA). Routine inhibitor tests were performed. The remaining plasma samples, from patients who previously tested positive for inhibitor, were then stored in cryovials at -70˚C until the validation study.

2.4 The Classic Bethesda Assay

The classic BA was performed according to the established method at the CMJAH using NHLS SOP: HAE0198. This entails the set of the following (see Figure 2.1):

a) Standard: 400 mcL of NPP was added to 400 mcL of Owren-Koller (STA® Owren-Koller, Diagnostica Stago, Asnières sur Seine, France) which served as the calibrator and the standard control.
b) Neat: 200 mcL of patient's plasma was added to 200 mcL of NPP (1:1).
c) Serial dilutions (see Figure 2.2) were set up by adding 200 mcL of Owren-Koller in all the test tubes labelled 1:2 to 1:64 (in doubling quantity, more if required). A volume of 200 mcL of patient's plasma was added to the 1:2 tube, tap-mixed and 200 mcL of the mixture pipetted out and put into the next dilution tube (1:4). Similar principle applies to the subsequent doubling dilutions – 200 mcL Owren-Koller and 200 mcL of the previous mixture, tap-mixed, move 200 mcL of the mixture to the next dilution tube. The extra 200 mcL after mixing of the final tube of desired dilution was discarded. Finally,

200 mcL of NPP was pipetted into all the dilution tubes.

All tubes were then mixed well, capped and incubated in a waterbath at 37˚C for 2 hours. Factor FVIII assays were then performed on the post-incubated specimen using the “standard” tube as the calibrator. A residual FVIII activity of 25% - 75% was read off the Bethesda chart and multiplied with the dilution factor to obtain the inhibitor levels in Bethesda unit (BU/mL).

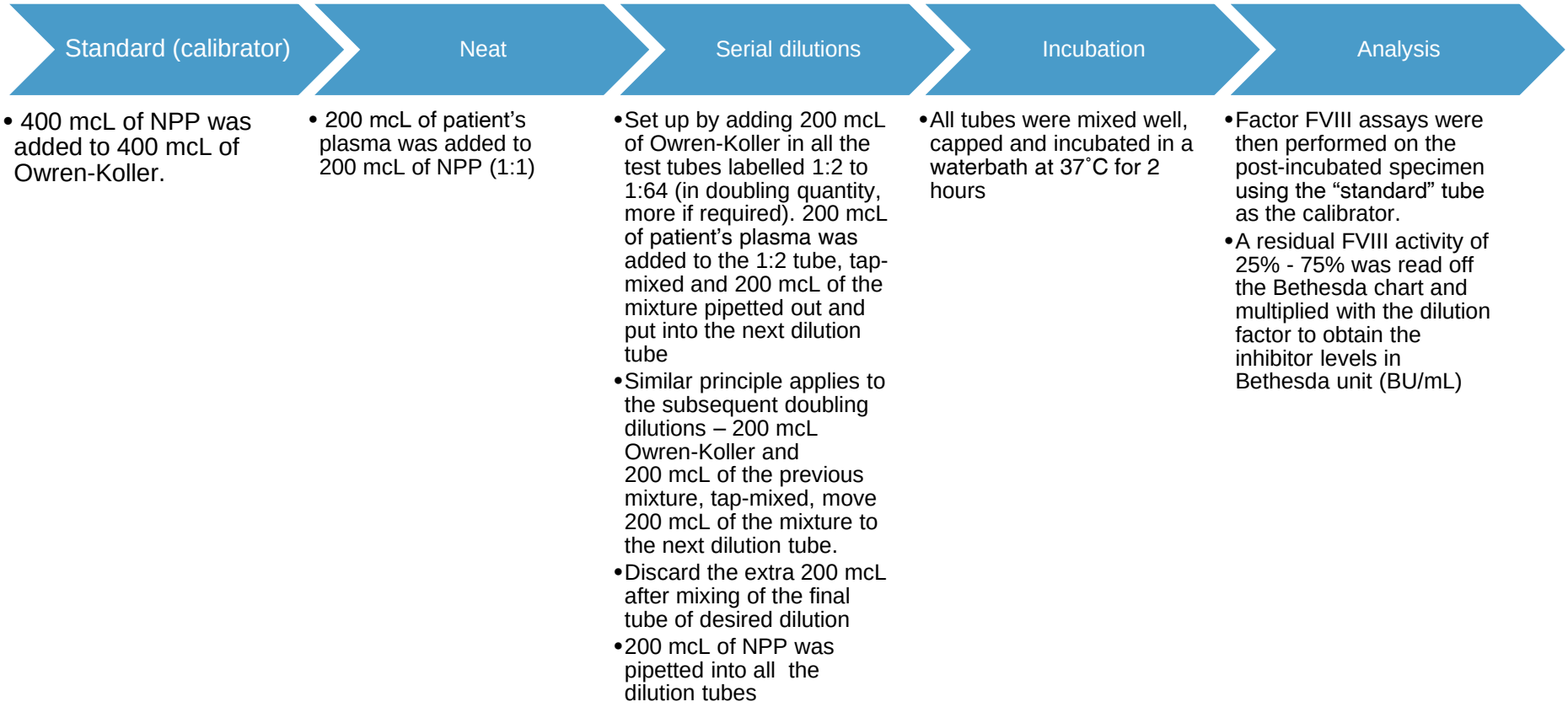


Figure 2.1. Flow diagram demonstrating the Bethesda Assay set up process.

Abbreviations: NPP, Normal pooled plasma; BU/mL, Bethesda unit per millilitre.

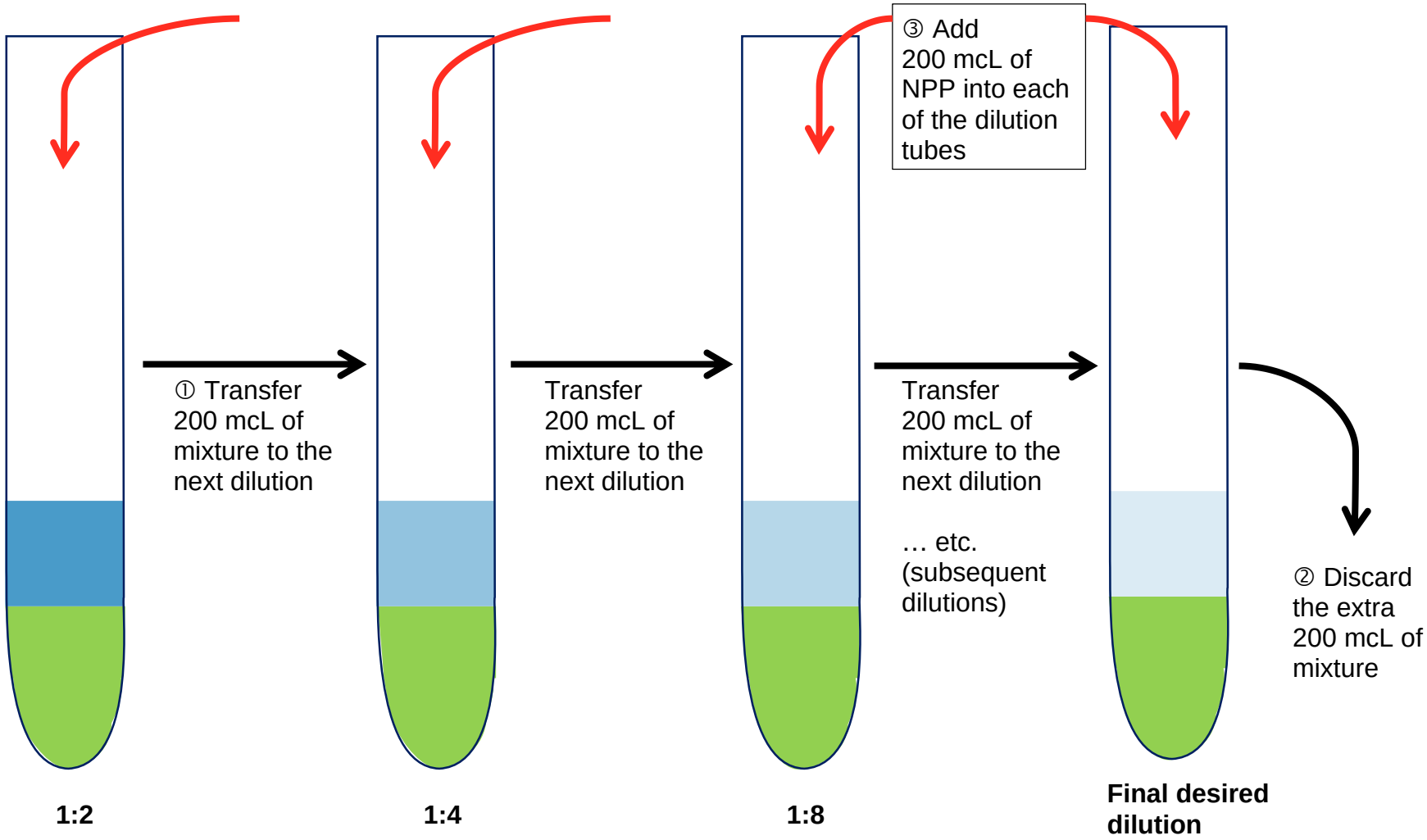


Figure 2.2. Flow chart demonstrating the setup of doubling dilutions.

The flow chart shows how to setup doubling dilutions. Abbreviation: NPP, normal pooled plasma in BA or buffered normal pooled plasma in NA. Green, buffering agents (Owren-Koller in BA, FVIII deficient plasma in NA); blue, patient's plasma.

2.5 Establishment of the Nijmegen Modification of the Bethesda Assay

2.5.1 pH measurement

All pH analyses of imidazole solution and plasma were performed on the pH/conductivity meter C561 (Consort, Turnhout, Belgium) in the cytogenetics branch of haematology laboratory at NHLS, CMJAH. The QC and maintenance for the pH meter were done according to NHLS SOPs namely NJHC0232 and FML0408.

2.5.2 Preparation of Imidazole buffer

All solid imidazole (Sigma-Aldrich, ≥99% (titration), crystalline, MW = 68.08, St. Louis, Missouri, USA) mass determinations were performed on the AE ADAM PGW Precision Balance (AE ADAM, Kingston, MK, UK) and Sartorius CPA 3245 (Sartorius, Göttingen, Germany) weight balance scales in the flow bench and the main laboratory respectively. The QC and maintenance of the scales were done according to NHLS SOPs namely GPL0062, FML0738, GPL2335 and NJHH0202.

2.5.3 FVIII inhibitor testing

All FVIII inhibitor analyses were performed on the STA-R Evolution® Expert Series (Diagnostica Stago, Asnières sur Seine, France) instrument in the coagulation bench of the haematology laboratory at the NHLS, CMJAH. The QC and maintenance of the instruments were performed according to the NHLS SOPs: GPL2472.

2.5.4 Reagents storage and preparation

The storage and preparation of the reagents were carried out according to the corresponding package inserts and NHLS SOP GPL2849.

- Partial thromboplastin time reagent (PTT Automate 5, Diagnostica Stago, Asnières sur Seine, France), FVIII deficient plasma (STA® Deficient FVIII, Diagnostica Stago, Asnières sur Seine, France), Owren-Koller buffer and $CaCl_2$ (STA- $CaCl_2$ 0.025M, Diagnostica Stago, Asnières sur Seine, France) were stored at 2-8˚C and were expected to remain stable until the expiration date on the box label.
- Partial thromboplastin time reagent was reconstituted with 5mL of distilled water with a 30-minute stabilization time at room temperature (18 - 25˚C) and mixed rigorously by turning the vial upside down 5-10 times to obtain a homogeneous solution before loading onto the analyser. On board stability was for 24 hours with the original perforated cap and the reconstituted reagent remained stable for 7 days at 2-8˚C in the original capped vial.
- The STA® Deficient FVIII contains lyophilized citrated human plasma from which FVIII has been removed by selective Immuno-adsorption. The factor VIII deficient plasma was reconstituted with 1mL of distilled water with a 30-minute stabilization time at room temperature and then the vial was swirled gently to obtain a homogeneous solution before loading onto the analyser. On board stability is 4 hours. The FVIII deficient plasma was verified as part of the validation of the Stago analyser. This is confirmed by using a positive and negative commercial control prior to undertaking a FVIII assay as part of routine laboratory quality control.

- Owren-Koller buffer and $CaCl_2$ were ready for use (no stabilization time needed) with an on board stability of 72 hours without cap.

2.5.5 The Normal Pooled Plasma

The Normal Pooled Plasma was purchased from the South African National Blood Service (SANBS) (SANBS, Roodepoort, Johannesburg, South Africa) as “frozen Negative Plasma pooled” (PRU014, SANBS, RSA). Upon receiving the frozen pooled plasma, the unit was thawed in a 37˚C water bath. One millilitre aliquots were immediately prepared in cryo tubes and stored at -70˚C until used. The NPP was tested for PT and PTT to ensure its normality and serve as a baseline for subsequent monitoring.

2.5.6 Preparing a 4M imidazole solution at a pH of 7.4

The 4M imidazole solution at pH of 7.4 was prepared by adding crystalline imidazole into sterile water (Sabex, Boucherville, QC, Canada) then buffered with 10N HCl by titration to a pH of 7.4 (without changing the concentration/total volume of the desired mixture). The 4M imidazole solution was then stored in a closed plastic container in a 2-8˚C fridge for up to two months and used as required.

2.5.7 Preparation of Buffered Normal Pooled Plasma / Citrol 1

2.5.7.1 Direct buffering of NPP/Citrol 1 with solid Imidazole

The NPP/Citrol 1 was buffered by adding crystalline imidazole (Sigma-Aldrich, St. Louis, MO, USA) to obtain a concentration of 0.1M. The mixture was then titrated

to a pH of 7.4 by adding 1N HCl slowly. The BNPP and BC were then aliquoted into Cryo tubes and stored at -70˚C until use.

2.5.7.2 Buffering of NPP/Citrol 1 using 4M Imidazole solution

The NPP/Citrol 1 was freshly buffered by adding the pre-prepared 4M imidazole solution at the ratio of 3.9mL NPP/Citrol1 to 0.1mL 4M imidazole solution in a plastic container. The mixture was then buffered with 10N HCl by titration to a pH of 7.4.

2.5.8 The Nijmegen Modification of the Bethesda Assay

The NA was performed by modifying the original BA SOP HAE0198.

Set-up (refer to Figure 2.3)

400 mcL of NPP and 400 mcL of FVIII deficient plasma was pipetted to the first plastic tube (labelled “STD”) which served as the calibrator and the standard control. 200 mcL of patient’s plasma and 200 mcL of BNPP/BC was pipetted into the second plastic tube to act as the “neat” (1:1). Doubling dilutions (ranging from 1:1 to 1:64, more if required) was set up by pipetting 200 mcL of FVIII deficient plasma along with 200 mcL of patient plasma into a third plastic tube labelled 1:2. Next, 200 mcL of FVIII deficient plasma was added to 200 mcL of 1:2 dilution into a fourth plastic tube labelled 1:4 and thereof. The extra 200 mcL after mixing of the final tube of desired dilution was discarded. Finally, 200 mcL of BNPP/BC was pipetted into each of the series of dilution tubes (see Table 2.1 and Figure 2.2).

All tubes were then mixed well, capped and incubated in the waterbath at 37˚C for 2 hours.

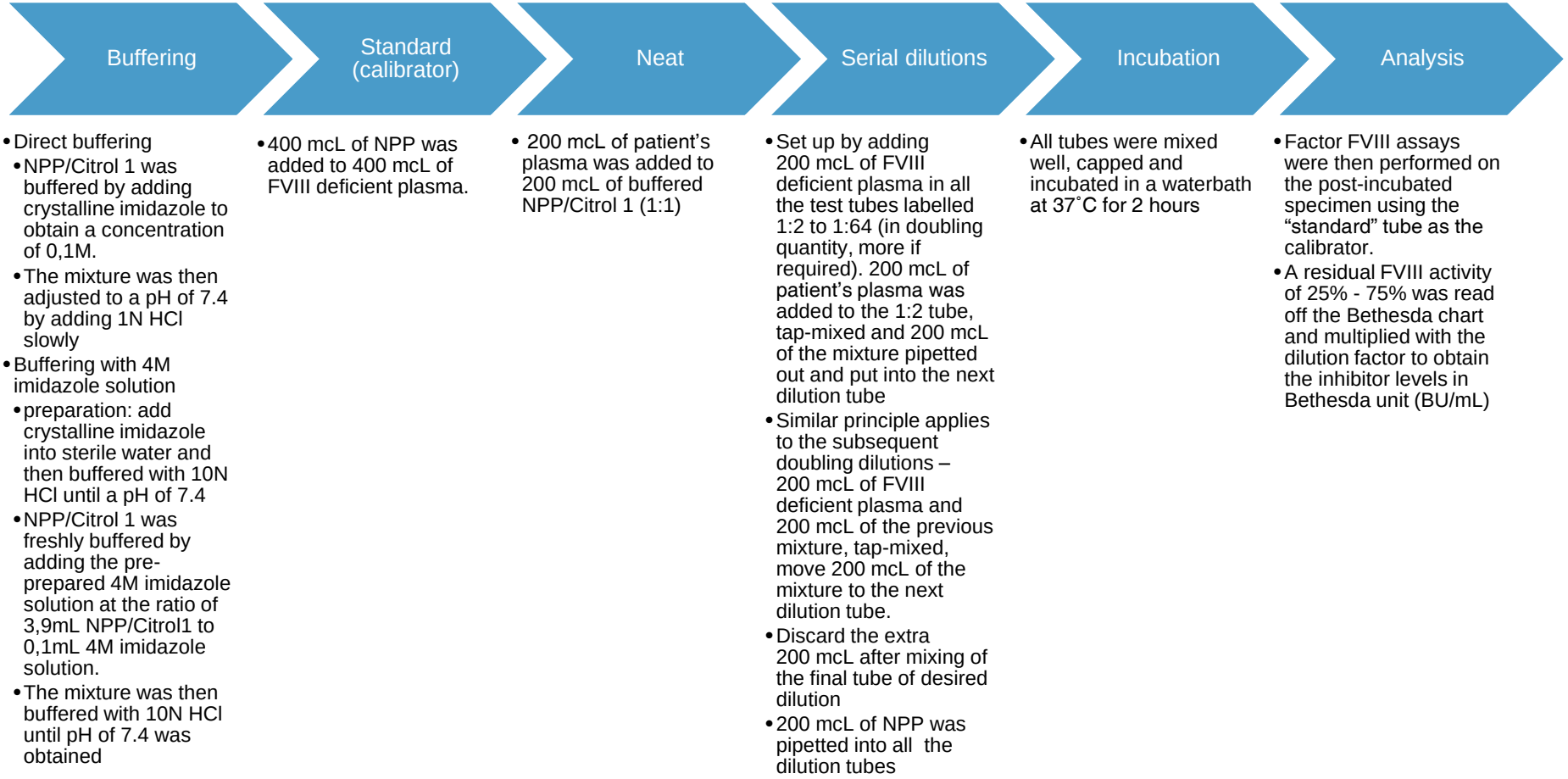


Figure 2.3. Flow diagram demonstrating the Nijmegen set up process

Abbreviations: NPP, Normal pooled plasma; Citrol 1, Dade® Citrol 1® Coagulation Control Level 1; HCl, Hydrochloric acid; M, mole per litre; N, normal.

Table 2.1. Scheme of Nijmegen Assay sample preparation.

Tube	STD	Neat (1:1)	1:2	1:4	1:8	1:16	1:32	1:64	etc..
F8 def (mcL)	200	X	200	200	200	200	200	200	
Patient plasma (mcL)	X	200	200	200 (1:2)	200 (1:4)	200 (1:8)	200 (1:16)	200 (1:32)	
Mix & transfer (mcL)	X	X	200	200	200	200	200	200	etc.… then mix & discard 200 mcL from last tube
BNPP/BC (mcL)	200	200	200	200	200	200	200	200	

Abbreviations: STD, standard control (calibrator); def, deficient; BNPP, buffered normal pool plasma; BC, buffered Dade® Citrol 1® Coagulation Control Level 1; X, add nothing in the tube; mcL, microlitre; etc., subsequent dilution tubes.

Post incubation

The STA-R Evolution® Expert Series was calibrated with the tube labelled “STD” with “FVIII INH" setting (assuming factor assay value of the standard control is 100%). After calibration is completed and passed, QC was performed by running a FVIII INH (essentially the same as factor assay which is a one-stage clot-based assay) on the same standard control to ensure the calibration of the analyser was correct and the result should be between 90-110%. Once QC passed, FVIII INH was then performed on the remaining incubated samples. The dilution of the samples that gave the residual factor within the range of 25%-75% were read off the Bethesda chart (see Table 2.2) and then multiplied by the dilution factor in order to obtain the inhibitor level in Nijmegen-Bethesda units (NBU).

Table 2.2. Bethesda Chart.

This chart is derived from the Bethesda graph which was based on the definition of the test plasma contains 1 BU/mL at 50% inhibition[89].

RESIDUAL FACTOR (%)	BETHESDA UNIT (BU/ML)	RESIDUAL FACTOR (%)	BETHESDA UNIT (BU/ML)
75	0.42	49	1.03
74	0.451	48	1.05
73	0.47	47	1.08
72	0.49	46	1.12
71	0.51	45	1.15
70	0.54	44	1.18
69	0.55	43	1.23
68	0.56	42	1.25
67	0.59	41	1.28
66	0.60	40	1.33
65	0.62	39	1.36
64	0.65	38	1.40
63	0.66	37	1.44
62	0.70	36	1.47
61	0.72	35	1.52
60	0.74	34	1.55
59	0.75	33	1.60
58	0.80	32	1.64
57	0.82	31	1.69
56	0.83	30	1.71
55	0.85	29	1.77
54	0.89	28	1.82
53	0.91	27	1.87
52	0.95	26	1.93
51	0.98	25	2.00
50	1.00		

Abbreviation: BU/mL, Bethesda unit per millilitre.

2.5.9 Control samples

For precision, accuracy and linearity analyses, commercial samples (with assigned inhibitor values) were used (see Table 2.3). These samples were prepared and validated by the External quality Control of diagnostic Assays and Tests (ECAT) Foundation (Dobbeweg 1, 2254 AG Voorschoten, The Netherlands) External Quality Assessment Programme (EQAP).

Table 2.3. ECAT control samples and expected values with 2SD

ECAT reference samples	Expected value	ECAT mean ±2SD
INH-24	0.5	0.0 - 1.2
INH-25	2.8	1.0 - 4.6
INH-26	6.1	2.1 - 10.1

Abbreviations: ECAT, ECAT foundation; SD, standard deviation; INH-24, -25, -26, ECAT control samples.

2.5.10 Precision Analysis

Precision is defined as the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions[90].

The precision analyses were assessed by performing the NA using BNPP/BC eleven times within a single run. One value was excluded per analysis and the standard deviation (SD), coefficient of variation (CV) and confidence interval (CI) were then calculated.

2.5.11 Accuracy Analysis

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found[90].

The accuracy analysis for NA was assessed by:

a) the results from the NA of three control samples with different concentrations were compared with the accepted reference values provided by the ECAT foundation;

b) the results from the NA using BNPP of 100 patient samples and NA using BC of 43 patient samples with a range of different concentrations (negative to high) were compared to the results obtained from the BA.

The difference between the NA measured value and the accepted true value (BA) together with the % bias, 95% confidence intervals were then calculated and represented in the Bland-Altman plot. Correlation studies between NA measured value and accepted true value with R^2, Y-intercept and slope were calculated and represented by linear regression analysis.

2.5.12 Linearity Analysis

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample [90].

The linearity analyses were performed by constituting nine diluted samples covering the interested range (≤5BU) from a control sample (with a known concentration) using FVIII deficient plasma as diluent. The results obtained from NA were then compared against the expected concentrations of the diluted samples. The results were then plotted with correlation coefficient, y-intercept, slope of the regression line and residual sum of square calculated.

2.5.13 Clinical data collection

A clinical correlation study was carried out concurrently with the method validation. The clinical data collection included a retrospective review of clinical files,

laboratory results and clinical trial data of the patients correlating with the available samples as well as known patients with inhibitors. Patients with von Willebrand disease, haemophilia B, acquired haemophilia or those who did not have a corresponding sample were excluded in the final analyses.

Parameters including demographics (age, gender, race), severity of the haemophilia, HIV status, presence of “transient” inhibitor, type of treatment (on demand or prophylaxis), type of replacement product, dosage given and exposure days were collected and compared to inhibitor development. The inhibitor levels were compared to bleeding frequency and response to the bypassing agents of the patients.

In a subset of patients (known inhibitor patients), the association between the presence of “transient” inhibitor and the development of high responding inhibitor were analyzed by going through the available patients’ records and response to treatment documented in the patients’ files.

2.5.14 Establishing the clinical significant cut-off value

The NA measured inhibitor values were correlated with the clinical presentation. The data was plotted in a 2 x 2 contingency table and true positive rate (TPR), false positive rate (FPR), positive predictive value (PPV), F1 score and accuracy (ACC) were calculated. The clinically significant cut-off value was determined by plotting the data into a Receiver Operating Characteristic (ROC) curve and the area under the curves (AUC) values were calculated.

2.5.15 Statistical Analysis

Accuracy analyses (linear regression and Bland-Altman plot) and linearity analyses (linear regression) were performed using GraphPad Prism® version 6 for Mac (GraphPad Software, San Diego, CA). Precision analyses (SD, %CV and 95% CI) and clinical data analyses were performed with Numbers version 3.2 (Apple Inc, Cupertino, CA, USA).

3 RESULTS

3.1. Laboratory validation:

The NA method validation was performed using both normal pooled plasma as well as Citrol 1 (which is commercially available and more standardized) with the possibility of replacing NPP with Citrol 1.

3.1.1 NA (Direct buffering of NPP with solid Imidazole)

3.1.1.1 Precision analysis

The results of precision analysis using BNPP are shown in Table 3.1.

Table 3.1. Precision analysis of NA using BNPP

Sample A	NA-BNPP
1	0.72
2	0.65
3	0.66
4	0.66
5	0.72
6	0.54
7	0.70
8	0.74
9	0.74
10	0.62

Abbreviations: sample A, random biological sample; NA, Nijmegen Assay; BNPP, buffered normal pooled plasma.

Precision analysis of NA using BNPP showed a SD of 0.06, %CV of 9.3% and a 95% CI of 0.68 ± 0.04.

3.1.1.2 Accuracy analysis

The result of accuracy analysis using BNPP with ECAT control samples are shown in Table 3.2.

Table 3.2. Accuracy analysis of NA using BNPP (control samples)

ECAT Reference sample	ECAT values (BU/mL)	BA (BU/mL)	NA with BNPP (NBU/mL)
INH-24-reference	0.5	1.05	1.03
INH-25-reference	2.8	3.86	5.44
INH-26-reference	6.1	7.48	7.48

Abbreviations: ECAT, ECAT foundation; INH-24, -25, -26, ECAT inhibitor control samples; BA, Bethesda Assay; NA, Nijmegen Assay; BNPP, buffered normal pooled plasma; BU/mL, Bethesda unit per millilitre; NBU/mL, Nijmegen-Bethesda unit per millilitre.

3.1.2 NA (Direct buffering of Citrol 1 with solid Imidazole)

3.1.2.1 Precision analysis

The results of precision analysis using BC are shown in Table 3.3.

Table 3.3. Precision analysis of NA using BC

SAMPLE A	NA-BC (NBU/mL)
1	0.75
2	0.74
3	0.98
4	0.83
5	0.72
6	0.65
7	0.70
8	0.85
9	0.89
10	0.83

Abbreviations: sample A, random biological sample; NA, Nijmegen Assay; BC, buffered Dade® Citrol 1® Coagulation Control Level 1; NBU/mL, Nijmegen-Bethesda unit per millilitre.

Precision analysis of NA using BC showed a SD of 0.1, %CV of 12.56 and 95% CI of 0.79 ± 0.02.

3.1.2.2 Accuracy analysis

The results of accuracy analysis using BC with the ECAT control samples are shown in Table 3.4.

Table 3.4. Analysis of NA using BC (control samples)

ECAT reference samples	ECAT values (BU/mL)	BA (BU/mL)	NA with BC (NBU/mL)
INH-24-reference	0.5	1.05	1.28
INH-25-reference	2.8	3.86	7.08
INH-26-reference	6.1	7.48	14.16

Abbreviations: ECAT, ECAT foundation; INH-24, -25, -26, ECAT inhibitor control samples; BA, Bethesda Assay; NA, Nijmegen Assay; BC, buffered Dade® Citrol 1® Coagulation Control Level 1; BU/mL, Bethesda unit per millilitre; NBU/mL, Nijmegen-Bethesda unit per millilitre.

3.1.3 NA (Buffering of NPP using 4M Imidazole solution)

3.1.3.1 Precision analysis

The results of precision analysis using BNPP are shown in Table 3.5.

Table 3.5. Precision analysis of NA using BNPP

INH-24	NA-BNPP (NBU/mL)
1	0.72
2	0.85
3	0.75
4	0.66
5	0.80
6	0.80
7	0.85
8	0.82
9	0.70
10	0.74

Abbreviations: INH-24, ECAT control sample; NA, Nijmegen Assay; BNPP, buffered normal pooled plasma; NBU/mL, Nijmegen-Bethesda unit per millilitre.

Precision analysis of NA using BNPP showed a SD of 0.06, %CV of 8.44 and 95% CI of 0.77 ± 0.05.

3.1.3.2 Accuracy analysis

The results of accuracy analysis using BNPP with control sample are shown in Table 3.6.

Table 3.6. Analysis of NA using BNPP (control sample)

ECAT reference sample	ECAT values (BU/mL)	BA (BU/mL)	NA with BNPP (NBU/mL)
INH-24-reference	0.5	0.95	0.65
INH-25-reference	2.8	3.86	3.64
INH-26-reference	6.1	12.16	8.24

Abbreviations: ECAT, ECAT foundation expected value; INH-24, -25, -26, ECAT inhibitor control samples; BA, Bethesda Assay; NA, Nijmegen Assay; BNPP, buffered normal pooled plasma; BU/mL, Bethesda unit per millilitre; NBU/mL, Nijmegen-Bethesda unit per millilitre.

All three control samples showed measured values within ECAT assigned values.

One hundred patient samples were analysed using BNPP buffered with 4M imidazole solution. The results are shown in Figure 3.1 and Figure 3.2.

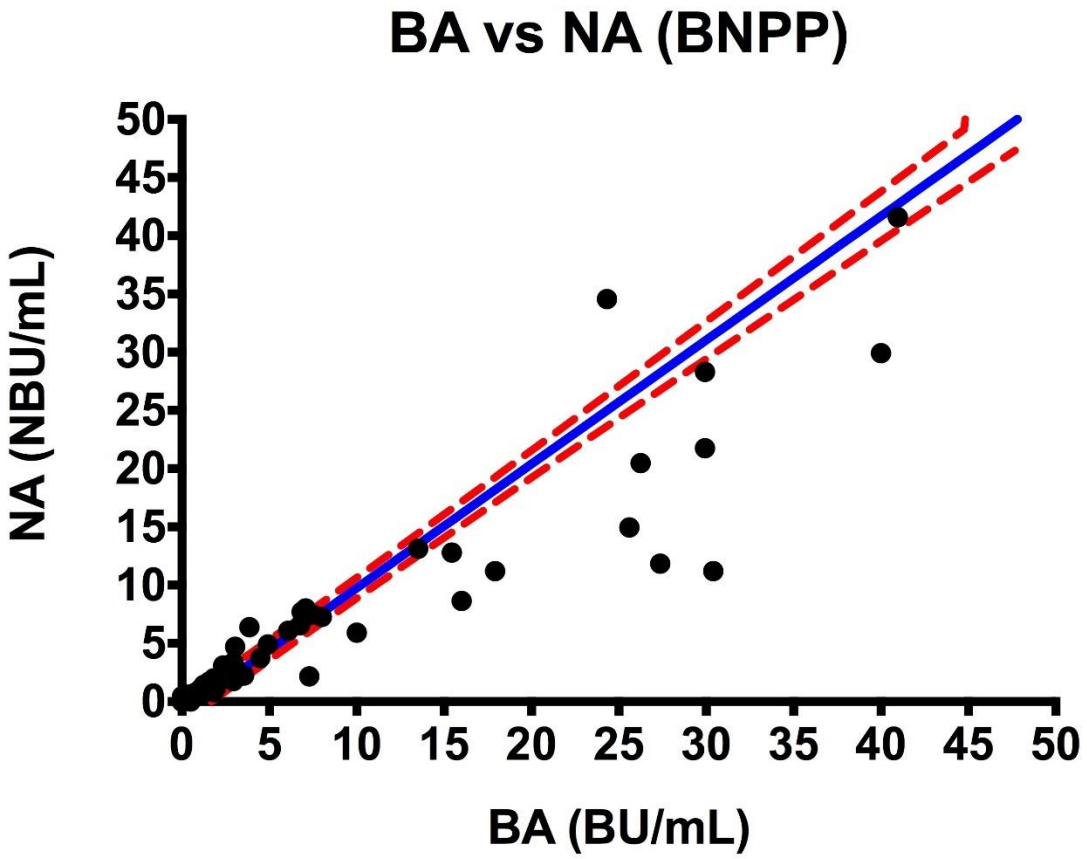


Figure 3.1. Linear regression graph demonstrating NA using BNPP against BA.

Abbreviations: BA, Bethesda Assay; NA, Nijmegen Assay; BU/mL, Bethesda unit per millilitre; NBU/mL, Nijmegen-Bethesda unit per millilitre; BNPP, buffered normal pooled plasma. Blue line represents linear regression between BA and NA with BNPP and the red dotted line represent the 95% confidence interval.

Correlation studies between NA (BNPP) and BA showed a $R^2 = 0.93$ (P-value <0.0001), Y-intercept = -0.89 and slope of 1.06 ± 0.03.

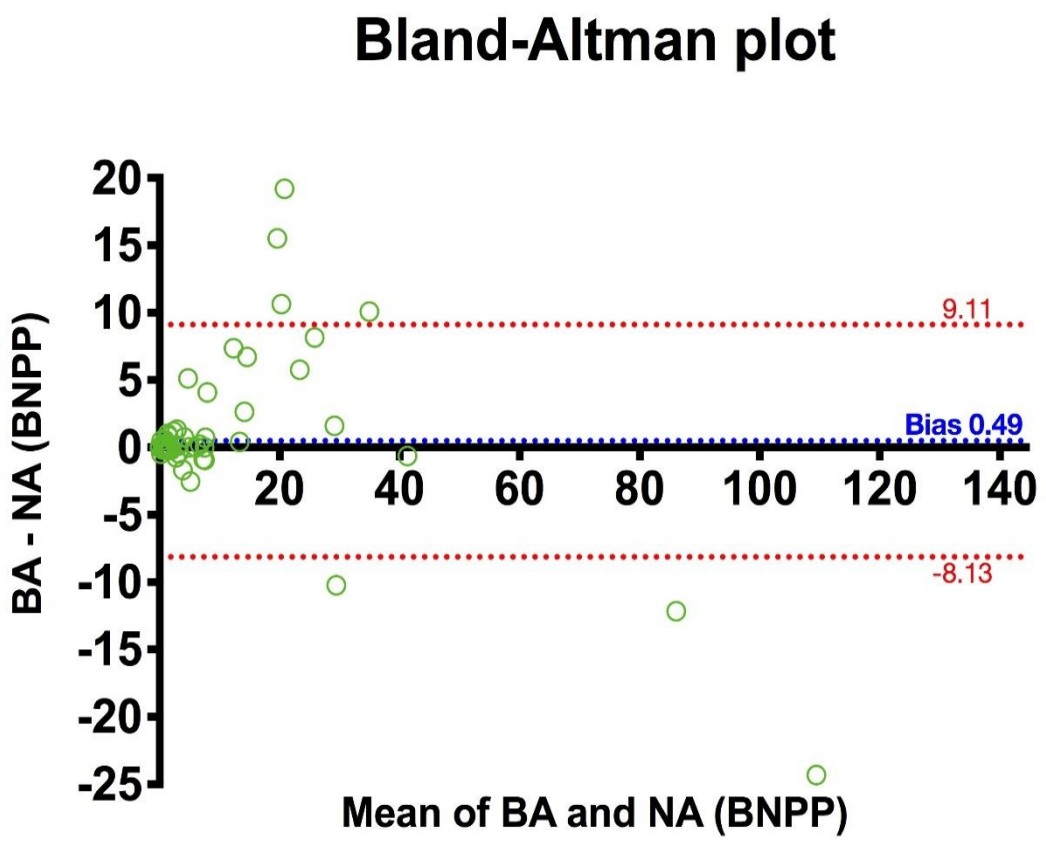


Figure 3.2. Bland-Altman plot demonstrating accuracy between BA and NA with BNPP.

Abbreviations: BA, Bethesda Assay; NA, Nijmegen Assay; BNPP, buffered normal pooled plasma. Blue dotted line represents the bias and red dotted lines represent 95% confident interval.

Comparative study using the Bland-Altman plot showed a bias = 0.49, SD of bias = 4.4 and 95% limits of agreement from -8.13 to 9.11.

In addition, the NA with BNPP provides a sensitivity of 100%, specificity of 94.5%, false positive rate of 5.5% and false negative rate of 0%.

3.1.3.3 Linearity analysis

The results of linearity analysis using BNPP are shown in Figure 3.3.

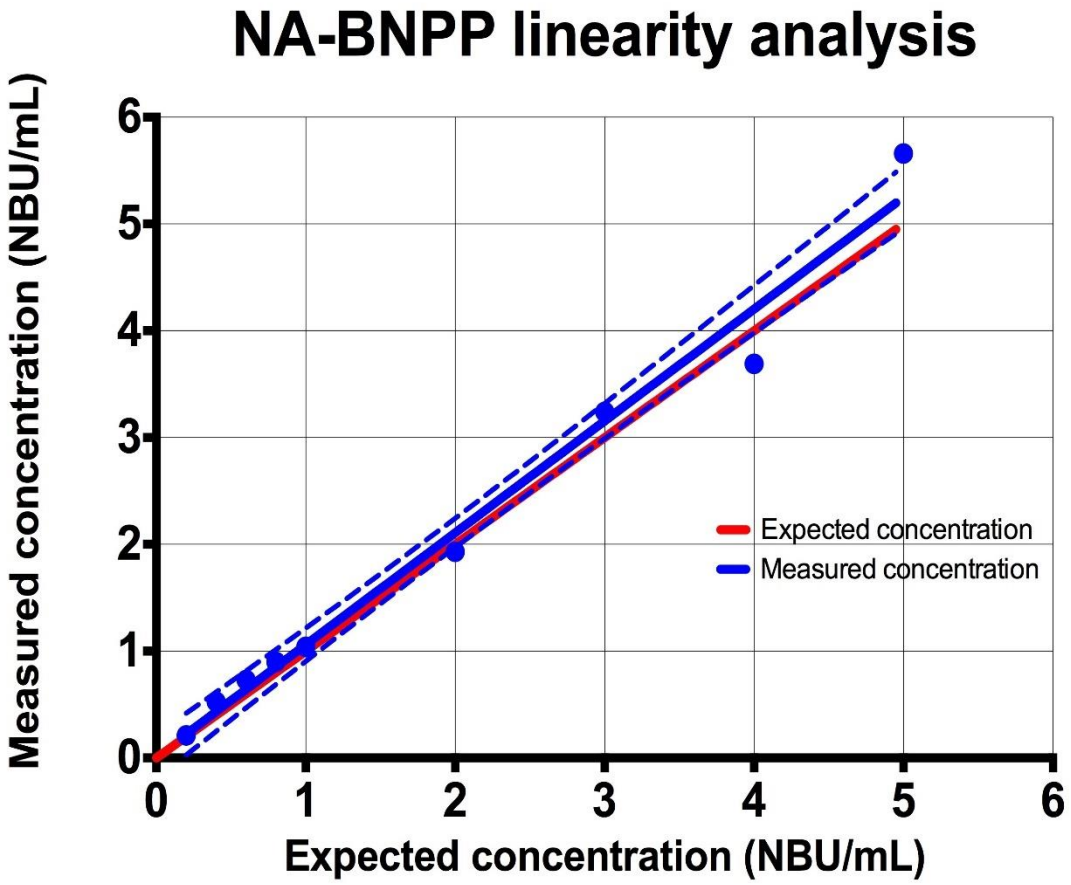


Figure 3.3. Linearity analysis of NA with BNPP.

Abbreviations: NA, Nijmegen Assay, BNPP, buffered normal pooled plasma, BU/mL, Bethesda unit per millilitre; NBU/mL, Nijmegen-Bethesda unit per millilitre.

Linearity analysis of NA (BNPP) in lower ranges (0.2 - 5 BU/mL) shows $R^2 = 0.98$

(P-value <0.0001), Y-intercept = 0.01 and slope = 1.05 ± 0.05.

3.1.4 NA (Buffering of Citrol 1 using 4M Imidazole solution)

3.1.4.1 Precision analysis

The results of precision analysis using BC are shown in Table 3.7.

Table 3.7. Precision analysis of NA using BC

ECAT INH 24	NA-BC (NBU/mL)
1	1.03
2	1.05
3	1.03
4	1.00
5	0.83
6	0.83
7	0.85
8	0.83
9	0.95
10	0.89

Abbreviations: INH-24, ECAT control sample; NA, Nijmegen Assay; BNPP, buffered normal pooled plasma; NBU/mL, Nijmegen-Bethesda unit per millilitre; max, maximum; min, minimal.

Precision analysis of NA with BC shows SD of 0.09, %CV of 10 and 95% CI of

0.93 ± 0.02.

3.1.4.2 Accuracy analysis

The results of accuracy analysis using BC with ECAT control samples are shown

in Table 3.8.

Table 3.8. Analysis of NA using BC (control sample)

ECAT reference samples	ECAT values (BU/mL)	BA (BU/mL)	NA with BC (NBU/mL)
INH-24-reference	0.5	0.95	1.03
INH-25-reference	2.8	3.86	4
INH-26-reference	6.1	12.16	10.24

Abbreviations: ECAT, ECAT foundation expected value; INH-24, -25, -26, ECAT inhibitor control samples; BA, Bethesda Assay; NA, Nijmegen Assay; BC, buffered Dade® Citrol 1® Coagulation Control Level 1; BU/mL, Bethesda unit per millilitre; NBU/mL, Nijmegen-Bethesda unit per millilitre.

In the 2 ECAT samples with mean <5BU (INH-24 and INH-25) showed measured results within the ECAT assigned values, however, INH-26 showed a measurement outside of the assigned value.

Forty-three patient samples were analysed using Citrol 1 buffered with 4M imidazole solution. The results are shown in Figure 3.4 and Figure 3.5.

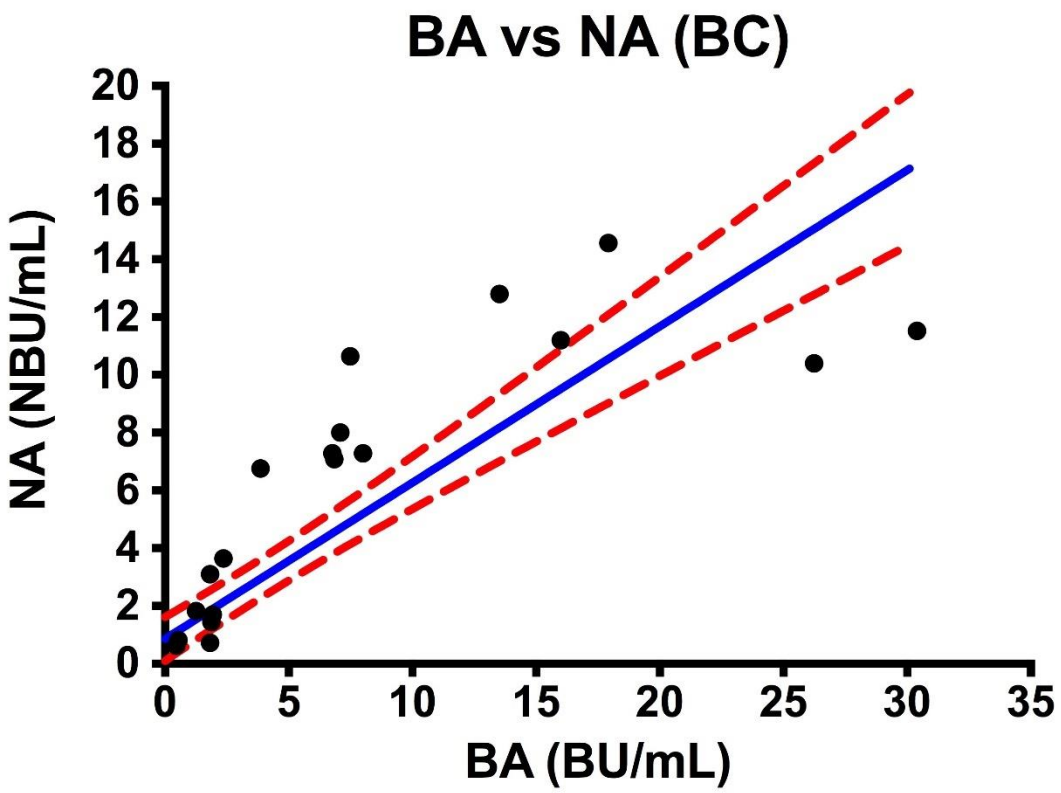


Figure 3.4. Linear regression graph demonstrating NA using BC against BA.

Abbreviations: BA, Bethesda Assay; NA, Nijmegen Assay; BU/mL, Bethesda unit per millilitre; NBU/mL, Nijmegen-Bethesda unit per millilitre; BC, buffered Dade® Citrol 1® Coagulation Control Level 1. Blue line represents linear regression between BA and NA with BC and the red dotted line represent the 95% confidence interval.

Correlation studies between NA (BC) and BA shows $R^2 = 0.76$ (P-value <0.0001),

Y-intercept = 0.86 and slope of 0.54 ± 0.05.

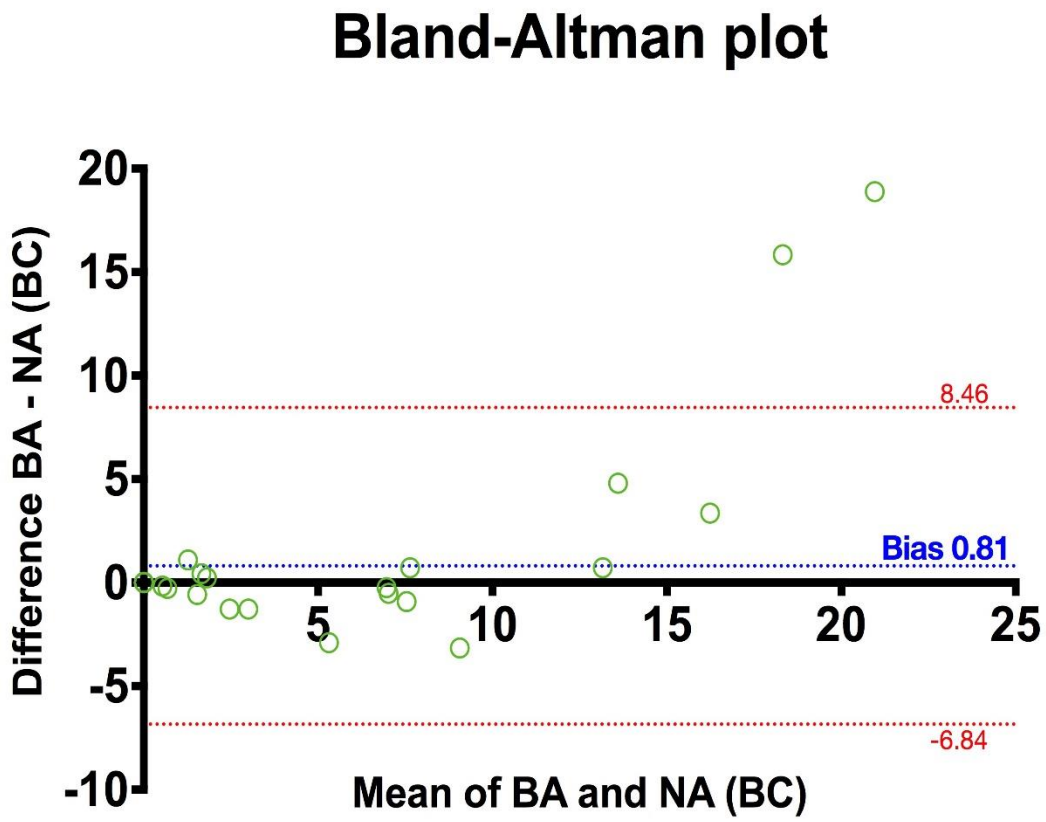


Figure 3.5. Bland-Altman plot demonstrating accuracy between BA and NA with BC.

Abbreviations: BA, Bethesda Assay; NA, Nijmegen Assay; BC, buffered Dade® Citrol 1® Coagulation Control Level 1. Blue dotted line represents the bias and red dotted lines represent 95% confident interval.

Comparative study using the Bland-Altman plot shows a bias = 0.81, SD of bias =

3.9 and 95% limits of agreement from -6.84 to 8.46.

3.1.3.3 Linearity analysis

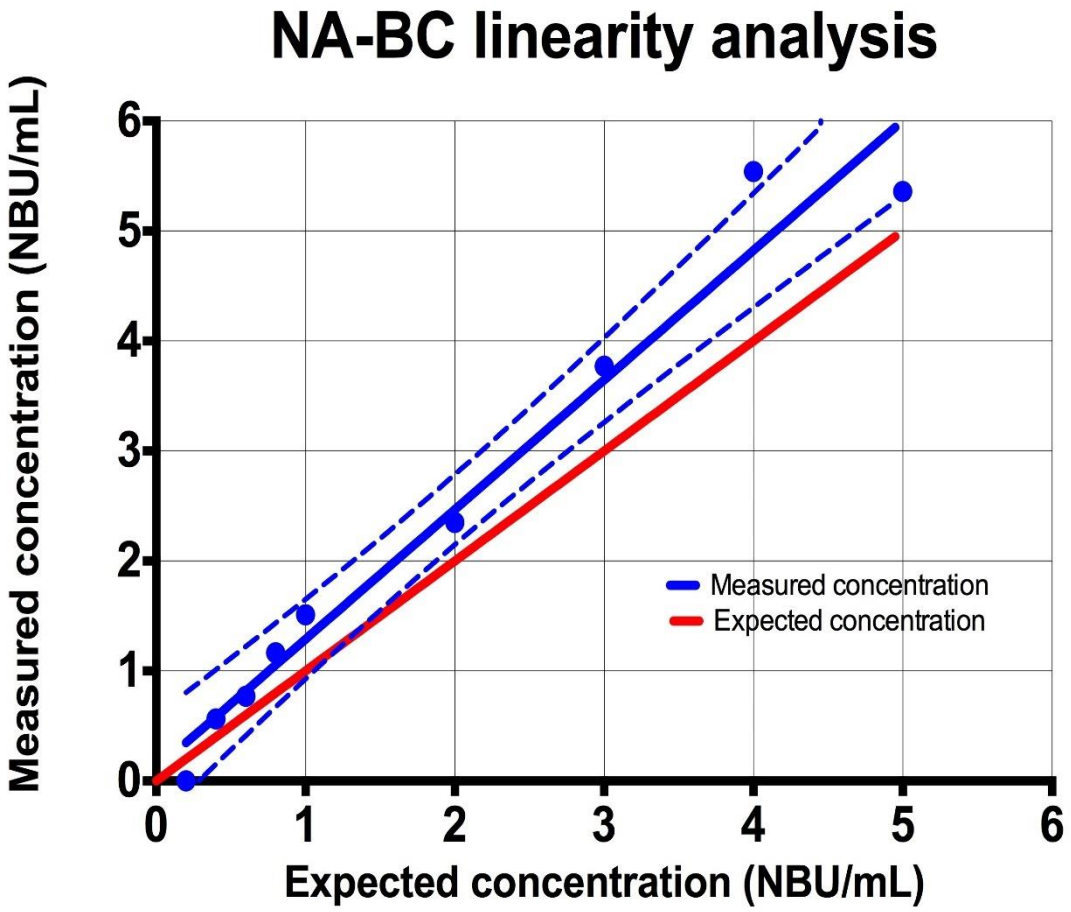


Figure 3.6. Linearity analysis of NA with BC.

Abbreviations: NA, Nijmegen Assay, BC, buffered Dade® Citrol 1® Coagulation Control Level 1; BU/mL, Bethesda unit per millilitre; NBU/mL, Nijmegen-Bethesda unit per millilitre.

Linearity analysis of NA (BC) in lower ranges (0.2 - 5 BU/mL) shows R2 = 0.97 (P-value <0.0001), Y-intercept = 0.11 and slope = 1.18 ± 0.08.

3.2. Clinical correlation studies

In the clinical correlation studies, a total of 82 patient files were reviewed. Of which, 10 patients did not have corresponding samples and were therefore

excluded. In addition, patients with acquired haemophilia, haemophilia B and von Willebrand disease are also excluded (see Figure 3.7).

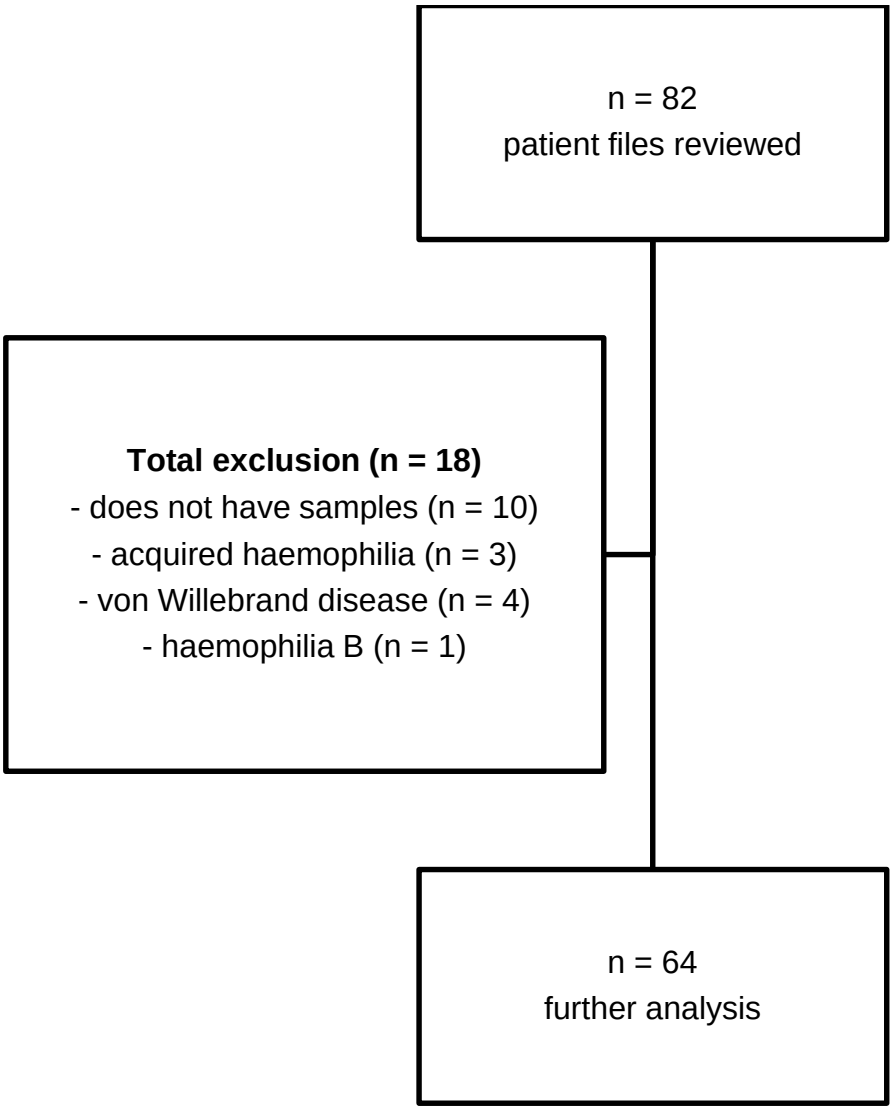


Figure 3.7. Flow diagram indicating the number of patient files reviewed, exclusion criteria and total number analyzed.

Table 3.9. Characteristics of patients analyzed.

Parameters	Number	Percentage (%)
Gender:		
Male	63	98,44
Female	1	1,56
Ethnicity:		
African	37	57,81
White	24	37,50
Coloured	2	3,13
Indian	1	1,56
Severity:		
Moderate	9	14,06
Severe	55	85,94
Age group:		
Paeds (less than 16)	21	32,81
Adults	43	67,19
HIV status:		
HIV +	4	6,25
HIV -	60	93,75
Known with inhibitor treated with a bypassing agent:		
Yes	20	31,25
No	44	68.75
Treatment strategy:		
Prophylaxis	6	9,38
On demand	58	90,63

The 6 patients described in Table 3.9 who received prophylactic therapy were all paediatric patients. In addition, patients treated with bypassing agents were all receiving on-demand treatment.

Of the analyzed patients, patients with high titre-inhibitors treated with bypassing agents comprised of approximately one third. Only 3 of the 20 high-titre inhibitor patients had a clear history of presence of transient inhibitor prior to the development of clinically significant inhibitor. The remaining 17 high-titre inhibitor patients have incomplete data regarding the presence of transient inhibitor as

most of these patients are adult and they were diagnosed before routine inhibitor testing/monitoring was implemented. All 44 patients with previously recorded low-titre, most likely “transient” inhibitor have achieved haemostasis with normal dose CFC and did not required increased dosage of CFC nor bypassing agents to date.

4 DISCUSSION

Although haemophilia is a rare disorder, the management of and expertise required to manage these patients is a costly undertaking. The development of inhibitors (in particularly high titre) is one of the most serious complications of CFC treatment rendering standard replacement therapy useless. These inhibitors have unpredictable kinetics as well as variable patient responses to different bypassing agents making the management of these patients challenging.

Historically, the gold standard of inhibitor determination is by the Bethesda assay which was developed in the 1970's. The nature (high sensitivity but lack of specificity) of Bethesda assay is deemed problematic particularly when it comes to low-titre inhibitors. In addition, with routine monitoring of inhibitors becoming more common, the phenomenon of transient inhibitors was discovered. In the 90's, the Nijmegen modification of the Bethesda Assay was introduced and was proven to be more reliable than BA in low-titre ranges.

Aim of the study

Multiple studies[82, 83, 91] have already been conducted and concluded that NA provides more reliable results in low titre ranges. However, LTA was in development recently[88] and was suggested that it may be superior for post ITI monitoring. Nevertheless, the primary objective of this study was to validate the NA against BA in a cohort of patients with inhibitors (regardless of titre) as well as determining a clinically significant cut-off value for our laboratory. Another objective of this study was to determine the significance of the low-titre (possibly

transient) inhibitors (i.e. whether they eventually become clinically significant requiring treatment with a bypassing agent).

4.1 Validation of NA using different buffered reagents

4.1.1 NA (Direct buffering with solid Imidazole)

Most studies conducted previously were utilizing NPP as buffered reagent. In this study, the NA has been validated with both buffered NPP and Citrol 1 against BA. The NA was initially set up according to the publication by Verbruggen[82] using direct buffering of fresh frozen pooled plasma/Citrol 1 with solid imidazole to a 0.1M concentration. The NPP/Citrol 1 was then adjusted to a pH of 7.4 with 1N HCl.

Precision analyses for both direct buffering with NPP or Citrol 1 were similar but NA (using BC) appeared to give us a higher mean, higher SD as well as higher %CV. When ECAT control samples (INH-24, -25 and -26) were analysed using NPP and Citrol 1 that were directly buffered, one level for NPP and all three for Citrol 1 were outside of the assigned values. This could possibly be explained by the variation or inaccuracy of measurement of imidazole powder and the dilutional effect with the use of 1N HCl during buffering (the volume required to achieve pH of 7.4 with 1N HCl was quite significant).

4.1.2 NA (using 4M Imidazole solution)

The use of 4M imidazole solution and a higher concentration of HCl (10N) to obtain pH of 7.4 seems to have eliminated the discrepancies in measurement. The precision analyses of NA buffered with 4M imidazole solution using NPP or Citrol 1

showed a better %CV when compared to the respective original (direct buffering) NA with NPP or Citrol 1.

Using the same ECAT control samples, NA with NPP obtained measured values within the assigned values using all three levels. Meanwhile, NA with Citrol 1 improved and only the high-titre level did not achieve a measured value within the assigned value. NA correlated well and was comparable with BA but the slope obtained with NPP was superior to Citrol 1. NA with NPP gave a smaller bias than Citrol 1, however both were within acceptable limits.

Linearity studies was only performed at the range of interest (i.e. low-titre inhibitor range of 0-5 BU/mL) revealing both NA with NPP and Citrol 1 were linear but NPP had a better slope being closer to the expected measurements than Citrol 1.

In general, NA with Citrol 1 appeared to give higher measurements than BA and NA with NPP. Another major difficulty experienced throughout this project was the QC and calibrating processes when using Citrol 1 in comparison to NPP. This could be explained by the fact that Citrol 1 is a lyophilized preparation. The FVIII in lyophilized preparations is less stable as compared to FVIII in fresh or frozen plasma (according to the package insert, the Citrol 1 is stable for 8 hours after reconstitution in closed vial at room temperature. Owing to the need for buffering as well as incubation, reconstituted Citrol 1 could not be kept in the original vial which could have contributed to FVIII instability. Moreover, FVIII can be inactivated during the buffering process resulting in an even lower FVIII level than NPP. For

the above reasons, the Citrol 1 arm of the study was terminated early due to technical difficulties and limited funding for reagents.

There is no other intra- or inter-laboratory data available in South Africa as this is the first NA set-up and validation performed.

4.1.3 Other observations

Of interest, 23 of 34 samples previously measured with lower-titre inhibitor when the sample was first submitted no longer have a measurable inhibitor. The significance of this discrepancy in measurement over time is uncertain. The remaining 11 samples with previously measured values, 9 of 11 samples were from known inhibitor patients treated with a bypassing agent demonstrating clinically significant low-titre inhibitors did not alter over time. Only 1 of 100 samples that showed no inhibitor on BA demonstrated a low-titre inhibitor on NA with NPP, but a measured value of 0.49 NBU/mL was nonetheless deemed negative when using the international cut off value of 0.6 NBU/mL.

In this study, the NA with NPP was comparable to BA and showed acceptable precision, accuracy and linearity but could not be demonstrated to be superior to the current BA.

4.2 Clinical correlation and low-titre transient inhibitors

Only 15% of the known inhibitor patients had transient inhibitors prior to the development of clinically significant inhibitors and required a change of treatment from CFC to bypassing agents.

The investigators could not conclusively demonstrate the presence of the transient inhibitor eventually becoming clinically significant. This phenomenon concurred with the suggestion of previous studies that the transient inhibitors represented the natural tolerance to the CFC given.

4.3 Limitation of the study

The samples were stored over several years and the acquired NPP was stored in -80˚C for over 1 month; the investigators could not determine how much, if any, sample degradation has occurred which may have partly affected the results. Owing to the limited amount of reagents, the Citrol 1 arm of the study was terminated early limiting the sample size to be less than the NPP arm. In addition, the accuracy analyses were not performed in duplicate.

Both the lack of previous transient inhibitor studies and the lack of available data in known high-titre inhibitor patients (before the diagnosis was made) hinder the interpretation of the significance of transient inhibitors.

ROC curves for NA with NPP and Citrol 1 could not be established owing to the limited sample size.

4.4 Recommendation

The NA with Citrol 1 showed inferior results compared to NA with NPP. In addition, owing to the technical difficulties experienced, NA with Citrol 1 is not recommended for routine inhibitor testing. The NA performed with NPP buffered

with 4M imidazole solution could be implemented in our laboratory and could replace the current BA.

The international agreed cut of value of 0.6 NBU/mL should be used.

Only NPP and Citrol 1 were considered as buffered reagents when we designed the study, other buffered reagents (such as National Bioproducts Institute Bioplasma FDP or STA-Pool Norm) should be investigated as alternative to NPP.

Prospective long-term studies are needed to determine the nature and significance of the transient inhibitor.

5 Conclusion

In this validation assay using patient and control samples, the NA performed better when using 4M imidazole solution for both NPP and Citrol 1 when compared to NA using solid imidazole direct buffering. The performance of the Nijmegen assay with buffered pool plasma was better than that of the same assay using buffered Citrol 1.

The performance of the NA with BNPP is comparable to the currently used BA. However, the investigators cannot demonstrate the NA to be superior in this study.

While not conclusive, the results of this study suggests that the presence of transient low-titre inhibitor does not predict the development of future clinically significant inhibitors.

APPENDICES

Appendix A Permission letter from CMJAH

GAUTENG PROVINCE
HEALTH
REPUBLIC OF SOUTH AFRICA

CHARLOTTE MAXEKE JOHANNESBURG ACADEMIC HOSPITAL

Enquiries:
Ms. O. Noge
Office of the Chief Executive Officer
(011): 488-3792
(011) 488-3753
06th February 2014

Dr. Yuen On Wan
Department of Haemotology
University of Witwatersrand

Dear Dr. Wan

RE: "Diagnostic and clinical evaluation of inhibitors in a Cohart of Haemophilia patients in a quaternary care centre"

Permission is granted for you to conduct the above research as described in your request provided:

1. Charlotte Maxeke Johannesburg Academic hospital will not in anyway incur or inherit costs as a result of the said study.
2. Your study shall not disrupt services at the study sites.
3. Strict confidentiality shall be observed at all times.
4. Informed consent shall be solicited from patients participating in your study.

Please liaise with the Head of Department and Unit Manager or Sister in Charge to agree on the dates and time that would suit all parties.

Kindly forward this office with the results of your study on completion of the research.

Approved / ~~not approved~~

Dr. M. Mofokeng
Acting Chief Executive Officer
6/02/2014

Appendix B Ethics clearance certificate

M130539

R14/49 Dr Yuen On Wan

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M130539

NAME: (Principal Investigator)	Dr Yuen On Wan
DEPARTMENT:	School of Pathology/Molecular Med & Haem. National Health Laboratory Services
PROJECT TITLE:	Diagnostic and Clinical Evaluation of Inhibitors in a Cohort of haemophilia Patients in a Quaternary Care Centre
DATE CONSIDERED:	31/05/2013
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof JN Mahlangu
APPROVED BY:	Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 31/05/2013

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

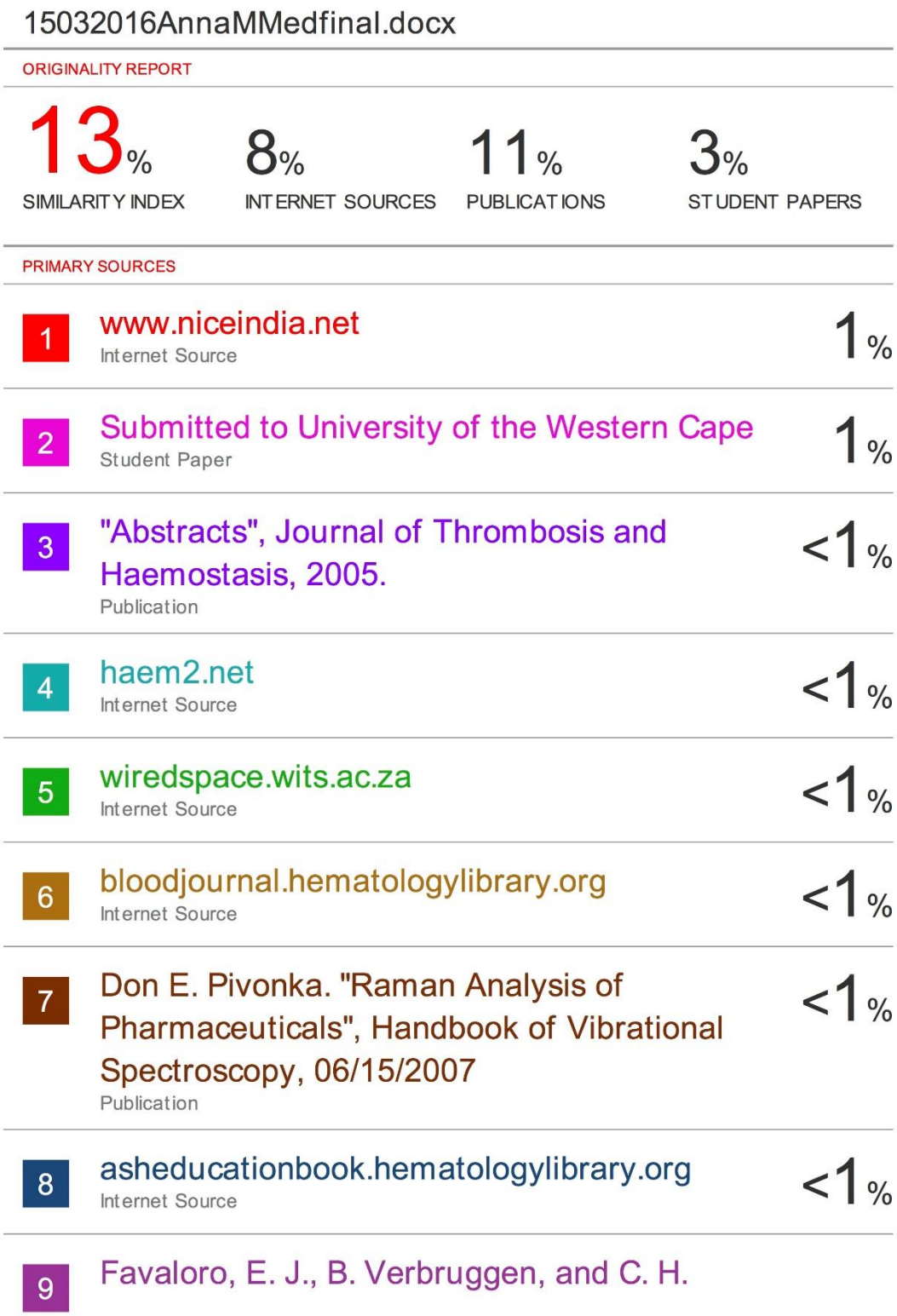
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature

Date 20/07/2014

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix C Turnitin result



15032016AnnaMMedfinal.docx

ORIGINALITY REPORT

13%	8%	11%	3%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	www.niceindia.net Internet Source	1%
2	Submitted to University of the Western Cape Student Paper	1%
3	"Abstracts", Journal of Thrombosis and Haemostasis, 2005. Publication	<1%
4	haem2.net Internet Source	<1%
5	wiredspace.wits.ac.za Internet Source	<1%
6	bloodjournal.hematologylibrary.org Internet Source	<1%
7	Don E. Pivonka. "Raman Analysis of Pharmaceuticals", Handbook of Vibrational Spectroscopy, 06/15/2007 Publication	<1%
8	asheducationbook.hematologylibrary.org Internet Source	<1%
9	Favaloro, E. J., B. Verbruggen, and C. H.	

REFERENCES

1. Owaidah TM. Hemophilia inhibitors prevalence, causes and diagnosis. In: Batorova A, editor. Hemophilia: InTech; 2012. p. 67-78.

2. White GC, Rosendaal F, Aledort LM*, et al.* Definitions in Hemophilia. Recommendation of the Scientific Subcommittee on Factor VIII and Factor IX of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. Thromb Haemost. 2001;85(3):560.

3. Oldenburg J, El-Maarri O. New insight into the molecular basis of hemophilia A. Int J Hematol. 2006;83(2):96-102.

4. Lakich D, Kazazian HH, Jr., Antonarakis SE*, et al.* Inversions disrupting the factor VIII gene are a common cause of severe haemophilia A. Nat Genet. 1993;5(3):236-41.

5. Lillicrap D. The molecular basis of haemophilia B. Haemophilia. 1998;4(4):350-7.

6. Delgado J, Jimenez-Yuste V, Hernandez-Navarro F*, et al.* Acquired Haemophilia: Review and Meta-Analysis Focused on Therapy and Prognostic Factors. Br Med J. 2003;121(1):21-35.

7. Franchini M, Favaloro EJ, Lippi G. Mild hemophilia A. J Thromb Haemost. 2010;8(3):421-32.

8. Kulkarni R, Soucie JM, Lusher J*, et al.* Sites of initial bleeding episodes, mode of delivery and age of diagnosis in babies with haemophilia diagnosed before the age of 2 years: a report from The Centers for Disease Control and Prevention's (CDC) Universal Data Collection (UDC) project. Haemophilia. 2009;15(6):1281-90.

9. Pollmann H, Richter H, Ringkamp H*, et al.* When are children diagnosed as having severe haemophilia and when do they start to bleed? A 10-year single-centre PUP study. Eur J Pediatr. 1999;158 Suppl 3:S166-70.

10. Small S, Rose PE, McMillan N*, et al.* Haemophilia and the kidney: assessment after 11-year follow-up. Br Med J (Clin Res Ed). 1982;285(6355):1609-11.

11. Eyster ME, Gill FM, Blatt PM*, et al.* Central nervous system bleeding in hemophiliacs. Blood. 1978;51(6):1179-88.

12. Ghosh K, Nair AP, Jijina F*, et al.* Intracranial haemorrhage in severe haemophilia: prevalence and outcome in a developing country. Haemophilia. 2005;11(5):459-62.

13. Ljung RC. Intracranial haemorrhage in haemophilia A and B. Br J Haematol. 2008;140(4):378-84.

14. Tarantino MD, Gupta SL, Brusky RM. The incidence and outcome of intracranial haemorrhage in newborns with haemophilia: analysis of the Nationwide Inpatient Sample database. Haemophilia. 2007;13(4):380-2.

15. Srivastava A, Brewer AK, Mauser-Bunschoten EP*, et al.* Guidelines for the management of hemophilia. Haemophilia. 2013;19(1):e1-e47.

16. Thompson AR, Bajaj SP, Chen SH*, et al.* "Founder" effect in different families with haemophilia B mutation. Lancet. 1990;335(8686):418.

17. Kazazian HH, Jr. The molecular basis of hemophilia A and the present status of carrier and antenatal diagnosis of the disease. Thromb Haemost. 1993;70(1):60-2.

18. Franchini M, Mannucci PM. Past, present and future of hemophilia: a narrative review. Orphanet J Rare Dis. 2012;7(1):1-8.

19. Allain JP, Frommel D. Antibodies to factor VIII. V. Patterns of immune response to factor VIII in hemophilia A. Blood. 1976;47(6):973-82.

20. Wight J, Paisley S. The epidemiology of inhibitors in haemophilia A: a systematic review. Haemophilia. 2003;9(4):418-35.

21. Ma AD, Carrizosa D. Acquired Factor VIII Inhibitors: Pathophysiology and Treatment. Hematology Am Soc Hematol Educ Program. 2006;2006(1):432-7.

22. Green D. Factor VIII inhibitors: a 50-year perspective. Haemophilia. 2011;17(6):831-8.

23. Dimichele D. Inhibitors: resolving diagnostic and therapeutic dilemmas. Haemophilia. 2002;8(3):280-7.

24. Berntorp E, Shapiro A, Astermark J*, et al.* Inhibitor treatment in haemophilias A and B: summary statement for the 2006 international consensus conference. Haemophilia. 2006;12:1-7.

25. Sawamoto Y, Shima M, Yamamoto M*, et al.* Measurement of anti-factor IX IgG subclasses in haemophilia B patients who developed inhibitors with episodes of allergic reactions to factor IX concentrates. Thromb Res. 1996;83(4):279-86.

26. Haya S, Moret A, Cid AR*, et al.* Inhibitors in haemophilia A: current management and open issues. Haemophilia. 2007;13:52-60.

27. Rothschild C, Gill J, Scharrer I*, et al.* Transient Inhibitors in the Recombinate Pup Study. Thromb Haemost. 2000;84(7):145-6.

28. Iorio A, Halimeh S, Holzhauer S*, et al.* Rate of inhibitor development in previously untreated hemophilia A patients treated with plasma-derived or recombinant factor VIII concentrates: a systematic review. J Thromb Haemost. 2010;8(6):1256-65.

29. Peyvandi F, Mannucci P, Garagiola I*, et al.* Source of Factor VIII replacement (plasmatic or recombinant) and incidence of inhibitory alloantibodies in previously untreated patients with severe hemophilia A: the multicenter randomized SIPPET study. 57th ASH Annual Meeting & Exposition; December 5-8; Orlando, FL2015.

30. Tagariello G, Iorio A, Matino D*, et al.* High rate of spontaneous inhibitor clearance during the long term observation study of a single cohort of 524 haemophilia A patients not undergoing immunotolerance. J Hematol Oncol. 2013;6:63.

31. van den Berg HM. Epidemiological aspects of inhibitor development redefine the clinical importance of inhibitors. Haemophilia. 2014;20 Suppl 4:76-9.

32. Mondorf W, Klinge J, Luban NLC*, et al.* Low factor VIII recovery in haemophilia A patients without inhibitor titre is not due to the presence of anti-factor VIII antibodies undetectable by the Bethesda assay. Haemophilia. 2001;7(1):13-9.

33. Xi M, Makris M, Marcucci M*, et al.* Inhibitor development in previously treated hemophilia A patients: a systematic review, meta-analysis, and meta-regression. J Thromb Haemost. 2013;11(9):1655-62.

34. Carcao M, Re W, Ewenstein B. The role of previously untreated patient studies in understanding the development of FVIII inhibitors. Haemophilia. 2015.

35. Darby SC, Keeling DM, Spooner RJD*, et al.* The incidence of factor VIII and factor IX inhibitors in the hemophilia population of the UK and their effect on subsequent mortality, 1977–99. J Thromb Haemost. 2004;2(7):1047-54.

36. Hay CRM, Palmer B, Chalmers E*, et al.* Incidence of factor VIII inhibitors throughout life in severe hemophilia A in the United Kingdom. Blood. 2011;117(23):6367-70.

37. Mauser-Bunschoten EP, Den Uijl IE, Schutgens RE*, et al.* Risk of inhibitor development in mild haemophilia A increases with age. Haemophilia. 2012;18(2):263-7.

38. Morado M, Villar A, Jiménez Yuste V*, et al.* Prophylactic treatment effects on inhibitor risk: experience in one centre. Haemophilia. 2005;11(2):79-83.

39. Morfini M, Haya S, Tagariello G*, et al.* European Study on Orthopaedic Status of haemophilia patients with inhibitors. Haemophilia. 2007;13(5):606-12.

40. Oldenburg J, Pavlova A. Genetic risk factors for inhibitors to factors VIII and IX. Haemophilia. 2006;12:15-22.

41. Kempton CL, White GC, 2nd. How we treat a hemophilia A patient with a factor VIII inhibitor. Blood. 2009;113(1):11-7.

42. Astermark J, Berntorp E, White GC*, et al.* The Malmö International Brother Study (MIBS): further support for genetic predisposition to inhibitor development. Haemophilia. 2001;7(3):267-72.

43. Carpenter SL, Michael Soucie J, Sterner S*, et al.* Increased prevalence of inhibitors in Hispanic patients with severe haemophilia A enrolled in the Universal Data Collection database. Haemophilia. 2012;18(3):e260-e5.

44. Viel KR, Ameri A, Abshire TC*, et al.* Inhibitors of Factor VIII in Black Patients with Hemophilia. New Engl J Med. 2009;360(16):1618-27.

45. Ter Avest PC, Fischer K, Mancuso ME*, et al.* Risk stratification for inhibitor development at first treatment for severe hemophilia A: a tool for clinical practice. J Thromb Haemost. 2008;6(12):2048-54.

46. Lochan A, Macaulay S, Chen WC*, et al.* Genetic factors influencing inhibitor development in a cohort of South African haemophilia A patients. Haemophilia. 2014;20(5):687-92.

47. Santagostino E, Mancuso ME, Rocino A*, et al.* Environmental risk factors for inhibitor development in children with haemophilia A: a case–control study. Br J Haematol. 2005;130(3):422-7.

48. van der Bom JG, Mauser-Bunschoten EP, Fischer K*, et al.* Age at first treatment and immune tolerance to factor VIII in severe hemophilia. Thromb Haemost. 2003;89(3):475-9.

49. Lorenzo JI, López A, Altisent C*, et al.* Incidence of factor VIII inhibitors in severe haemophilia: the importance of patient age. Br J Haematol. 2001;113(3):600-3.

50. Sharathkumar A, Lillicrap D, Blanchette VS*, et al.* Intensive exposure to factor VIII is a risk factor for inhibitor development in mild hemophilia A. J Thromb Haemost. 2003;1(6):1228-36.

51. Kempton CL, Soucie JM, Miller CH*, et al.* In non-severe hemophilia A the risk of inhibitor after intensive factor treatment is greater in older patients: a case–control study. J Thromb Haemost. 2010;8(10):2224-31.

52. Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol. 2007;81(1):1-5.

53. Kurnik K, Bidlingmaier C, Engl W*, et al.* New early prophylaxis regimen that avoids immunological danger signals can reduce FVIII inhibitor development. Haemophilia. 2010;16(2):256-62.

54. Hermans C, Astermark J, De Moerloose P. Exposure to factor VIII and prediction of inhibitor development: exposure days vs. danger days, or both? J Thromb Haemost. 2012;10(10):2194-6.

55. Bray GL, Gomperts ED, Courter S*, et al.* A multicenter study of recombinant factor VIII (recombinate): safety, efficacy, and inhibitor risk in previously

untreated patients with hemophilia A. The Recombinate Study Group. Blood. 1994;83(9):2428-35.

56. Aznar JA, Moret A, Ibanez F*, et al.* Inhibitor development after switching of FVIII concentrate in multitransfused patients with severe haemophilia A. Haemophilia. 2014;20(5):624-9.

57. Gouw SC, van der Bom JG, Auerswald G*, et al.* Recombinant versus plasma-derived factor VIII products and the development of inhibitors in previously untreated patients with severe hemophilia A: the CANAL cohort study. Blood. 2007;109(11):4693-7.

58. Gouw SC, van der Bom JG, Ljung R*, et al.* Factor VIII Products and Inhibitor Development in Severe Hemophilia A. New Engl J Med. 2013;368(3):231-9.

59. Fischer K, Iorio A, Hollingsworth R*, et al.* FVIII inhibitor development according to concentrate: data from the EUHASS registry excluding overlap with other studies. Haemophilia. 2016;22(1):e36-8.

60. Kreuz W, Ettingshausen CE, Auerswald G*, et al.* Epidemiology of inhibitors and current treatment strategies. Haematologica. 2003;88(6):Erep04.

61. Gruppo RA, Brown D, Wilkes MM*, et al.* Comparative effectiveness of full-length and B-domain deleted factor VIII for prophylaxis – a meta-analysis. Haemophilia. 2003;9(3):251-60.

62. Kaufman RJ, Pipe SW, Tagliavacca L*, et al.* Biosynthesis, assembly and secretion of coagulation factor VIII. Blood Coagul Fibrinolysis. 1997;8 Suppl 2:S3-14.

63. Santagostino E. A new recombinant factor VIII: from genetics to clinical use. Drug Des Devel Ther. 2014;8:2507-15.

64. Hay CR, Palmer BP, Chalmers EA*, et al.* The incidence of factor VIII inhibitors in severe haemophilia A following a major switch from full-length to B-domain-deleted factor VIII: a prospective cohort comparison. Haemophilia. 2015;21(2):219-26.

65. Migliore E, Allione A, Dutto L*, et al.* Acquired factor VIII inhibitor in patient infected with HIV: a casual association or a prone immunological setting? Haemophilia. 2009;15(6):1334-5.

66. Rivoisy C, D'Oiron R, Cherin M*, et al.* Acquired haemophilia A associated with HIV infection: a rare disease. AIDS. 2014;28(6):931-2.

67. Zeichner SB, Harris A, Turner G*, et al.* An Acquired Factor VIII Inhibitor in a Patient with HIV and HCV: A Case Presentation and Literature Review. Case Rep Hematol. 2013;2013:7.

68. Hultin MB. Acquired inhibitors in malignant and nonmalignant disease states. Am J Med. 1991;91(5, Supplement 1):S9-S13.

69. Green D, Lechner K. A survey of 215 non-hemophilic patients with inhibitors to Factor VIII. Thromb Haemost. 1981;45(3):200-3.

70. Morrison AE, Ludlam CA. Acquired Haemophilia and its management. Br J Haematol. 1995;89(2):231-6.

71. Gringeri A, Mantovani LG, Scalone L*, et al.* Cost of care and quality of life for patients with hemophilia complicated by inhibitors: the COCIS Study Group. Blood. 2003;102(7):2358-63.

72. Witkop M, Guelcher C, Forsyth A*, et al.* Treatment outcomes, quality of life, and impact of hemophilia on young adults (aged 18-30 years) with hemophilia. Am J Hematol. 2015;90 Suppl 2:S3-s10.

73. Kulkarni R, Aledort LM, Berntorp E*, et al.* Therapeutic choices for patients with hemophilia and high-titer inhibitors. Am J Hematol. 2001;67(4):240-6.

74. Collins PW, Chalmers E, Hart D*, et al.* Diagnosis and management of acquired coagulation inhibitors: a guideline from UKHCDO. Br J Haematol. 2013;162(6):758-73.

75. Collins P, Baudo F, Huth-Kuhne A*, et al.* Consensus recommendations for the diagnosis and treatment of acquired hemophilia A. BMC Res Notes. 2010;3:161.

76. Janbain M, Leissinger CA, Kruse-Jarres R. Acquired hemophilia A: emerging treatment options. J Blood Med. 2015;6:143-50.

77. Hay CR, DiMichele DM. The principal results of the International Immune Tolerance Study: a randomized dose comparison. Blood. 2012;119(6):1335-44.

78. Mariani G, Kroner B. Immune tolerance in hemophilia with factor VIII inhibitors: predictors of success. Haematologica. 2001;86(11):1186-93.

79. Di Minno MN, Di Minno G, Di Capua M*, et al.* Cost of care of haemophilia with inhibitors. Haemophilia. 2010;16(1):e190-201.

80. Kasper CK, Aledort L, Aronson D*, et al.* Proceedings: A more uniform measurement of factor VIII inhibitors. Thromb Diath Haemorrh. 1975;34(2):612.

81. Verbruggen B. Diagnosis and quantification of factor VIII inhibitors. Haemophilia. 2010;16(102):20-4.

82. Verbruggen B, Novakova I, Wessels H*, et al.* The Nijmegen modification of the Bethesda assay for factor VIII:C inhibitors: improved specificity and reliability. Thromb Haemost. 1995;73(2):247-51.

83. Verbruggen HW, Novakova IRO, Wessels JMC*, et al.* Frequency of inhibitors in haemophiliacs. Lancet. 1992;339(8804):1301.

84. Verbruggen B, van Heerde WL, Laros-van Gorkom BA. Improvements in factor VIII inhibitor detection: From Bethesda to Nijmegen. Semin Thromb Hemost. 2009;35(8):752-9.

85. Verbruggen B, Dardikh M, Polenewen R*, et al.* The factor VIII inhibitor assays can be standardized: results of a workshop. J Thromb Haemost. 2011;9(10):2003-8.

86. Favaloro EJ, Bonar R, Kershaw G*, et al.* Laboratory identification of factor VIII inhibitors in the real world: the experience from Australasia. Haemophilia. 2010;16(4):662-70.

87. Favaloro EJ, Verbruggen B, Miller CH. Laboratory testing for factor inhibitors. Haemophilia. 2014;20 Suppl 4:94-8.

88. Dardikh M, Albert T, Masereeuw R*, et al.* Low-titre inhibitors, undetectable by the Nijmegen assay, reduce factor VIII half-life after immune tolerance induction. J Thromb Haemost. 2012;10(4):706-8.

89. Laffan M, Manning R. Investigation of haemostasis. In: Lewis SM, Bain BJ, Bates I, editors. Dacie and Lewis Practical Haematology. 11th ed. London: Churchill Livingstone Elsevier 2012. p. 423.

90. International conference on the harmonization of technical requirements for the registration of pharmaceuticals for human use. ICH harmonized tripartite guideline validation of analytical procedures: text and methodology Q2 (R1). 2005.

91. Giles AR, Verbruggen B, Rivard GE*, et al.* A detailed comparison of the performance of the standard versus the Nijmegen modification of the Bethesda assay in detecting factor VIII:C inhibitors in the haemophilia A population of Canada. Association of Hemophilia Centre Directors of Canada. Factor VIII/IX Subcommittee of Scientific and Standardization Committee of International Society on Thrombosis and Haemostasis. Thromb Haemost. 1998;79(4):872-5.