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GENETIC FACTORS INFLUENCING INHIBITOR DEVELOPMENT IN A COHORT OF SOUTH AFRICAN HAEMOPHILIA A PATIENTS

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GENETIC FACTORS INFLUENCING INHIBITOR DEVELOPMENT

IN A COHORT OF SOUTH AFRICAN

HAEMOPHILIA A PATIENTS

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, in partial fulfillment of the requirements for the degree of Master of Medicine in Medical Genetics

Johannesburg, 2013

DECLARATION

I, Anneline Lochan, declare that this research report is my own work. It is being submitted for the degree of Master of Medicine in Medical Genetics, at the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

……

……day of ……,2013.

DEDICATION

To my family, a sincere thank you for your unconditional love, support and motivation during my degree. You have always been my pillar of strength.

ABSTRACT

Haemophilia A (HA) is a an X-linked bleeding disorder that manifests due to a mutation in the *F8* gene encoding the coagulation factor VIII (FVIII) protein. Therapeutic management of HA involves intravenous FVIII infusions which are either plasma derived or recombinant concentrates that are administered to prevent or manage bleeding episodes promptly. A critical complication of repeated FVIII replacement therapy is the production of FVIII neutralising inhibitors which affect the coagulation potential of the replacement therapy, thus compromising the ability to manage bleeding episodes. The genetic and environmental factors predisposing to inhibitor development remain uncertain, and require improved understanding to provide optimal patient care and surveillance.

The study firstly aimed to characterise a cohort of South African HA patients in terms of clinical severity, ethnicity, int22 mutation status and inhibitor development; secondly, to explore whether the genetic factors (clinical severity, ethnicity, int22 mutation status, *F8* gene haplotype) influence inhibitor development.

A total of 229 probands who had diagnostic HA testing at the Molecular Genetics Laboratory, Division of Human Genetics, of the National Health Laboratory Services (NHLS) and School of Pathology, University of the Witwatersrand, Johannesburg, were included in the study. The majority of patients (91%) in the cohort had severe HA. There were a similar proportion of black and white patients in the cohort. There was a 13% incidence of inhibitor development in the cohort of which 72% were black and 28% were white patients. To investigate the influence of genetic factors on inhibitor development only the probands with known inhibitor status

were included (n=216). It was established that 36% (77/216) of patients were int22 positive of which 20% (15/77) were reported to be inhibitor positive while 10% (14/139) of the int22 negative patients (n=139) were shown to be inhibitor positive. Therefore, the int22 positive patients had a two-fold higher incidence of inhibitor development than int22 negative patients. *F8* gene haplotype analysis revealed that the H1 and H2 haplotypes were the most common in the cohort while the H3 and H5 haplotypes were only reported in black patients. Black patients were shown to have a higher prevalence of inhibitor development within each haplotype, thus suggesting that factors other than *F8* gene haplotype are important in inhibitor development.

Overall, black int22 positive probands had a significantly higher prevalence (p=0.04) of inhibitor development than white int22 positive and negative patients in the cohort which is suggestive that ethnicity and *F8* gene mutation may play a more major role in inhibitor development compared to *F8* gene haplotype. Hence, there is a need to identify other genetic factors that may predispose HA patients to inhibitor development.

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LIST OF ABBREVIATIONS

aPTT	Activated partial thromboplastin time
ARMS-PCR	Amplification Refractory Mutation System –Polymerase Chain Reaction
BU	Bethesda unit
BU/mL	Bethesda units per milliliter
DNA	Deoxyribonucleic acid
F8	*Factor VIII* gene
FVIII	Factor VIII protein
HA	Haemophilia A
HIV	Human immunodeficiency virus
HPA	Haemophilia A database patient file code
int1	Intron 1 inversion
int22	Intron 22 inversion
INR	International normalised ratio
kb	Kilobases
µg/ml	Micrograms per millilitre
mRNA	Messenger ribonucleic acid
NHLS	National Health Laboratory Services
pdFVIII	Plasma derived FVIII
rFVIII	Recombinant FVIII
SNPs	Single nucleotide polymorphisms
vWF	von Willebrand factor
Xq28	Chromosome X, region 2 band 8

CHAPTER 1: INTRODUCTION

Haemophilia A (HA) is a bleeding disorder due to mutations in the *factor VIII* (*F8*) gene encoding the coagulation factor VIII (FVIII) protein. This hereditary coagulation disorder has a prevalence of approximately 1:5000 live male births worldwide, without ethnic or geographic predilection (Konkle, Josephson, Fletcher et al., 2011). Effective management of bleeding episodes with FVIII infusion therapy prevents or reduces secondary complications such as pain, chronic joint disease and disability (Manco-Johnson, Abshire, Shapiro et al., 2007).

Although FVIII concentrate replacement therapy in HA has proven to be effective, a critical complication of treatment is inhibitor development to the FVIII protein (Powell, 2009). Inhibitor predisposing genetic factors are multiple and include, among others, the type of *F8* gene mutations, ethnicity, a family history of inhibitor development and FVIII haplotype mismatch. Knowledge of genetic risk factors for inhibitor development is important not only in predicting inhibitor risk but also in planning exposure to rapidly evolving FVIII replacement therapy (Maclean, Richards, Williams et al., 2011).

All research and data reviewed below is based on international studies. There are currently no published studies characterising genetic risk factors for inhibitor development in the South African HA population. Therefore, this study aims to characterise and correlate HA disease severity, intron 22 inversion mutation status, ethnicity, inhibitor development and FVIII haplotype in a South African HA cohort.

1.1 HAEMOPHILIA A

Haemophilia A (HA) is a bleeding disorder that is characterised by the absence or defective functioning of the FVIII protein. The severity of the disease, age of diagnosis and frequency of bleeding episodes are related to the level of FVIII activity (Antonarakis, 1995).

1.1.1 Mode of inheritance

Haemophilia A is an X-linked recessive disorder. Females who have an affected son and one other affected maternal relative are referred to as obligate carriers of a *F8* gene disease-causing mutation. Carrier females have a 1 in 2 chance (50%) of transmitting the *F8* gene mutation in each pregnancy. Sons of carrier females that inherit the *F8* disease-causing mutation will manifest the condition; whereas daughters who inherit the mutant gene will be carriers and are usually asymptomatic, but are at an increased risk for bleeding with major injuries, surgery or tooth extractions and occasionally bleed spontaneously. Affected males transmit the mutation to all of their daughters and none of their sons (Konkle et al., 2011).

1.1.2 Clinical characterisation of Haemophilia A

The clinical severity of HA is classified as mild, moderate and severe in relation to *in vitro* FVIII clotting activity (Konkle et al., 2011). Figure 1.1 illustrates the approximate frequencies of each clinical phenotype of HA as reported in earlier studies and have since been confirmed in numerous single cohort and multi-centred international studies (Antonarakis, 1995).

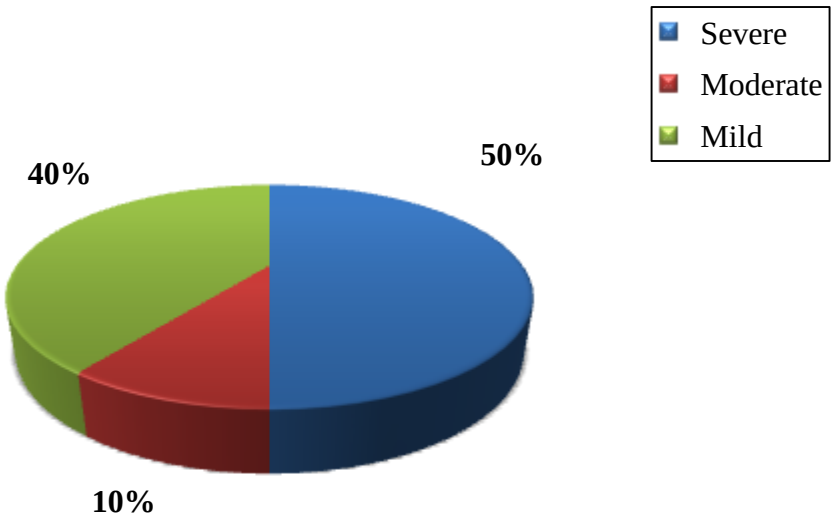


Figure 1.1 The frequency of Haemophilia A clinical phenotypes (Antonarakis, 1995).

Approximately 50% of HA patients have the severe phenotype with <1% of normal FVIII activity. Clinically these patients have frequent spontaneous joint or deep muscle bleeding and are usually diagnosed within the first year of life (Antonorakis, 1995). The most frequent presenting symptoms in untreated toddlers and children are subcutaneous haematomas and bleeding from minor mouth injuries. Head injuries may result in intracranial bleeding and, rarely, affected infants have extra- or intracranial bleeding following birth. Untreated individuals with severe HA may have two to five spontaneous bleeding episodes per month (Konkle et al., 2011).

On average 10% of patients exhibit moderate HA with 1-5% of normal FVIII activity. This class of patients experience abnormal or prolonged bleeding after minor injuries and rarely bleed spontaneously. The average age of diagnosis is five to six years (Antonorakis, 1995).

The frequency of bleeding in untreated individuals varies from one episode per month to one per year. The signs and symptoms of bleeding in patients with moderate HA are similar to those of severe HA patients (Konkle et al., 2011).

About 30-40% of HA patients have a mild phenotype with 6-35% of normal FVIII activity. These patients do not have spontaneous bleeding, but abnormal bleeding occurs after major trauma or surgical procedures. They are usually diagnosed later in life (Antonarakis, 1995). Untreated individuals with mild HA may have up to one episode of bleeding per year but may bleed only once every ten years (Konkle et al., 2011). Table 1.1 summarises the clinical classification of HA in relation to FVIII clotting activity and the clinical manifestations.

Bleeding into the joints and deep muscle haematomas results in pain and limping before swelling appears. The most common sites of spontaneous bleeding are the joints but other sites such as the gastrointestinal tract, kidneys and the brain may also be involved (Konkle et al., 2011).

Table 1.1 Clinical classification of Haemophilia A according to FVIII clotting activity levels and the clinical manifestations (Konkle et al., 2011).

Clinical classification	FVIII clotting activity	Clinical manifestation
Severe	< 1% of normal activity	Spontaneous bleeds - Haemarthrosis - Subcutaneous haematoma - Deep muscle haematoma - Brain/Intracranial - Gastrointestinal - Renal/Haematuria Prolonged bleeding after minor trauma/surgery - Tooth extraction - Mouth injury - After surgical procedures
Moderate	1-5% of normal activity	Prolonged bleeding after minor trauma or surgical procedures at similar clinical sites as stated above
Mild	6-35% of normal activity	No spontaneous bleeds Prolonged bleeding after major trauma or surgical procedures

1.1.2.1 Manifestation of HA in carrier females

Occasionally, carrier females may manifest with symptoms of HA. There are various explanations for this occurrence and the most likely being that of skewed X-inactivation. X-

inactivation is a process that usually occurs randomly whereby either one of the two X chromosomes in a female have an equal chance of being inactivated. If the X chromosome carrying the mutant HA allele, in a carrier female, remains active due to a non-random process, this is known as skewed X-inactivation (Turnpenny & Ellard, 2007).

Other explanations include numerical X-chromosome abnormalities when a HA carrier female has a single X-chromosome (i.e Turner syndrome) and X-autosome translocations that involves an exchange of chromosomal material between an autosome and an X chromosome and the X chromosome involved in the translocation remains activate to maintain expression of the autosomal genes thereby allowing an X-linked recessive disorder such as HA to manifest in a carrier female (Turnpenny & Ellard, 2007).

Approximately 10% of HA carrier females have a FVIII clotting activity below 35% of the normal range, irrespective of the clinical severity in the family. Such carrier females are at risk of abnormal bleeding similar to that of a male with mild HA (Plug, Mauser-Bunschoten, Brocker-Vriends et al., 2006). With pregnancy, use of oral contraception, exercise and chronic inflammation, there is a notable increase in plasma FVIII protein levels detected on FVIII coagulation assays (Konkle et al., 2011). Symptomatic carrier females may be protected by the increase of FVIII levels during pregnancy, however, obstetric complications may ensue once FVIII levels reduce to baseline levels and delayed oozing may occur and result in post partum haemorrhage (Lee, Pavord, Bolton-Maggs et al., 2006).

1.1.3 Function of the Factor VIII protein

Through a series of studies of the coagulation process, the essential role of FVIII as a plasma cofactor in the blood coagulation cascade was established (Mann, Nesheim, Church et al., 1990). As illustrated in Figure 1.2, the coagulation cascade consists of an extrinsic and an intrinsic pathway both of which enter into a common pathway during the process of clot formation. Within the intrinsic pathway, FVIII circulates in the plasma as a non-covalent complex with von Willebrand factor (VWF). Through the binding of VWF to subendothelial matrix proteins and adherent platelets, FVIII is allowed to concentrate at sites of vascular injury. This FVIII-VWF complex enhances FVIII synthesis and protects it from proteolytic degradation. The release of FVIII from the VWF complex within the intrinsic pathway enables FVIII to bind to the phospholipid membranes of injured cells and activated platelets. FVIII, together with factor VII from the extrinsic pathway, accelerates the cleavage of factor X by factor IX by several thousand fold, ultimately resulting in clot formation (Sherwood, 2007).

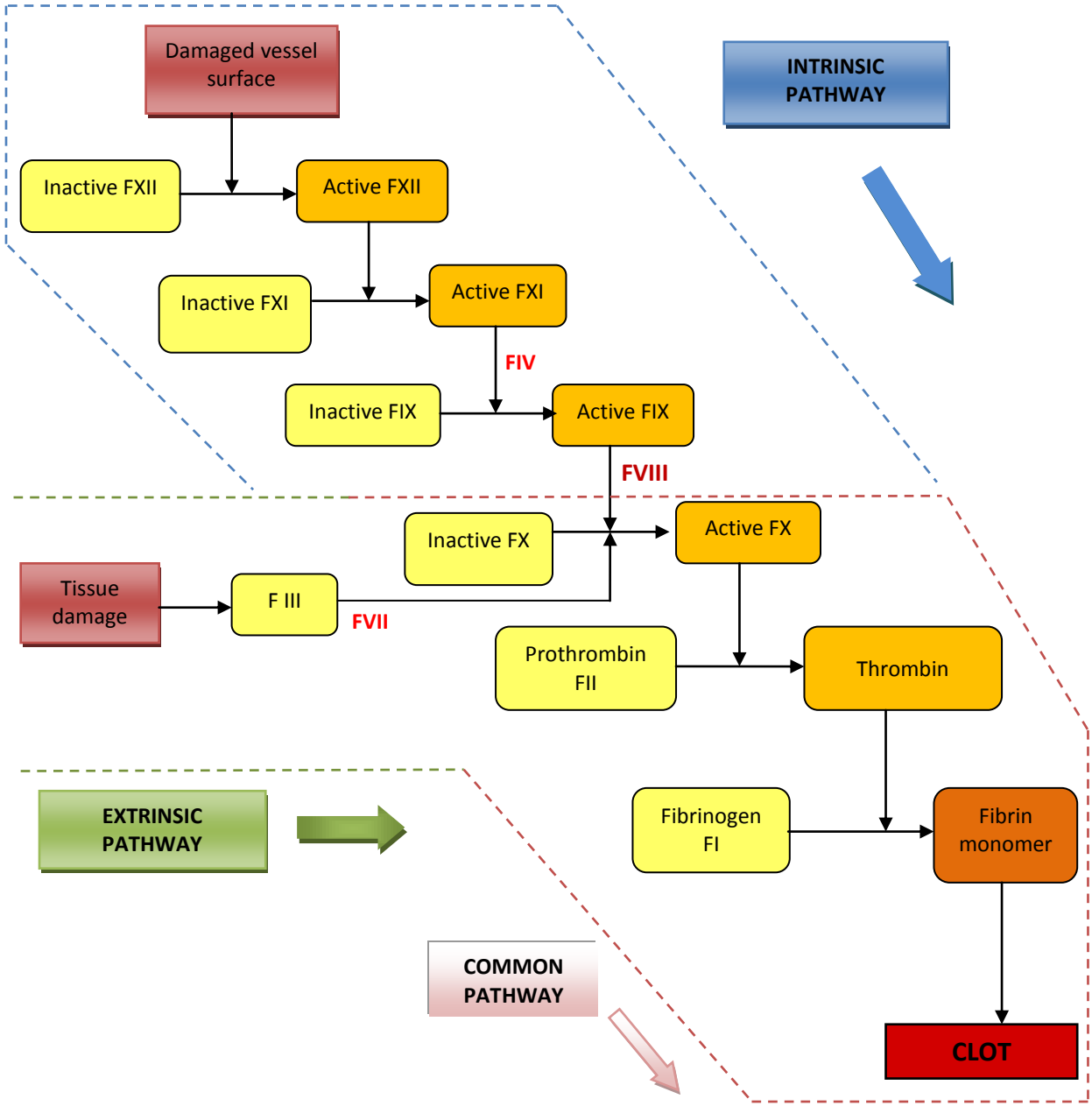


Figure 1.2 Schematic diagram of the coagulation cascade illustrating the role of FVIII (Sherwood, 2007).

1.1.4 Diagnosis of Haemophilia A

To establish a diagnosis of HA in individuals suspected of having a bleeding disorder, coagulation screening tests and FVIII assays are performed. The coagulation screening tests include: platelet count and platelet function analysis or bleeding time, activated partial thromboplastin time and prothrombin time. The above mentioned tests may be within the low to normal range in individuals with HA, therefore, specific coagulation factor assays need to be performed to determine the level of FVIII clotting activity. These include the standard 'one-stage' FVIII activity assay, the 'two-stage' FVIII activity assay and the chromogenic FVIII assay (Konkle et al., 2011).

The most commonly performed assay is the one-stage FVIII activity assay which involves the addition of a diluted patient sample to a FVIII deficient plasma sample which has an excess of all other essential clotting factors, thus allowing one to assess the ability of the patient sample to correct or shorten the delayed clotting of the FVIII deficient plasma. The two-stage FVIII activity assay requires the addition of a normal donor sample to a diluted patient sample with reagents that allow for prothrombinase formation. The prothrombinase levels produced are proportional to the FVIII concentration within the patient sample. The chromogenic assay, which is preferred over the two-stage FVIII assay, involves the addition of a reaction mixture (consists of different factor concentrates except FVIII) to a diluted patient sample as a FVIII source. The clotting activity is calculated and is directly proportional to the level of FVIII in the patient sample (Verbruggen, Meijer, Novakova et al., 2008). Normal FVIII clotting activity ranges between 50%-150% (Konkle et al., 2011). The severity of HA is classified as described in section 1.1.2.

In HA, individuals have FVIII levels usually lower than 30%-35% in the presence of a normal VWF level (Konkle et al., 2011). Very low concentrations of FVIII of approximately 0.2 µg/ml of plasma may be sufficient for procoagulation in normal individuals but a substantial reduction of more than 70%-80% of FVIII plasma levels results in an increased susceptibility to bleeding (Hoyer, 1994).

1.1.5 Molecular pathogenesis

In 1984 the *F8* gene was isolated and sequenced. The gene is located on the distal end of the long arm of the X chromosome (Xq28), spans 186 kb of genomic DNA and comprises 26 exons coding for the FVIII protein (Gitschier, Wood, Goralka et al., 1984).

Since the cloning of the *F8* gene, several studies have been conducted to characterise the molecular basis of HA. The spectrum of *F8* gene mutations published by Tuddenham, Cooper, Gitschier et al. (1991) comprises point mutations, deletions, insertions, gross rearrangements (inversion mutations), frameshift, nonsense and missense mutations. These findings demonstrated the high mutational heterogeneity of the *F8* gene. Small gene defects include point mutations, small insertions, small deletions, frameshift, missense, nonsense and splice site mutations. Gross rearrangements of the *F8* gene include large deletions, the intron 22 inversion (int22) and the intron 1 inversion (int1) mutations (Tuddenham, Schwaab, Seehafer et al., 1994).

Although the disease-causing mutations in the majority of mild and moderate HA patients were identified with early gene sequencing, the underlying molecular cause remained

unknown in a substantial percentage of patients with severe HA (Higuchi, Kazazian, Kasch et al., 1991). Shortly thereafter, the mRNA of the *F8* gene was used to identify the unknown mutations in severe HA patients and almost 50% of these patients were shown to have a common inversion mutation of intron 22 (int22) (Naylor, Green, Rizza et al., 1992).

Figure 1.3 below is an illustration of the mechanism of the inversion of intron 22 of the *F8* gene. As described by Bolton-Maggs & Pasi (2003), within intron 22 of the *F8* gene, there are two additional genes, namely, *F8A* and *F8B*. There are two extragenic homologues of the intragenic *F8A* gene that are positioned at the telomeric end (one proximal and one distal homologue, denoted by the letter A in Figure 1.3). The intron 22 inversion arises through loop formation and homologous recombination between the intragenic homologue (*F8A*) and either the proximal or distal extragenic homologues. This results in an inversion of intron 22 and the *F8* gene is disrupted with introns 1 to 22 being removed from the normal position into an inverted orientation.

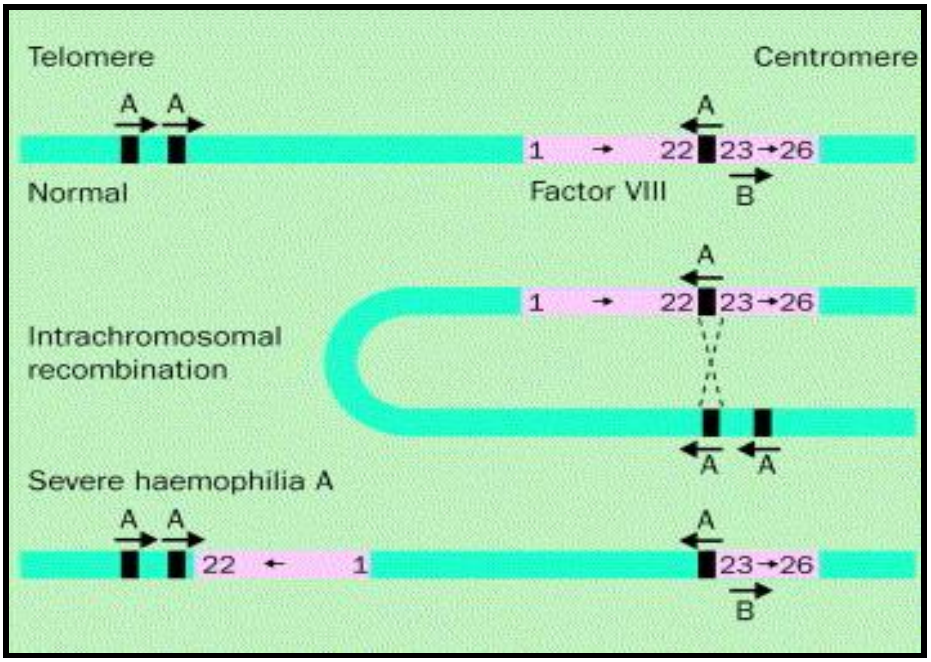


Figure 1.3 Schematic representation of the intron 22 inversion mutation of the *F8* gene (Bolton-Maggs &Pasi, 2003)

Two main types of the int22 mutation were identified by Lakich, Kazazian, Stylianos et al. (1993). A rare variant rearrangement pattern of int22, type 3, has also been observed in approximately 1% of individuals affected with HA (Antonorakis, 1995). (Table 1.2)

Table 1.2 Frequency of the types of int22 mutations in the *F8* gene.

Type of the int22 mutation	Frequency
Type 1 (Distal inversion)	83%
Type 2 (Proximal inversion)	16%
Type 3 (Rare variant rearrangement)	1%

International HA population studies (Andrikovics, Klein, Bors et al., 2003) have since confirmed these findings and recommended that routine screening should be carried out for the int22 mutations in all cases of severe HA. Data published on the *F8* gene mutations identified in a large cohort of HA patients (Margaglione, Castaman, Morfini et al., 2008) revealed that 52% of the severe HA patients had an int22 mutation, thus confirming the similar finding in earlier studies by Andrikovics et al. (2003).

1.1.6 Genotype-phenotype correlation

Although the clinical severity of HA is classified according to FVIII activity, nearly 10-15% of patients with the same causative mutation differ in phenotypic expression and the basis for this difference is not completely understood (Franchini, Montagnana, Targher et al., 2009).

Characterisation of the genotype in severe HA patients in a recent cohort study established and confirmed the int22 mutation to be the common causative mutation (Repesse, Slaoui, Ferrandiz et al., 2011). These findings were later supported by the observations in a single centre cohort study since 55% of the severe HA patients had the common int22 mutation (Gouw, van der Bom, van den Berg et al., 2011).

Further on, a systematic review and meta-analysis was conducted on published data from 30 studies, to determine the relative risks of inhibitor development in relation to the *F8* gene mutation class in patients with severe HA (Gouw, van den Berg, Oldenburg et al., 2012). Figure 1.4 below illustrates the distribution of mutation classes identified in severe HA patients from the above mentioned meta-analysis.

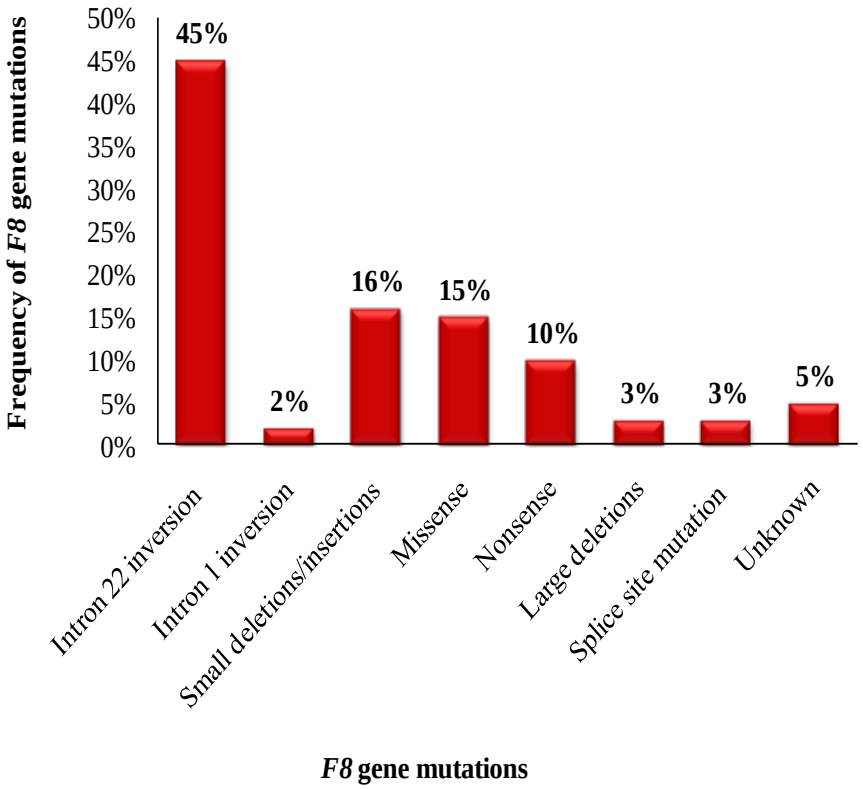


Figure 1.4 Frequencies of *F8* mutation classes in a cohort of severe Haemophila A patients (Gouw et al., 2012).

It has been demonstrated that 50% of patients with severe HA have large *F8* gene defects with the int22 mutation accounting for 45% of these causative mutations, while small gene defects were found in 44% of severe HA patients and approximately 5% of patients had an unknown causative mutation. Similar to previous studies, it was confirmed that the int22 mutation accounts for approximately 45% of the causative mutations in severe HA patients (Gouw et al., 2012).

The second most common mutation found in approximately 2-5% of patients with severe HA is an inversion mutation of int1 of the *F8* gene (Bagnell, Waseem, Green et al., 2002). Bagnell et al. (2002) also demonstrated by FVIII mRNA analysis, that homologous recombination is the causative mechanism for inversion mutations of int1 and int22 of the *F8* gene, as presented previously by Naylor et al (1995).

Generally, it has been established that the majority of moderate and mild HA cases result from small *F8* gene defects and rarely are found to have the int1 or int22 inversion mutations (Miller, Benson, Ellingsen et al., 2012). Although some small gene defects such as splice site and missense mutations are commonly seen in moderate and mild HA cases, they also cause severe HA depending on their location in the *F8* gene and the resultant amino acid change (Margaglione et al., 2008). Therefore, in the majority of severe HA cases large gene defects are found to be the causative mutations whereas moderate and mild HA cases are known to be frequently caused by small gene defects. Ultimately, these study findings were suggestive that some genotype-phenotype correlation does exist in HA.

1.1.7 Management of patients with Haemophilia A

The leading cause of death in affected individuals is intracranial haemorrhage and the major cause of disability in these individuals is chronic joint disease (Luck, Silva, Rodriguez-Merchan et al., 2004). The quality of life and life expectancy of individuals with HA has improved greatly with patient education and surveillance, and FVIII replacement (on-demand and prophylactic) therapy. Research conducted to determine the molecular basis of HA, as well as the structure and function of the FVIII protein, has lead to various developments in the

therapeutic management of HA (Lenting, van Mourik & Mertens, 1998).

1.1.7.1 Intravenous FVIII concentrate infusion therapy

Prior to the availability of intravenous FVIII therapy, the median life expectancy of individuals severely affected with HA was 11 years of age, whereas presently, those patients who receive adequate FVIII therapy have a life expectancy of approximately 63 years (Darby, Kan, Spooner et al., 2007). Not only does adequate FVIII therapy increase the life expectancy of individuals affected with HA, it also improves their quality of life with regard to their physical and mental well being (Gringeri, Mantovani, Scalone et al., 2003).

The intravenous FVIII infusions are either plasma derived or recombinant concentrates that are administered to manage or prevent arthropathy and bleeding episodes promptly (Konkle et al., 2011). Prophylactic intravenous FVIII infusions almost completely prevent primary manifestations of spontaneous bleeding and reduce the number of bleeding episodes in HA patients (Feldman, Pai, Rivard et al., 2006). FVIII replacement therapy is discussed in further detail in section 1.2.2.4. The multidisciplinary approach taken to effectively manage HA patients has proven to have an immense negative economic and social impact on available resources (Gringeri et al., 2003).

1.1.7.2 Complications of FVIII replacement therapy

A critical and challenging complication of repeated FVIII replacement therapy is the production of FVIII neutralising alloantibodies, commonly known as inhibitors (Powell, 2009). These inhibitors neutralise infused FVIII concentrates during the propagation phase of blood coagulation thus affecting the coagulation potential of the FVIII replacement therapy, which compromises the ability to manage bleeding episodes and increases the risk of morbidity and mortality (Hay, Brown, Collins et al., 2006a). Inhibitor formation has been recognised in 15-20% of all HA patients (Powell, 2009). About 20-30% of patients with severe HA have been shown to develop inhibitors against the FVIII protein (Coppola, Santoro, Tagliaferri et al., 2010).

A specialised approach is taken in management of these patients with the aim of reducing inhibitor levels. This includes induction of immune tolerance, which is aimed at eradication of FVIII inhibitors and plasma exchange treatment (Gringeri et al., 2003). Care for patients with inhibitor development involves the infusion of recombinant or plasma derived bypassing coagulation agents to either manage or prevent bleeding episodes or larger doses of FVIII to induce immune tolerance (Kulkarni, Aledort, Berntop et al., 2001). A multicentre prospective study carried out by Gringeri et al in 2003, analysed the cost of care and health related quality of life for patients with HA complicated by inhibitor development. The cost analysis was based on the factors listed in Table 1.3 below.

Table 1.3 Factors affecting the cost of care in patients with Haemophilia A and inhibitor development (Gringeri et al., 2003).

Factors affecting the cost of care
• Number and sites of bleeding episodes • Coagulation factor replacement therapy • Physician visits • Minor and major surgical procedures • Hospitalisations • Concomitant infections • Laboratory and other diagnostic examinations • Rehabilitation procedures

FVIII bypassing agents, such as an activated prothrombin complex concentrate, are able to bypass the factor VIII–dependent step in the coagulation cascade (refer to Figure 1.2) and promote haemostasis by enhancing thrombin/clot generation (Astermark, Donfield, DiMichele et al., 2007). The expense associated with such bypassing agents is considerable and is a significant liability to the patients, families and haemophilia care centres. Results of this study provided support for previous retrospective studies which showed that there is a higher amount of resources used in comparison to inhibitor negative HA patients, with therapeutic agents being the most costly aspect of care.

1.1.7.2.1 Factor VIII protein inhibitors

Inhibitor production is a complex multifactorial interaction that involves the degradation of FVIII by antigen presenting cells which is then recognised by $CD4_+$ T cells. This leads to $CD4_+$ cell mediated synthesis of FVIII antibodies in the B cells. Up and down regulation of antibody production by the B cells depends on the $CD4_+$ T cell subsets which determine the pathogenicity of the FVIII inhibitors (Astermark, Lacroix-Desmazes & Reding, 2008). These inhibitory antibodies neutralise plasma FVIII protein by either preventing FVIII-VWF complex formation and binding of FVIII protein to the phospholipid membranes of injured cells or by preventing activation of FVIII by other protein factors in the coagulation cascade (Krudysz-Amblo, Parhami-Seren, Butenas et al., 2009).

1.1.7.2.2 Quantification of FVIII inhibitors

Identification and quantification of FVIII inhibitors is done using the Nijmegen modified Bethesda assay (Kasper, Aledort, Aronson et al., 1975). One Bethesda unit (BU) represents the amount of antibody in patient plasma that neutralises 50% of FVIII activity in normal plasma (Kasper et al., 1975). A titre of 0.6 BU/mL or higher is reported as inhibitor positive. Low titre inhibitors are below 5 BU/mL and high titre inhibitors are above 5 BU/mL of plasma (White, Rosendaal, Aledort et al., 2001). The inhibitor phenotype is determined by the inhibitor titre which is either low or high (Oldenburg, Schroder, Brackmann et al., 2004). The Nijmegen modified Bethesda method was found to have a lower sensitivity, especially to low-titre inhibitors. A fluorescence-based immunoassay with a higher specificity and sensitivity to low-titre and non-neutralising FVIII inhibitors has recently been developed (Krudysz-Amblo et al., 2009).

1.1.7.3 Surveillance of patients with Haemophilia A

Surveillance of individuals diagnosed with HA includes follow up at health care centres to review their history (signs and symptoms) of bleeding episodes, to evaluate their joints and muscles, to monitor for infectious diseases such as the hepatitis C virus and the human immunodeficiency virus (HIV) which may be transmitted via the FVIII infusion therapy, to screen for inhibitor development, adjust management if necessary and provide continual education to the patient and their care givers. In addition, a very important aspect of management in HA is genetic counselling which is offered to the patient and their family members to improve understanding of this genetic condition as well as to provide information to at risk relatives (Konkle et al., 2011).

1.1.8 Haemophilia A in South Africa

On clinical suspicion of a bleeding disorder from a comprehensive bleeding history and physical examination, the following screening tests are performed as warranted: platelet counts, international normalised ratio (INR) and activated partial thromboplastin time (aPTT). The following confirmatory tests are then performed: specific factor assays, inhibitor assays, platelet function tests and von Willebrand factor assays if required (Mahlangu & Gilham, 2008). FVIII inhibitor titres are quantified in Bethesda units using the Nijmegen modified Bethesda assay (Mahlangu & Gilham, 2008).

Diagnostic mutation testing for HA is offered by the Molecular Genetics Laboratory, Division of Human Genetics, of the National Health Laboratory Services (NHLS) and School of

Pathology, University of the Witwatersrand, Johannesburg. The NHLS conducts HA diagnostic mutation testing for the majority of South Africa. Mutation testing, since 1997, has predominantly involved testing for the int22 mutation in the *F8* gene by the Southern blot technique in moderate and severe HA patients. A PCR technique has recently been introduced (2011) to detect the int22 mutation in the *F8* gene. If the int22 mutation is not detected in the proband, then linked marker analysis is undertaken in the family to identify the high risk X chromosome. More recently, further mutation analysis to detect the *F8* gene inversion mutation in int1 by PCR analysis and mutations in exon 14 by sequencing has been offered. The majority of mutations have been found in exon 14 as this is the largest exon in the *F8* gene and accounts for close to 49% of mutations (Mitchell, 2009).

It has been found that the int22 mutation accounts for approximately 32% of *F8* gene mutations in white patients with severe HA and 43% in black patients with severe HA (Mitchell, 2009). The presence of a founder Afrikaner mutation, in exon 14 of the *F8* gene, in approximately 14% of severe white HA patients was also reported by Mitchell in the same study (2009).

With regard to treatment of patients with HA, plasma derived FVIII concentrate is used predominantly as it is more cost effective and available than recombinant FVIII concentrates. Of significance, the majority of blood donors in South Africa are of white ethnicity (verbal communication: Professor J. Mahlangu).

1.2 FACTORS INFLUENCING INHIBITOR DEVELOPMENT

The predisposing factors to inhibitor development still remain unclear, but a survey conducted with the objective of developing a clinical prediction model for inhibitor development concluded that both genetic and environmental factors increased the risk of inhibitor development in patients with severe HA (van den Berg & Chalmers, 2009).

1.2.1 Genetic factors predisposing to inhibitor development

The genetic or non-modifiable risk factors are represented in Figure 1.5.

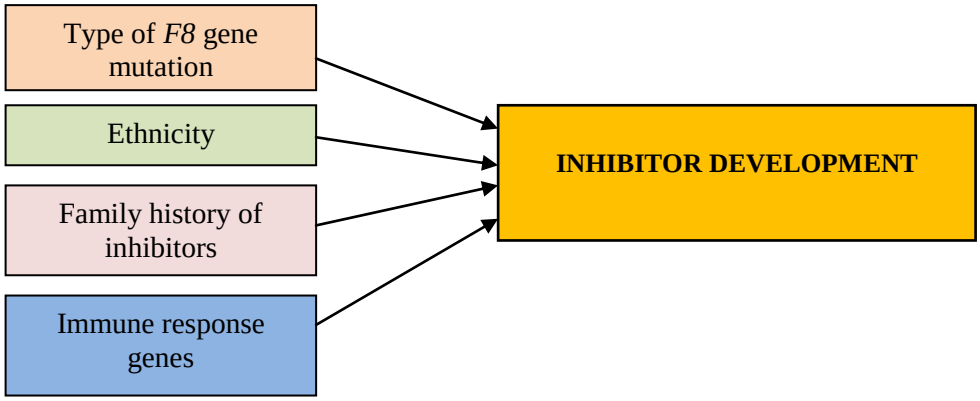


Figure 1.5 Genetic factors influencing inhibitor development in Haemophilia A patients (Maclean et al., 2011).

1.2.1.1 *F8* gene mutation type

The type and location of the *F8* gene mutation has been proven to be a significant risk factor and an influential determinant in the development of clinically relevant inhibitors (Gianelli & Brownlee, 1986; Gouw et al., 2011).

It was established that HA patients with severe *F8* gene mutations (e.g. large deletions, nonsense mutations, int22 mutation), known as null mutations, have an up to ten times higher incidence of inhibitor development in comparison to those with less severe *F8* gene defects (e.g. missense mutations, small deletions, small insertions, splice-site mutations) (Astermark, Berntop, White et al., 2001; Oldenburg, El-Maarri & Schwaab, 2002). Severe *F8* gene mutations were also shown to confer a significant risk for inhibitor development in comparison to other variables such as treatment related factors (Maclean et al., 2011).

The int22 inversion mutation has been shown to be the most prevalent mutation in patients with severe HA (Gouw et al., 2011) and between 21% to 34% of patients positive for the int22 mutation have reported inhibitor development (Saint-Remy, Lacroix-Desmazes, Oldenburg, 2004; Schroder, El-Maarri, Schwaab et al., 2006). Although previous studies in different ethnic groups reported an overall inhibitor prevalence of 9% for the int1 mutation, more recent studies have shown a much higher prevalence of 26% (Schroder et al., 2006; Gouw et al., 2012).

To explain this increased risk of inhibitor development, it has been postulated that severe *F8* gene defects result in either complete absence or lower circulating endogenous FVIII levels in

comparison to less severe gene defects which result in higher endogenous FVIII levels. Ultimately the exogenous FVIII may lead to an immune response in patients with severe gene defects and immune tolerance induction in patients with less severe gene defects (Gouw et al., 2011).

1.2.1.2 Ethnicity

Earlier studies observed a two-fold higher incidence of inhibitor development in African-American HA patients compared to white HA patients (Astermark et al., 2001). A recent study has provided a possible explanation for this phenomenon. Four nonsynonymous single nucleotide polymorphisms (SNPs) were identified in the *F8* gene: one in exon 10 (G1679A), two in exon 14 (A2554G and C3951G), and one in exon 25 (A6940G). These four SNP's encode four amino acid substitutions and their resultant amino acid residues as represented in Table 1.4 A. The naturally occurring allelic combinations (haplotypes) of these four SNPs encode six wild type FVIII proteins, named H1 through to H6 as represented in Table 1.4 B. The combination of either of the amino acid residues at positions 484 (R or H), 776 (R or G), 1241 (D or E) or 2238 (M or V), results in one of these six haplotypes (Viel, Ameri, Abshire et al., 2009). Although the six haplotypes are combinations of the four non-synonymous SNP'S, the nomenclature or numbering system used to designate the six wild-type FVIII proteins is their amino acid residue locations (Viel et al., 2009).

A segment of DNA at a given position on a particular chromosome is known as a locus. Alternative variants of a gene at a particular locus are known as alleles, which may be a wild-type (common) allele or a variant (mutant) allele. A set of closely linked alleles at a given loci

or cluster of loci are referred to as a haplotype and these alleles tend to be inherited together and are not likely to be separated by recombinant events (Nussbaum, McInnes, Willard, 2007).

Table 1.4 *F8* gene non-synonymous SNP's, their amino acid substitutions, residues, haplotypes and wild-type FVIII proteins (Viel et al., 2009).

A

Non-synonymous SNP's	Amino acid substitution	Amino acid residue
G1679A	Arg484His	R484H
A2554G	Arg776Gly	R776G
C3951G	Asp1241Glu	D1241E
A6940G	Met2238Val	M2238V

B

Haplotype	Wild-type FVIII protein
H1	R-R-D-M
H2	R-R-E-M
H3	R-R-E-V
H4	H-R-E-M
H5	R-R-D-V
H6	R-G-E-M

The H1 and H2 haplotypes are found amongst all racial groups and in recombinant FVIII (rFVIII) concentrates. Only haplotypes H1 and H2 are found in the white population while H3, H4 and H5 are found in the black population only. The H6 haplotype was only found in people of Chinese descent. It was suggested that mismatched FVIII replacement therapy could be a predisposing risk factor for inhibitor development. Replacement therapy could therefore increase the risk of inhibitor development if the HA patient is of a different ethnicity to the blood donor due to *F8* gene haplotype variation (Viel et al., 2009).

Though it has been proposed that black patients have a higher inhibitor prevalence due to *F8* gene haplotype mismatch therapy (Viel et al., 2009), recent findings from two large cohort studies did not support this hypothesis as black patients with the H1 haplotype showed a significantly higher inhibitor prevalence than white patients with the same haplotype, suggesting that the *F8* gene haplotype may not play a role in inhibitor development (Miller et al., 2012; Schwarz, Astermark, Menius et al,. 2012).

1.2.1.3 Family history of inhibitors

Although a significant risk of inhibitor development of 48% has been reported in families with a positive inhibitor history and other family studies have shown a higher inhibitor concordance (either high- or low- responding inhibitors) in siblings in comparison to extended relatives and unrelated patients (Gill, 1999; Astermark et al., 2001), concordance is not 100% (Hay, 2006b). An overall inhibitor concordance between siblings with severe HA was found to be over 78%. Concordance of inhibitor development between first degree relatives with the int22 mutation

was reported to be close to 64% in the Malmo International Brother Study (Astermark, Oldenberg, Escobar et al., 2005).

1.2.1.4 Immune response genes

The observation of discordance among first degree relatives implies that other variables (genetic/environmental) are involved in increasing the risk profile of the HA patient with regard to inhibitor development. It was postulated that genetic variations in the immune system (major histocompatibility complex human leukocyte antigen genes) may be a contributing factor since the causative genetic defect is expected to be similar in siblings and extended relatives (Chambost, 2010). These genetic markers or variations merit further studies in order to identify at risk family members (Astermark et al., 2005). Recently, polymorphisms in different immunoregulatory genes were found to be determinants in risk modification of FVIII inhibitors by either increasing or decreasing risk in HA patients (Lozier, Rosenberg, Goeder et al., 2011).

1.2.2 Environmental factors predisposing to inhibitor development

The environmental or modifiable non-genetic risk factors associated with inhibitor development are illustrated in Figure 1.6.

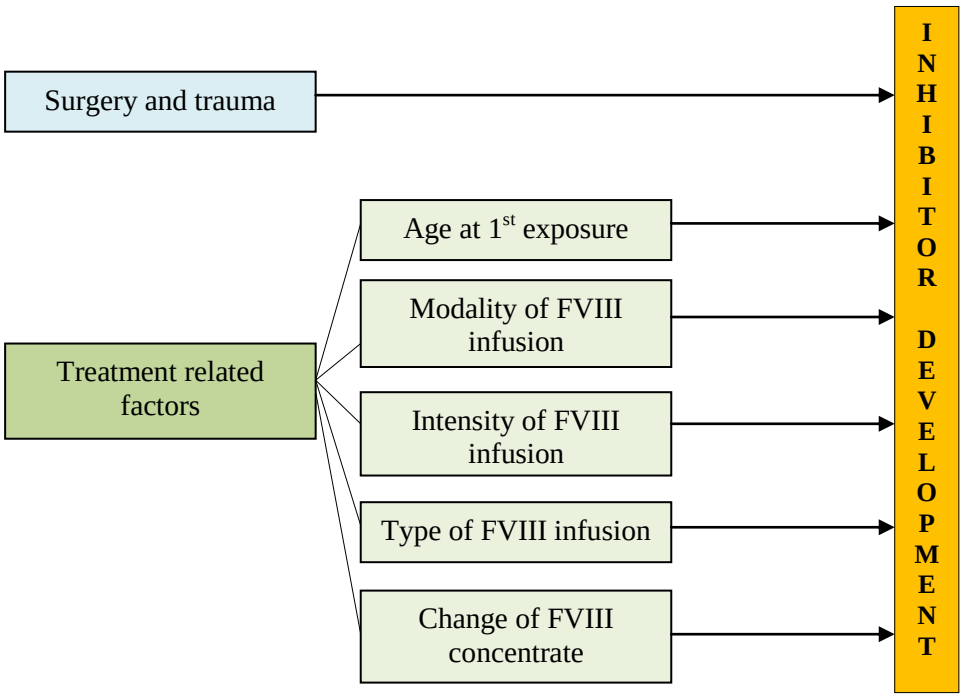


Figure 1.6 Non-genetic risk factors influencing inhibitor development in Haemophilia A patients (Chambost, 2010).

1.2.2.1 Early exposure to FVIII replacement therapy

An assessment of the relationship between age of onset of FVIII therapy and the incidence of inhibitors in severe HA patients revealed that a higher incidence occurred in patients starting therapy early in life, except neonates, as they tend to develop an immune tolerance (Lorenzo, Lopez, Altisent et al., 2001; van der Bom, Mauser-Bunschoten, Fischer et al., 2003). A similar trend was observed in the CANAL cohort study (Gouw et al., 2007b). In the above mentioned study (Gouw et al., 2007b) patients with inversion mutations of the *F8* gene were excluded. Hence further review of the literature was conducted which had shown that there was a higher inhibitor incidence with early exposure to FVIII therapy despite *F8* gene mutation type and interestingly children with the int22 mutation and other severe molecular defects of the *F8* gene have a significantly higher incidence of high titre inhibitors and they required earlier onset of FVIII therapy than those with less severe *F8* gene defects (Chalmers, Brown, Keeling et al., 2007).

Overall, in the above mentioned studies, a correlation was shown to exist between early exposure to FVIII therapy and inhibitor incidence in severe HA patients in that early exposure to FVIII therapy predisposes patients to inhibitor development.

1.2.2.2 Prophylactic FVIII replacement therapy

Several studies have explored the effects of prophylactic treatment on inhibitor risk and the overall observations provide evidence that patients on prophylaxis have a lower risk of developing inhibitors than those receiving on-demand therapy (Morado, Villar, Jimenez et al.,

2005). A 60% reduction in inhibitor prevalence was reported in the CANAL cohort study and other similar studies (Gouw, van der Bom, van den Berg et al., 2007b; van den Berg & Chalmers, 2009; Zakarija, Harris, Rademaker et al., 2011) which indicates that an early prophylaxis regimen avoids immunological danger signals (events that trigger an immune response such as trauma, infection, vaccinations, bleeding episodes) and rather allows for the development of immune tolerance to FVIII protein (Kurnik, Bidlingmaier, Engl et al., 2010).

Not only is the inhibitor prevalence reduced with prophylactic FVIII therapy but it has also been proven to show marked reduction in the frequency of haemarthrosis with a better quality of life for HA patients (Hoots, Ebbesen, Konkle et al., 2008).

1.2.2.3 Intensity of FVIII replacement therapy

The intensity of treatment involves the dose and frequency of FVIII infusions as well as the administration in times of trauma or surgical procedures as prophylaxis. A four fold increased risk of inhibitor development has been observed after intensive exposure to exogenous FVIII in mild HA patients (Sharathkumar, Lillicrap, Blanchette et al., 2003) and this association was emphasised by another study (Gouw et al., 2007). It therefore was proposed that early low-dose prophylaxis in a controlled clinical setting may considerably reduce the prevalence of inhibitors (Kurnik et al., 2010).

1.2.2.4 Plasma derived and recombinant FVIII concentrates

Over a ten year period (1990–2000), the CANAL cohort study was performed among severe HA patients who had received either rFVIII or plasma derived FVIII (pdFVIII) therapy.

According to the data analysed, it was concluded that inhibitor development was not statistically different between the two types of therapy administered, but that some pdFVIII products may provide a lower risk for inhibitors. It was also shown that intensive exposure to FVIII therapy increases the risk of inhibitors (Gouw, van der Bom, Auerswald et al., 2007a). Although studies have been performed in heterogeneous study populations to investigate whether rFVIII concentrates pose a higher risk for inhibitor development than pdFVIII concentrates, it still remains unclear and controversial as to whether FVIII product types have an effect on inhibitor development (Ettingshausen and Kreuz , 2006). More recent studies support these findings; however, there appears to be ambiguity and discrepancies that exist in present and past studies (Franchini, Tagliaferri, Mengoli et al., 2011; Maclean et al., 2011).

Overall, a complete understanding of the genetic and environmental predisposing factors to inhibitor development remains uncertain. It is essential to identify HA patients with a high risk profile to developing inhibitors so that individualised treatment regimens may be offered. In turn, management strategies would improve patient surveillance and care, minimise incidence of inhibitor formation and optimise therapeutic benefits.

1.3 MOTIVATION FOR RESEARCH

At present in South Africa, there are no current published data on the characteristics of the cohort of South African HA patients or the prevalence of inhibitor development to the FVIII infusion therapy currently being used to manage HA patients. Therefore, there exists a definite need for studies to be conducted amongst a local South African HA cohort to determine the correlation between inhibitor production and some of the above mentioned genetic and environmental predisposing risk factors.

Insight into the predisposing factors to inhibitor formation is of considerable value in order to predict which patients are at high risk of developing inhibitors, to develop a clinical prediction model for inhibitor development and to provide appropriate management of bleeding episodes.

The information generated from such a study could also have important implications for the type of FVIII replacement therapy used in the management of South African HA patients.

1.4 AIMS

The study aimed to characterise a South African HA cohort of patients, all of whom had int22 mutation testing, and investigate some of the genetic factors that influence inhibitor development.

1.5 OBJECTIVES

1) To characterise the cohort of South African HA patients in terms of clinical severity, ethnicity, int22 mutation status and inhibitor development.
2) To explore whether genetic factors (ethnicity, int22 mutation status, *F8* gene haplotype) influence inhibitor development.

CHAPTER 2: SUBJECTS AND METHODS

An existing cohort of HA patients in the Division of Human Genetics, National Health Laboratory Services (NHLS) in Johannesburg, South Africa, formed the basis for this study. This was a retrospective study that involved the analysis of patient files, HA molecular and clinical databases and prospective molecular analysis of the *F8* gene haplotypes. Some clinical data were obtained from the patients or the referring clinicians. This chapter describes the subjects; the inclusion and exclusion criteria used to achieve the desired study sample, and describes the methods implemented to accomplish the objectives of this study. An application for ethics clearance was submitted to the Human Research Ethics Committee (Medical), Faculty of Health Sciences, University of the Witwatersrand, and approval for this study was granted: reference number M10248 (Appendix A).

2.1 SUBJECTS

2.1.1 Source of data

A molecular database of HA patients has been compiled at the Division of Human Genetics NHLS. This database includes details of South African patients who have had testing for the common int22, int1 and exon 14 mutations in the *F8* gene. All *F8* gene mutation analysis has been performed at the Molecular Genetics Laboratory, Division of Human Genetics, NHLS and School of Pathology, the University of the Witwatersrand, Johannesburg. Table 2.1 summarises the various sources from which data were gathered.

Individuals who have *F8* gene mutation analysis are assigned a HA file code and are entered into the molecular database. The files of related individuals are linked to the same HA file code. This molecular database of HA patients only contains mutation analysis information and does not contain clinical information on the severity of disease, ethnicity and FVIII inhibitor status. Many of these patients have also been counselled by genetic counsellors (Division of Human Genetics, NHLS, Johannesburg) and the clinical information obtained is captured into a clinical HA database.

Therefore, a retrospective genetic counselling file and clinical database analysis was also conducted to obtain the clinical information required for this study. The genetic counselling files and database contains information on the affected individuals' severity of disease, ethnicity, family pedigree and notes documented during the genetic counselling session.

Clinical management and FVIII inhibitor status of affected individuals is not always recorded in the counselling files. Genetic counselling file records are kept at the Division of Human Genetics, NHLS and are stored in a separate alphabetically organised filing cabinet.

When information on FVIII inhibitor status could not be obtained from either the genetic counselling file or database, the medical professional involved in management of the proband, family members of the affected proband or the affected proband themselves were contacted telephonically to gather this information (Table 2.1).

Table 2.1 List of the sources used for data collection

Sources of data
❖ Molecular database at the Division of Human Genetics, NHLS, Johannesburg
❖ Genetic counselling database at the NHLS, Division of Human Genetics, Johannesburg
❖ Genetic counselling patient files
❖ Medical professionals involved in clinical management of the HA patients from provincial clinical departments and private medical practices in South Africa
❖ Family members involved in the care of an individual affected with HA
❖ From the individual affected with HA

2.1.1.1 Inclusion criteria

The inclusion criteria to achieve the desired study sample allowed for selection of individuals who had been clinically diagnosed with HA by a health care professional and had molecular analysis of their *F8* gene performed between the January 1994 and April 2011. Patients classified with mild, moderate and severe HA were incorporated into the study cohort.

Only a small number of individuals of Asian, Indian and Coloured ancestry were recorded in the databases and they were as a result excluded from the study. Therefore, only white and black affected male probands were included in this study in order to investigate if ethnicity, as a genetic factor, may play a role in inhibitor development. One affected male proband per family was included in the study. The proband selected was the first male in a family who had diagnostic *F8* gene mutation analysis.

There were a total of 861 entries in the molecular database at the Division of Human Genetics, NHLS in Johannesburg. The entries included male and female individuals that had *F8* gene mutation analysis for the int22 mutation, as well as samples for prenatal diagnostic testing. (Figure 2.1)

2.1.1.2 Exclusion criteria

The exclusion criteria listed in Table 2.2 were applied to the individuals recorded on the HA molecular database and from the file based review.

Table 2.2 List of criteria used to exclude patients from the study

Exclusion criteria
• Individuals tested before January 1994 and after April 2011 • Unaffected and affected family members of a proband • Females tested • Prenatal diagnostic samples • Individuals who were not white or black

The selection process used to achieve the final study sample is depicted in Figure 2.1.

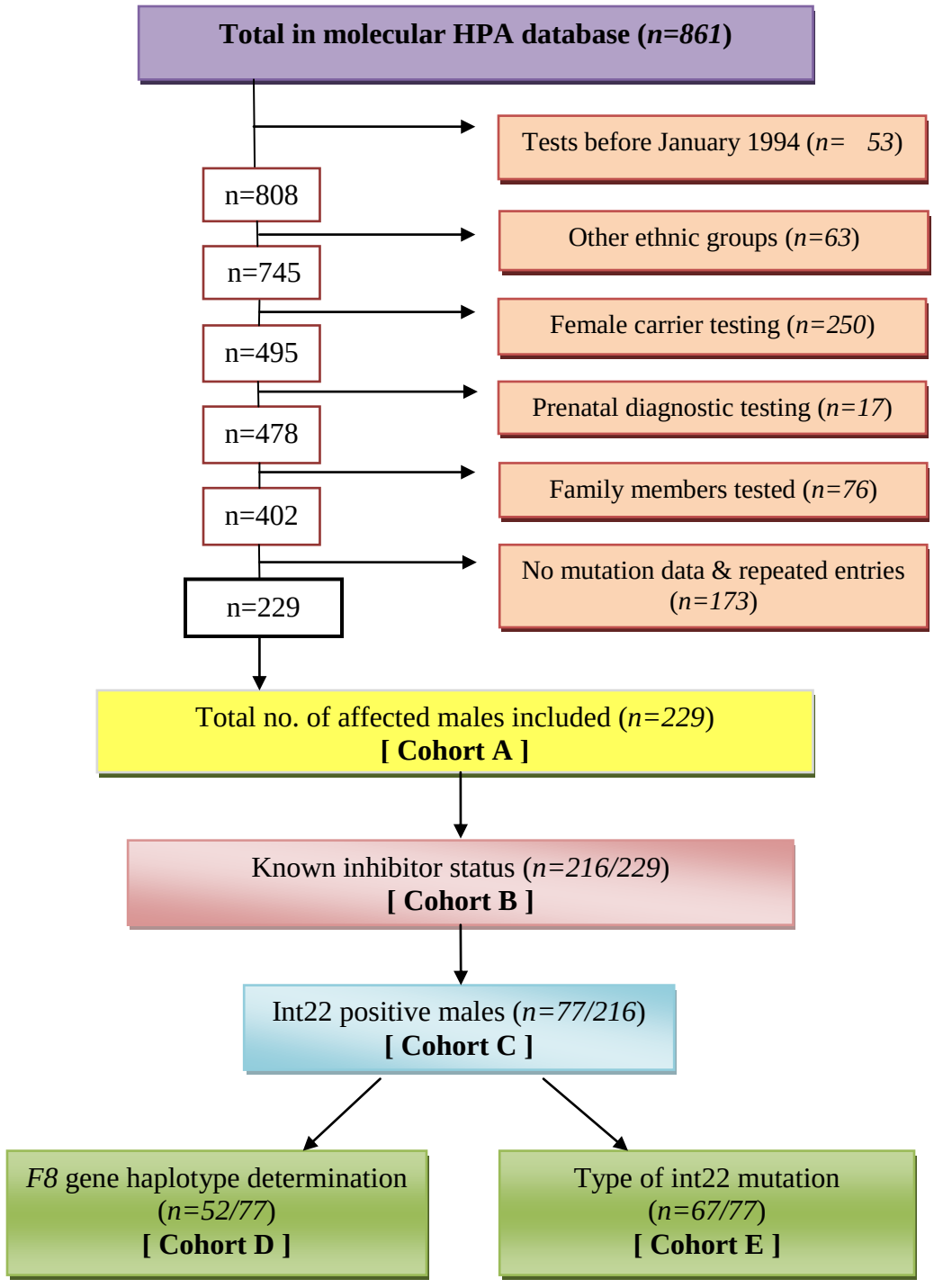


Figure 2.1 Flow diagram of the study sample selection process

2.2 METHODS

2.2.1 Data collection

A data collection sheet (Appendix B) was compiled in an Excel spreadsheet to record the relevant information from the different data sources listed in Table 2.1.

2.2.1.1 File and database review

The HA molecular database review involved selection of black and white male probands who met the inclusion criteria mentioned in section 2.1.2. Data were collected on the 229 male probands that were selected for the study. These 229 probands were assigned to Cohort A. Their int22 mutation status was recorded as either positive (including int22 mutation type) or negative in the data collection sheet.

Thereafter, a file based review was performed on the 229 male probands that were selected from the HA molecular database. The file based review involved collection of information pertaining to the severity of disease, ethnicity and FVIII inhibitor status if documented. The information gathered was then used to accomplish the first objective of this study, namely, to characterise the local cohort of South African HA patients.

As there was a lack of information on inhibitor status on numerous patients, medical professionals involved in the care of the male probands from the following hospitals were contacted telephonically and via electronic mail in an attempt to obtain this information:

- Charlotte Maxeke Johannesburg General Hospital
- Steve Biko Academic Hospital
- Chris Hani Baragwanath Hospital
- George Mukhari Hospital, Garankuwa
- Tygerberg Hospital
- Groote Schuur Hospital
- Red Cross Children's Hospital
- Pietersburg Hospital
- Universitas Hospital
- Private General Practitioners

Of the 229 (Cohort A) patients included in the study, information on inhibitor development was obtained on 216 (Cohort B). A total of 82 of the 229 probands were positive for the int22 mutation but inhibitor status was only known in 77 of the 82 probands. These 77 probands were assigned to Cohort C. Two separate analyses were conducted on Cohort C, firstly prospective *F8* gene haplotype determination was performed on 52 probands (refer to section 2.2.1.2) and these probands were assigned to Cohort D and secondly the type of int22 mutation was determined from the molecular database and the counselling files and these probands were assigned to Cohort E.

2.2.1.2 *F8* gene haplotype determination

Of the 77 int22 positive probands selected for *F8* gene haplotype determination (Figure 2.1), 25 probands were excluded as the stored DNA specimens were either of insufficient quantity or of poor quality. Only the int22 positive patients were used for this analysis due to insufficient availability of data for the int1 and exon 14 positive patients. Therefore a total of 52 probands (Cohort D) had *F8* gene haplotype analysis done. The *F8* gene haplotype analysis was performed at the Human Genetics Molecular Genetics Laboratory, NHLS in Johannesburg by a medical scientist (research assistant).

Anonymized *F8* gene analysis was therefore performed on 52 of the 82 int22 positive probands. Four known nonsynonymous SNPs in the *F8* gene (Viel et al., 2009), one in exon 10 (G1679A; p.Arg484His), two in exon 14 (A2554G; p.Arg776Gly and C3951G; p.Asp1241Glu) and one in exon 25 (A6940G; p. Met2238Val), were genotyped to determine the FVIII haplotype in the int22 positive probands. The mutation nomenclature assigned to the four nonsynonymous SNPs are based on their nucleotide positions in the *F8* gene complementary DNA (Viel et al., 2009).

Each DNA specimen was subjected to four polymerase chain reactions (PCR) using a Tetra-primer ARMS-PCR technique to genotype each of the four SNP's. The alleles were differentiated based on the PCR product size. Since only male patients were screened and the *F8* gene is located on the X chromosome, a hemizygous allele call was expected. The combination of the four alleles yielded a haplotype. The technique applied for the SNP

analysis was adapted from Ye, Dhillon, Ke et al. (2001).

2.2.2. Data analysis

The data collected, as described in section 2.2.1.1 and 2.2.1.2, were exported into an Excel spreadsheet (Appendix B). As this was a quantitative and comparative study, the frequencies and p-values of the captured data were calculated using the STATISTICA program version 10.0 MR1. The Fisher's Chi-squared test was used with a confidence interval of 95% to determine significance and probability values (a p value of <0.05 was taken to indicate significance). All percentage and frequency calculations were rounded off to the nearest whole percent. A statistician from the Faculty of Health Sciences, the University of the Witwatersrand, was consulted to verify the statistical techniques employed.

2.2.2.1 Characterisation of the cohort of South African HA patients

Cohort A was characterised according to the severity of HA, ethnicity, int22 mutation status, type of int22 mutation and inhibitor development. The process of characterisation is demonstrated in Figure 2.2.

2.2.2.2 Comparative analysis of the cohort of South African HA patients (Cohort B, C, D, E)

Further comparative statistical analysis was performed on the data collected using the Chi-squared test, to determine the influence of the various genetic characteristics on FVIII inhibitor development in the cohort of South African HA patients. As mentioned in section

2.2.1.1, only 216 (Cohort B) of the 229 male probands had known inhibitor statuses. Therefore, only these 216 probands were included in the comparative analysis between int22 mutation positive and int22 mutation negative black and white patients. This was performed to ascertain whether *F8* gene mutation type and ethnic background influences inhibitor development in the cohort of South African black and white HA patients. Figure 2.2 also illustrates the process of data collection for the comparative analysis of Cohort B, C, D and E.

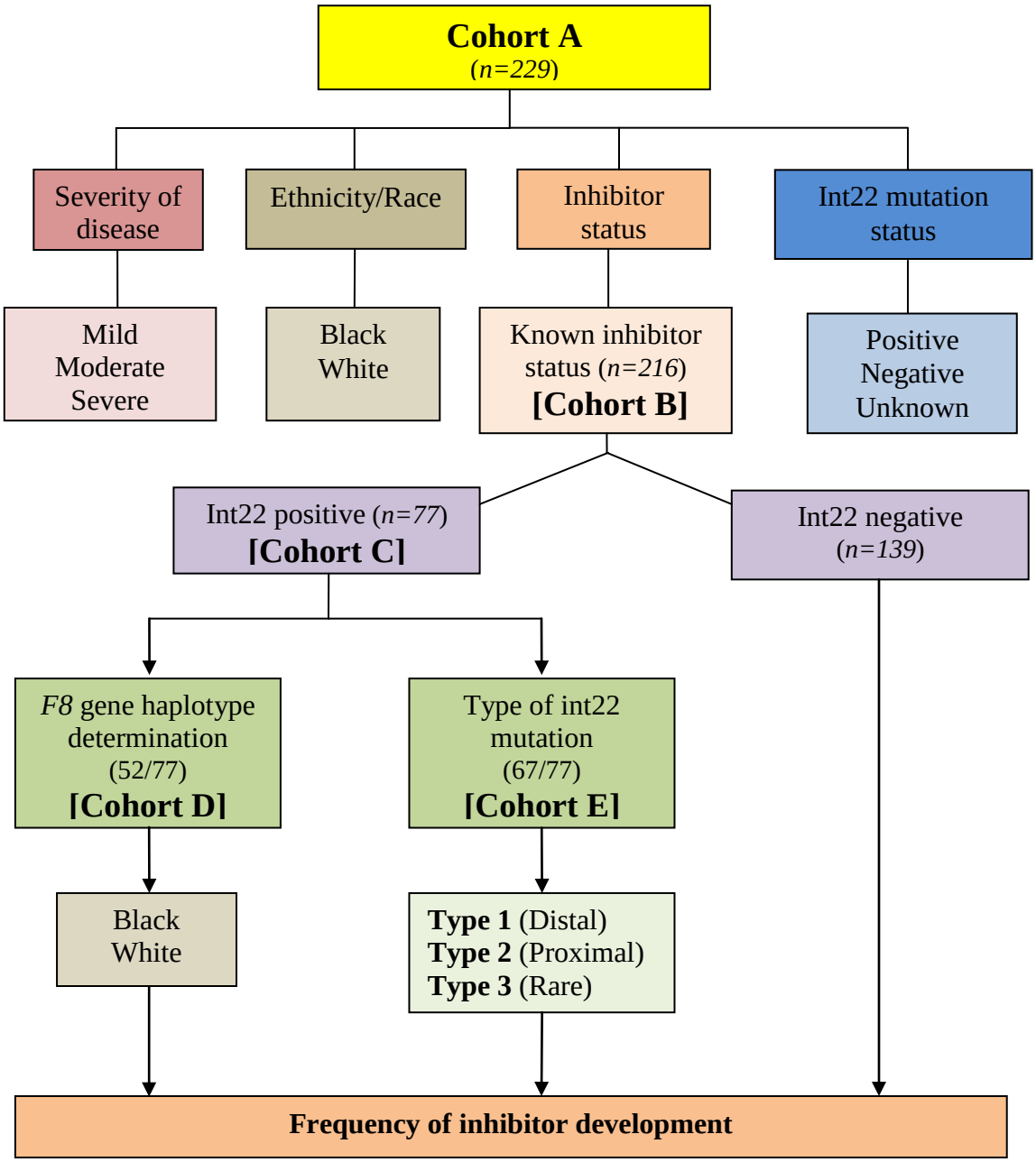


Figure 2.2 Flow diagram of the process of characterisation of the cohort of South African Haemophilia A patients and the methods used to determine the frequency of inhibitor development in black and white probands.

CHAPTER 3: RESULTS

From the patient file, as well as the molecular and clinical database review, a total of 229 male probands met the inclusion criteria for the present study. The results generated from the data analysis are presented in two main sections within this chapter. The first section includes results obtained from characterisation of Cohort A and the second section includes results obtained from a comparative analysis between the genetic factors and inhibitor development in Cohorts B, C, D and E.

As mentioned earlier (section 2.2.1.2), the 229 male probands were assigned to Cohort A. Of these 229 male probands, 216 had known inhibitor status and were assigned to Cohort B. Of the 216 probands in Cohort B, 77 were reported to be positive for the int22 mutation with known inhibitor status and were assigned to Cohort C and 52 of these probands were assigned to Cohort D for determination of their *F8* gene haplotype and 67 of the probands from Cohort C were assigned to Cohort E to assess their type of int22 mutation.

3.1 CHARACTERISATION OF THE COHORT OF SOUTH AFRICAN HAEMOPHILIA A PATIENTS

Cohort A (n=229) was characterised according to their severity of disease, ethnicity, int22 mutation status and inhibitor status. The information required to characterise Cohort A was obtained from all of the sources listed in Table 2.1.

3.1.1 Severity of Haemophilia A

The proportion of individuals affected with mild, moderate and severe HA in Cohort A is illustrated in Figure 3.1. Of the 229 individuals, the majority had severe HA (207/229) followed by mild (12/229) then moderate HA (10/229).

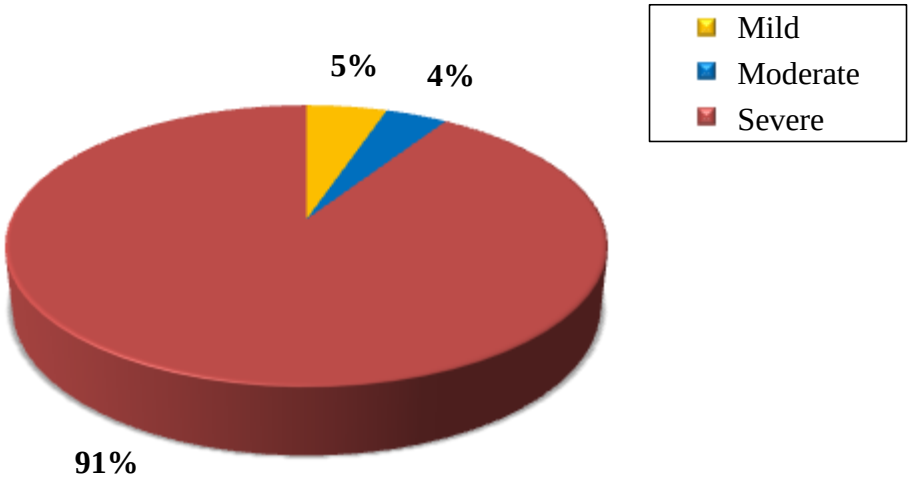


Figure 3.1 Percentage of severities of Haemophilia A in Cohort A.

3.1.2 Ethnicity

There were a similar proportion of black and white patients in Cohort A. One hundred and sixteen (51%) of the 229 individuals affected with HA were identified to be black and 113/229 (49%) were white.

3.1.3 Intron 22 inversion mutation status

Figure 3.2 is a graphical representation of the frequency of int22 mutation positive and negative male probands in Cohort A. Eighty two (36%) of the 229 male probands in cohort A were found to be positive for the int22 mutation, irrespective of ethnicity. The remaining 147 (64%) of the 229 male probands were int22 negative.

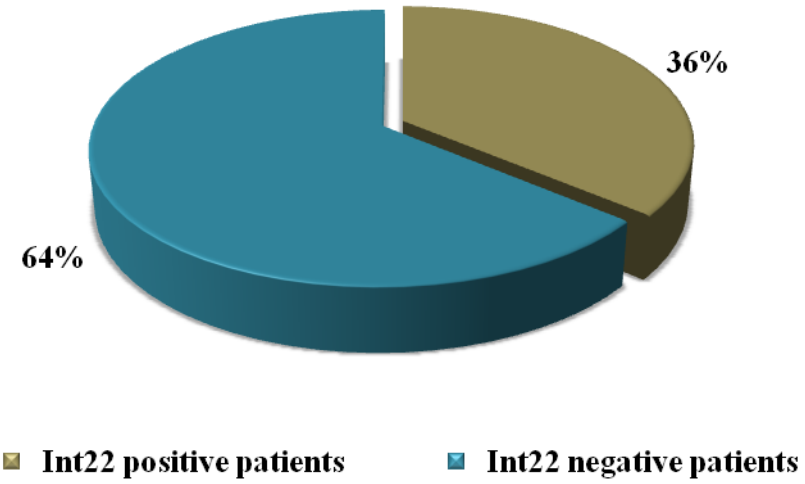


Figure 3.2 Percentage of the int22 mutation in Haemophilia A patients in Cohort A.

3.1.4 Inhibitor development

Figure 3.3 represents the frequency of inhibitor development in patients from Cohort A (n=229). The majority of probands had a known inhibitor status (94%; 216/229) and these patients were used to accomplish the second objective of the study (Cohort B) (refer to section 1.5). An overall frequency of 13% inhibitor development was observed in Cohort A.

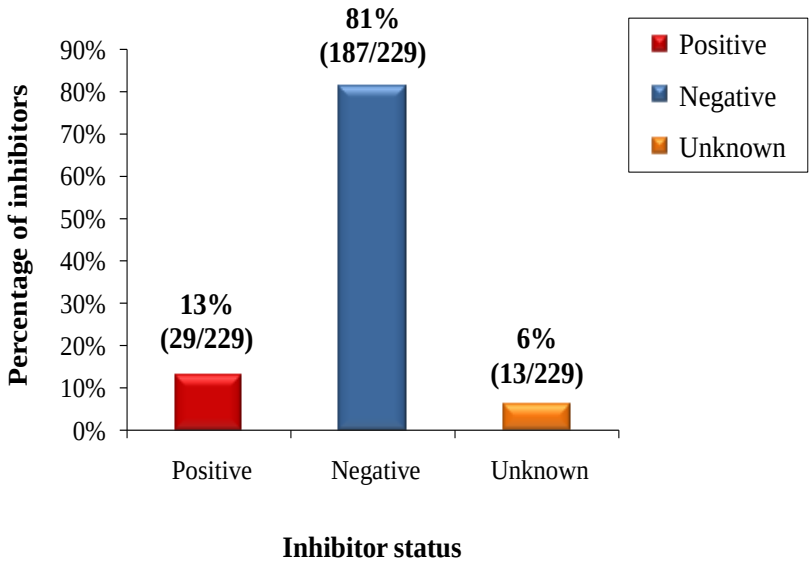


Figure 3.3 Inhibitor status in Cohort A.

Information on those patients with an unknown inhibitor status was not obtainable as there was no active clinical follow up of these patients. A summary of the characteristics of Cohort A is presented in Table 3.1.

Table 3.1 Characteristics of patients in Cohort A.

	Cohort A (*n=229*)					
	Black *n = 116*		**White *n = 113***		**Total in Cohort**	
	n	**%**	***n***	**%**	***n***	**%**
Severity of HA						
Mild	5	4	7	6	12	5
Moderate	3	3	7	6	10	4
Severe	108	93	99	88	207	91
Int22 mutation						
Positive	46	40	36	32	82	36
Negative	70	60	77	68	147	64
Inhibitor status						
Positive	21	18	8	7	29	13
Negative	93	80	94	83	187	81
Unknown	2	2	11	10	13	6

3.2 INVESTIGATION OF THE GENETIC FACTORS INFLUENCING FVIII INHIBITOR DEVELOPMENT

Cohort B (n=216) was used to perform a comparative analysis to determine if an association exists between the *F8* gene mutation type, ethnic background, *F8* gene haplotype status and inhibitor development.

3.2.1 *F8* gene mutation type and inhibitor development

The int22 mutation status and inhibitor status of the 216 probands (Cohort B) was determined. The resultant data were used to establish if an association exists between *F8* gene mutation type and inhibitor development. The frequencies of inhibitor positive and negative male probands were then determined within each of the int22 positive and negative groups, as illustrated in Figure 3.4.

Ten percent of patients within the int22 negative group (14/139) were reported to be inhibitor positive while 20% of the int22 positive patients (15/77) were shown to be inhibitor positive.

Although there was a two-fold increase in inhibitor development observed in the int22 positive group compared to the int22 negative group of patients, the association between inhibitor development and positive int22 mutation status was only of borderline significance (p=0.05; CI=95%).

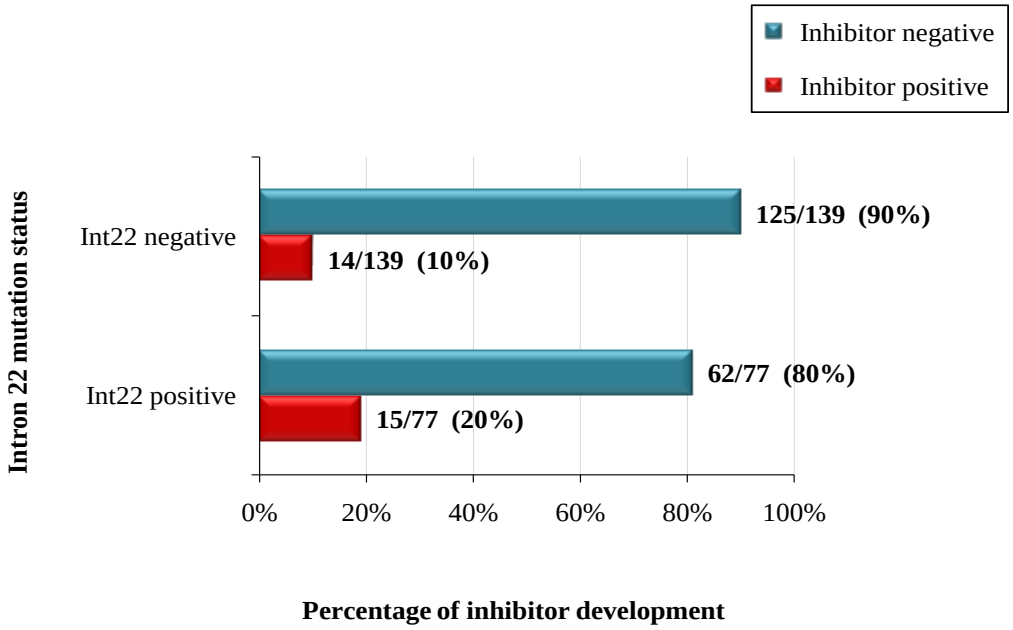


Figure 3.4 Percentage of inhibitor development in int22 positive and negative Haemophilia A patients in Cohort B.

3.2.2 Ethnicity and inhibitor development

Of the 216 probands, 114 (53%) were black and 102 (47%) were white. Figure 3.5 depicts the association between ethnicity and inhibitor development in Cohort B. It was established that 21 (18%) of the black probands and 8 (8%) of the white probands were inhibitor positive.

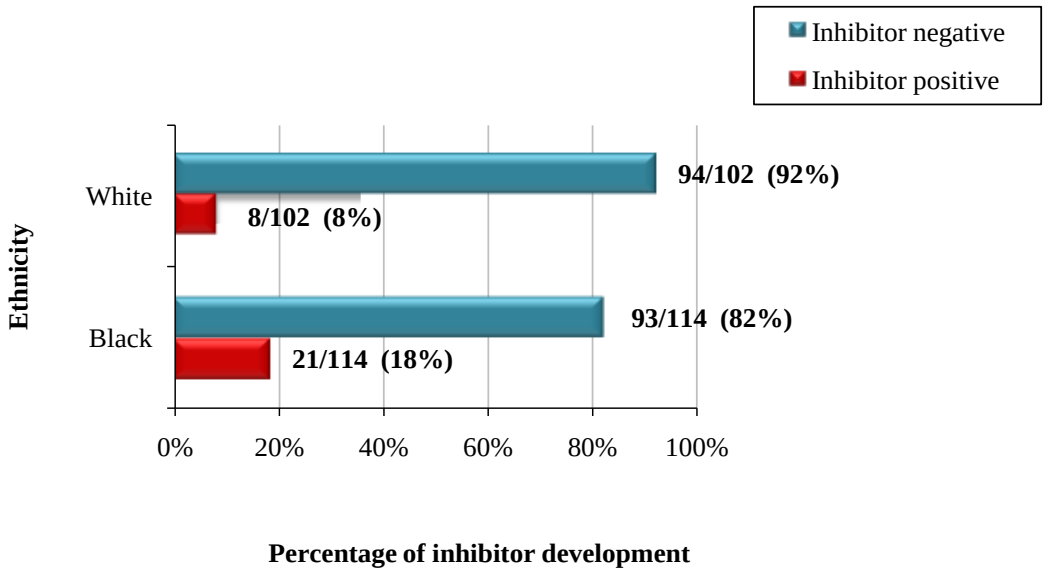


Figure 3.5 Percentage of inhibitor development in black and white Haemophilia A patients in Cohort B.

Overall, of the 29 (13%) inhibitor positive probands in Cohort B, 72% (21/29) were black patients and 28% (8/29) were white. This result showed a significant difference (p=0.02; CI= 95%) in inhibitor development, in that black HA patients were more likely to develop inhibitors than white HA patients.

3.2.3 *F8* gene mutation type, ethnicity and inhibitor development

To determine if an association exists between genetic factors and inhibitor development, the number of black and white int22 positive and int22 negative probands were established and then the frequency of inhibitor development within each ethnic group was ascertained. Figure 3.6 represents the analytic process described above and some of the results obtained.

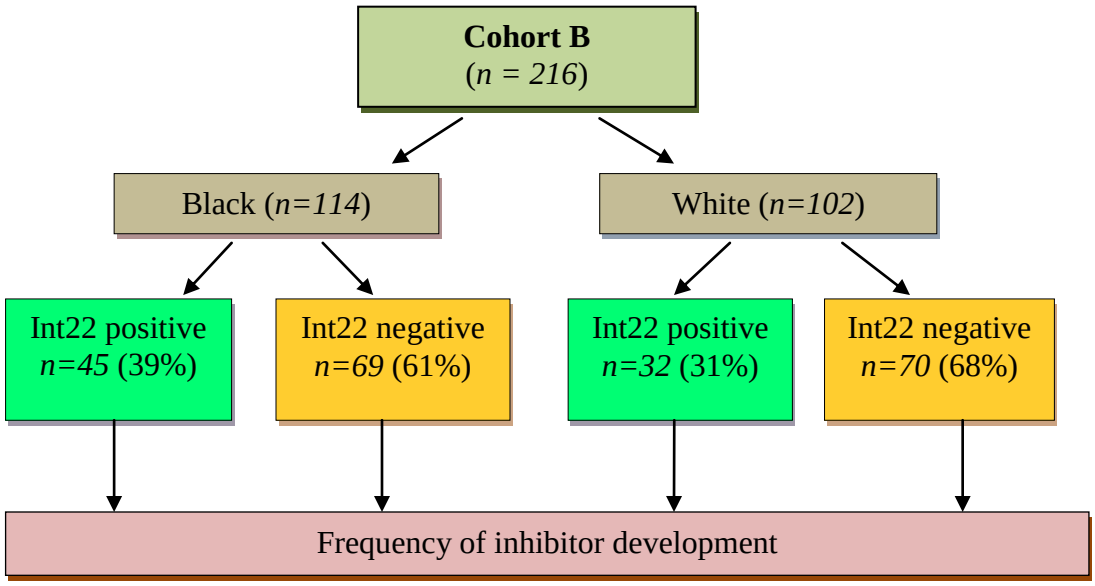


Figure 3.6 The process to determine the percentage of inhibitor development in Cohort B with relation to ethnicity and *F8* gene int22 mutation status.

Of the 216 probands in Cohort B, 77 (36%) were int22 mutation positive. Although there is a higher percentage of black probands who are positive for the int22 mutation compared to

white probands in Cohort B, this difference is not statistically significant (p=0.21; CI=95%).

As there was a similar number of black and white probands within each of the int22 positive and negative groups and no statistical significance was shown to exist (p=0.2; CI=95%) between the int22 mutation status and ethnicity, this allowed for further analysis of the inhibitor development among the black and white int22 positive and negative probands. The results are illustrated in Figure 3.7 and Figure 3.8 below.

In the group of int22 positive probands, there was a significantly higher inhibitor development observed in black probands compared to white probands (p=0.04; CI=95%). This is represented in Figure 3.7.

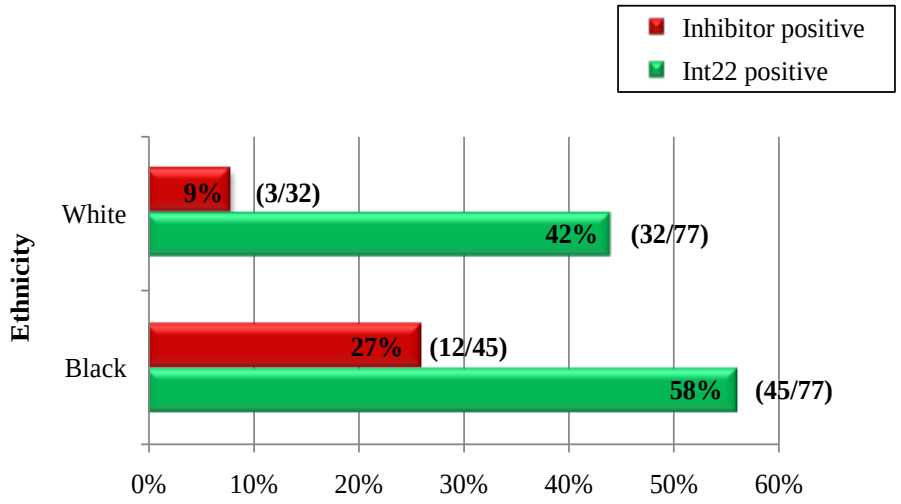


Figure 3.7 Percentage of inhibitor development in black and white int22 positive Haemophilia A probands in Cohort B.

Among the int22 negative probands, a significant association was also shown to exist between inhibitor development, *F8* gene mutation and ethnicity, in that black probands had a two-fold higher inhibitor development than white probands (p=0.017;CI=95%), as represented in Figure 3.8 below.

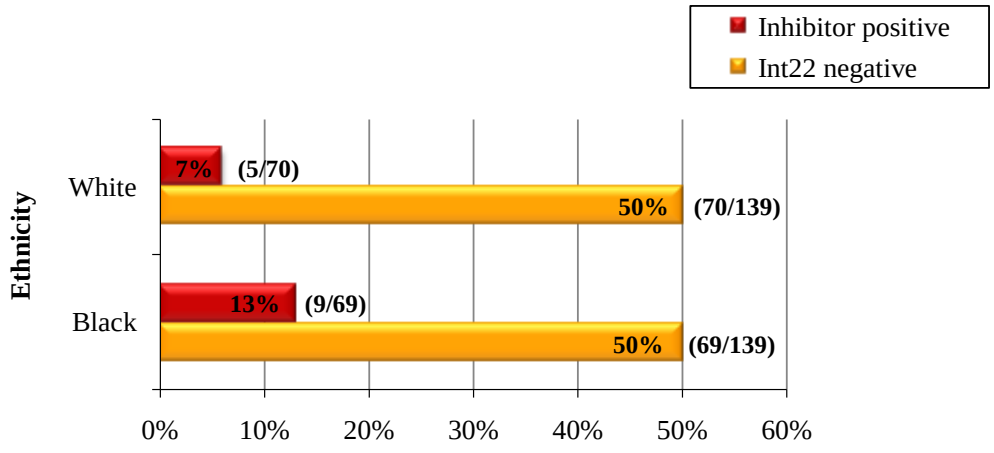


Figure 3.8 Percentage of inhibitor development in black and white int22 negative Haemophilia A probands in Cohort B.

When comparing inhibitor development among white HA patients in Cohort B, although there was a higher frequency of inhibitor development reported in the int22 positive patients compared to the int22 negative patients, the difference was not shown to be of statistical significance (p=0.06; CI=95%). However, among the black HA patients in Cohort B, the int22 positive patients were shown to have a significantly higher frequency of inhibitor development than the int22 negative patients (p=0.02; CI=95%). Therefore, the black patients were shown to have a significantly higher frequency of inhibitor development than white patients in Cohort B, regardless of their int22 mutation status. Overall, black int22 positive patients were shown to have the highest frequency of inhibitor development in the cohort of South African HA patients.

3.2.4 *F8* gene haplotype and ethnicity

As described in section 2.2.1.2, a total of 52 int22 positive probands assigned to Cohort D had SNP analysis of the *F8* gene. The four SNP's were analysed to determine the probands' haplotype, namely H1 through to H6. In Cohort D (n=52), 32 (62%) probands were black and 20 (38%) probands were white.

Of the 52 probands (Cohort D) that had *F8* gene SNP analysis, 25 (48%) had the H1 haplotype, 19 (36%) had the H2 haplotype, 6 (12%) had the H3 haplotype and 2 (4%) had the H5 haplotype. The frequencies of the haplotypes in black and white patients are represented in Table 3.2.

H1 and H2 were the most common haplotypes identified in the int22 positive group. The data generated revealed that a significant difference exists between *F8* gene haplotype and ethnicity in the cohort (p=0.01; CI=95%). The H1 and H2 haplotypes are found in both black and white individuals but H1 was seen most commonly in white individuals whereas the H2 haplotype was more common in black individuals. The H3 and H5 haplotypes were only observed in blacks. It was established that none of the 52 probands had the H4 or H6 haplotypes.

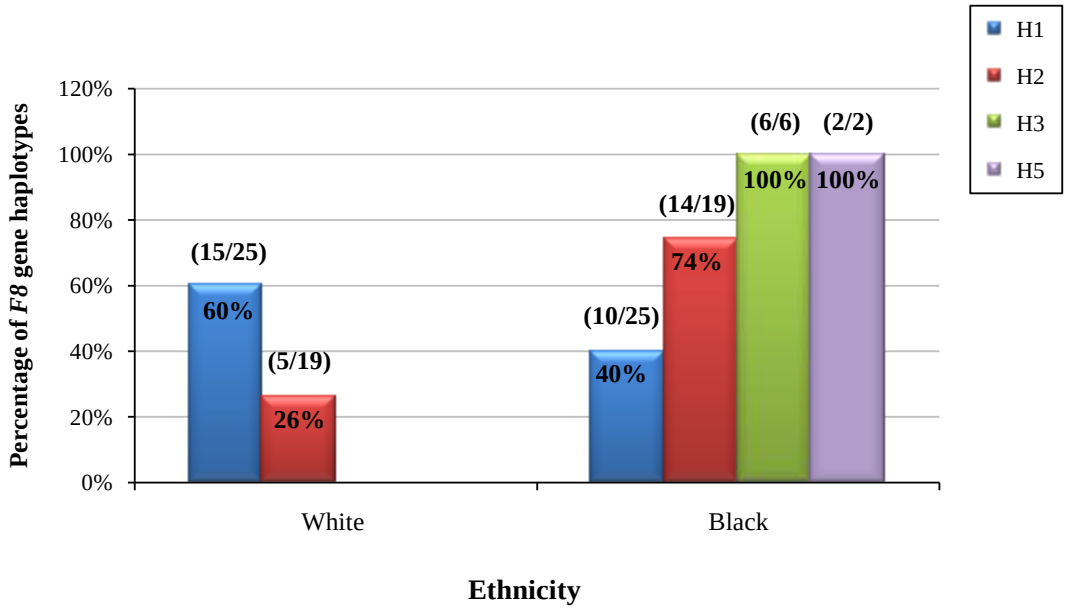


Figure 3.9 Relative frequency of each *F8* gene haplotype in the int22 positive black and white probands (Cohort D).

3.2.5 *F8* gene haplotype and inhibitor development

The 52 probands (Cohort D) were also used for this part of the comparative analysis to determine if an association exists between inhibitor development in black and white HA patients with a specific *F8* gene haplotype. The results are represented in Figure 3.10. Overall, nine of the 52 (17%) probands were reported to be inhibitor positive, of which eight (89%) were black. Of these eight inhibitor positive black probands, two (25%) had the H1 haplotype, three (38%) had the H2 haplotype, two (25%) had the H3 haplotype and one (12%) had the H5 haplotype. Only one inhibitor positive white proband was identified in Cohort D, and was observed to have the H2 haplotype.

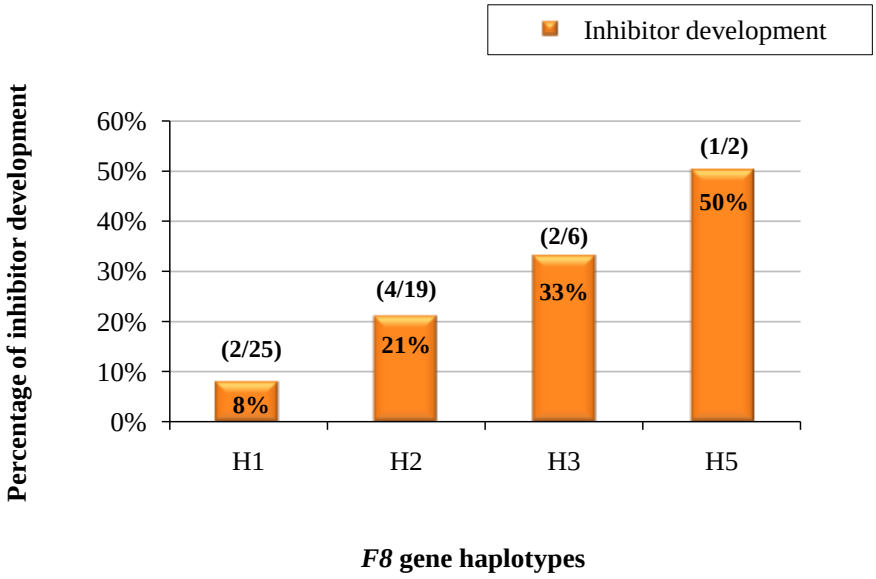


Figure 3.10 Frequency of inhibitor development for each *F8* gene haplotype in Cohort D.

Due to the small number of patients included in the *F8* gene haplotype analysis, the H1 and H2 haplotypes were grouped and the H3 and H5 haplotypes were grouped. This was done as rFVIII and pdFVIII replacement therapy products are enriched with FVIII proteins with the H1 or H2 haplotype whereas the H3 and H5 haplotypes are generally not used in FVIII replacement therapies (Viel et al, 2009) as they are rare in white patients.

The H1/H2 haplotype group had a total of 44 probands with 24 (55%) black and 20 (45%) white probands with 14% (6/44) inhibitor development. The H3/H5 haplotype group had a total of 8 probands, all of whom were black probands and there was 38% (3/8) inhibitor development observed (refer to Figure 3.9 and 3.10).

Figure 3.11 is a graphical representation of the percentage of inhibitor development in black and white probands for each *F8* gene haplotype. With particular regard to inhibitor development in black versus white probands, there was a significantly higher incidence of inhibitor development in black probands in the H1/H2 haplotype group (p=0.04; CI=95%) with an overall 21% (5/24) inhibitor development in the black probands compared to 5% (1/20) inhibitor development in the white probands in Cohort D.

Overall, the H3/H5 haplotype group was only reported in black patients. Although the above results suggest that the H3/H5 haplotype group have a higher prevalence of inhibitor development of 38% compared to the H1/H2 haplotype group with 14% inhibitor development, the sample sizes were very small and therefore the differences in frequency of inhibitor development between the H1/H2 and H3/H5 haplotype groups were not shown to be of statistical significance (p=0.2; CI= 95%).

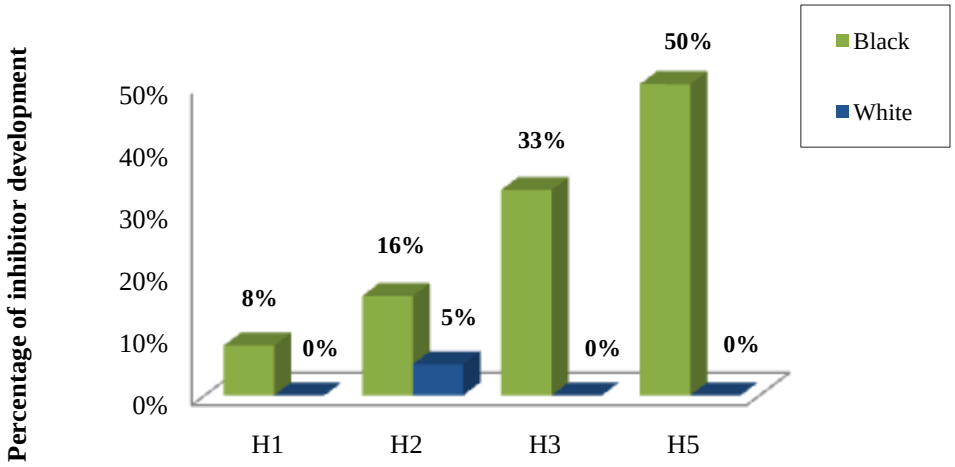


Figure 3.11 Percentage of inhibitor development in black and white HA probands for each *F8* gene haplotype (Cohort D).

A summary of the results from the comparative analysis between the frequency of inhibitor development in black and white HA patients in relation to their *F8* gene haplotype is represented in Table 3.2.

Table 3.2 Frequency of factor VIII inhibitor development in black and white HA patients in relation to their *F8* gene haplotype (Cohort D).

	Cohort D (n=52)									
	Black (n=32)				White (n=20)				Total in Cohort	
			Inhibitor +				Inhibitor +			
	n	%	n	%	n	%	n	%	n	%
***F8* gene haplotype**										
H1	10	31	2	6	15	75	0	0	25	48
H2	14	44	3	10	5	25	1	5	19	36
H3	6	19	2	6	0	0	0	0	6	12
H5	2	6	1	3	0	0	0	0	2	4
Total	32	100	8	25	20	100	1	5	52	100

3.2.6 Type of int22 mutation and inhibitor development

Of the 77 int22 positive probands in Cohort C, 10 probands had an atypical rearrangement of intron 22 which could not be confirmed to be one of the three main types of the int22 mutations and were therefore excluded from this analysis. Hence, a total of 67 int22 positive probands were assigned to Cohort E to determine which of the three types of int22 mutation were present and the frequency of inhibitor development for each type. The type 1 (distal inversion) mutation was observed in 58% (39/67) of the probands of which 25 (64%) were black and 14 (36%) were white. The type 2 (proximal inversion) was observed in 34% (23/67) of probands of which 12 (52%) were black and 11 (48%) were white. The type 3 (rare variant rearrangement of int22) in 8% (5/67) of the probands of which 4 (80%) were black and 1 (20%) were white.

The frequency of inhibitor development reported for each type of int22 mutation in the black and white HA probands is illustrated in Table 3.3. There was an overall frequency of 19% inhibitor development in Cohort E. The difference in the percentage inhibitor development between the three types of int22 mutations was not statistically significant (p=0.95; CI=95%).

Table 3.3 Frequency of inhibitor development for each type of int22 mutation in Cohort E.

	Frequency of type of int22 mutation (*n* = *67*)		**Inhibitor positive**	
	n	**%**	***n***	**%**
Type 1 **(Distal inversion)**	39	58	8	21
Type 2 **(Proximal inversion)**	23	34	4	17
Type 3 **(Rare int22 inversion)**	5	8	1	20
TOTAL	67	100	13	19

3.3 SUMMARY OF FINDINGS

The aim of this study was to characterise and correlate HA disease severity, intron 22 inversion mutation status, ethnicity, inhibitor development and FVIII haplotype in a South African HA cohort. The results generated from the study allowed for accomplishment of this aim. A summary of the findings of the present study are listed below:

- There were an almost equivalent number of black and white patients in the HA cohort.
- The majority of the patients (91%) in the South African cohort had severe HA.
- There is an overall 13% incidence of inhibitor development in the HA cohort of which 72% were black and 28% were white patients.
- Approximately a third (36%) of the patients in the HA cohort were positive for the int22 mutation.
- Type 1 (distal inversion) is the most common type of the int22 mutation in the HA cohort, followed by Type 2 (proximal inversion) then Type 3 (rare variant rearrangement).
- The overall frequency of inhibitor development for each type of int22 mutation was similar.
- Int22 positive patients had a two-fold higher prevalence of inhibitor development than int22 negative patients.

- Black int22 positive patients had the highest frequency of inhibitor development compared to black int22 negative, white int22 positive and white int22 negative patients.
- The H1 and H2 haplotypes were the most common in the entire cohort.
- The H3 and H5 haplotypes were only reported in black patients.
- Black patients had a higher prevalence of inhibitor development in the H1/H2 and the H3/H5 haplotype groups.

CHAPTER 4: DISCUSSION

The present study performed on a small cohort of patients affected with HA in South Africa, allowed for characterisation of and comparative analysis between some of the genetic factors that are known to influence inhibitor development against the FVIII protein. Although the study sample only included HA probands that had *F8* gene molecular testing performed at the Division of Human Genetics, National Health Laboratory Service, Johannesburg, there were several patients that were referred for molecular analysis from provincial hospitals and general practitioners beyond the borders of the Gauteng Province. Therefore, the results generated from the study cohort do to some extent represent the characteristics of patients affected with HA throughout South Africa.

As it has been long established that patients with severe HA and gross *F8* gene defects are at a higher risk of inhibitor development than those with mild or moderate HA and small gene defects, the current study focused mainly on severe HA patients positive for the *F8* gene int22 mutation (Hay, 2006b).

4.1 CHARACTERISTICS OF THE COHORT OF HAEMOPHILIA A PATIENTS IN SOUTH AFRICA

4.1.1 The frequency of each class of HA disease severity in Cohort A

In the present study cohort comprising an almost equivalent number of black and white patients affected with HA, 91% of the cohort was reported to have the severe phenotype. Although the majority of Cohort A had severe HA, which is also a finding of several single and multi-centred international studies (Naylor et al., 1992; Lakich et al., 1993; Andrikovics et al., 2003), Cohort A had a much higher frequency of patients with severe HA compared to the reported frequency of an international study (Antonorakis, 1995).

The most likely reasons for this finding is either that the severe HA patients seek health care more often than patients with mild and moderate HA phenotypes and are thus more likely to receive diagnostic services; that mild and moderate HA patients are not as easily diagnosed as severe patients as their symptoms are not as dramatic and are therefore missed for a longer period of time; patients do not have easy access to health care facilities or they are not referred for diagnostic molecular analysis. The present study was performed in South Africa which is a developing country and the limited presence of and access to specialized health care services or resources does warrant consideration with regard to diagnosis of HA patients.

In earlier studies, performed in the United Kingdom, United States of America and several other international countries, characterisation of the cohort included the mild and moderate HA patients, whereas, more recent studies are mainly focused on patients with severe HA, as

these patients frequently require health care services and FVIII prophylaxis or on demand therapy which is often complicated by FVIII inhibitor development. Therefore, earlier literature on HA cohort characterisation studies was reviewed to compare the findings of the present study.

4.1.2 The frequency of the int22 mutation in Cohort A

Within the cohort of South African HA patients, which included all classes of HA disease severity, the majority of patients were reported to be int22 negative while 36% of the probands were reported to be positive for the int22 mutation. On the contrary, earlier international studies reported the frequency of the int22 mutation to be close to 50% (Naylor et al., 1992). As recent studies for the most part report on the genotype-phenotype correlation in severe HA patients, further analysis of the data was performed to determine the frequency of the int22 mutation in severe HA patients in the present study cohort.

Approximately 45% of severe patients are reported to be positive for the int22 mutation in international studies (Gouw et al., 2012), which is close to the 39% severe int22 positive probands detected through this study.

Additionally, within this group of int22 positive probands, while there was a slightly higher frequency of black probands (40%) reported to be int22 positive compared to white probands (32%), the difference was not of statistical significance (refer to Table 3.1). Similar findings with regard to the frequency of black and white int22 positive patients in the South African cohort were established in a previous study. The presence of a founder Afrikaner mutation in approximately 26% of severe white patients most probably accounts for the lower frequency

of the int22 mutation in white patients (Mitchell, 2009).

4.1.3 The frequency of the type of int22 mutation

The type 1 (distal inversion) int22 mutation was found to be the most common type of inversion of intron 22 in the South African HA cohort which is a similar finding to that of international cohort studies (Antonarakis, 1995; Gouw et al., 2011), although the observed frequency in the present study was lower. There was a two-fold higher frequency of the type 2 int22 mutation observed in South African HA patients (34%) compared to international studies (16%) and the type 3 int22 mutation was the least frequent type of inversion reported but a higher frequency (8%) than the 1% frequency reported by Antonorakis (1995), (refer to Table 1.2 and section 3.2.6).

Therefore, the type 2 and 3 int22 mutation were observed at a higher frequency in the present study compared to international studies and all three types of the int22 mutation were more common in black probands compared to whites, especially the type 1 and 3 int22 mutation. These findings may be related to the small sample size although there were an almost equivalent number of black and white int22 positive probands in the cohort.

4.1.4 The frequency of inhibitor development

Similar findings to those of international HA cohorts were observed in the South African HA cohort with regard to the frequency of inhibitor development (Powell, 2009). The South African HA cohort had an overall rate of 13% inhibitor development which was consistent

with the inhibitor frequency reported in other countries. It was also established that 15% (29/195) of the severe HA patients, in the present study, were inhibitor positive compared to the 20-30% inhibitor development noted in severe patients reported internationally (Coppola et al., 2010).

As listed in Table 3.1, of the 13% of inhibitor positive probands, the black probands have a nearly three-fold higher incidence of inhibitor development (18%) compared to the white probands (7%), irrespective of genotype and clinical phenotype. This finding was higher than the two-fold increase observed in the Malmo International Brother Study performed by Astermark et al. (2001). Black probands were shown to have a significantly higher incidence of inhibitor development ($p=0.02$) compared to white probands and this has been a consistent finding throughout the study irrespective of int22 mutation status (refer to section 3.2.2 and 3.2.3). Possible explanations for the higher incidence of inhibitor development in black probands may be related to genetic variations on immunoregulatory genes as postulated by Lozier et al. (2011) or environmental factors related to FVIII replacement therapy.

4.2 GENETIC FACTORS INFLUENCING FVIII INHIBITOR DEVELOPMENT

There are many genetic and environmental factors thought to be related to FVIII inhibitor development, but uncertainty still remains as to the influence these individual factors have on inhibitor development especially in severe HA patients. Therefore, the comparative analysis drawn between the different genetic factors characterised earlier in Cohort A was of paramount importance to allow us to enhance our understanding of this critical complication

seen in HA patients in a South African cohort. From Cohort A, only 216 patients had a known inhibitor status and were included in the comparative analysis (Cohort B).

4.2.1 The int22 mutation and frequency of inhibitor development

In the present study, 20% of int22 positive probands were inhibitor positive. This is supportive of findings by Saint-Remy et al. (2006) and Schroder et al. (2006) who reported a 21% to 34% inhibitor incidence in int22 mutation positive patients.

There was an overall two-fold higher frequency of inhibitor development observed in the int22 positive group of patients compared to the int22 negative group. However, the present study sample size was small which may account for the borderline statistical significance (p=0.05; CI=95%) of the analysis. Despite two thirds of the cohort being int22 negative, there were more inhibitor positive patients among the int22 positive probands. This finding supports that of several international studies in that severe *F8* gene defects which result in either complete absence or lower FVIII levels ultimately leading to an immune response in the form of inhibitor development (refer to section 1.2.1.1).

With regard to the type of int22 mutation, even though there was an overall frequency of 19% inhibitor development observed in this group (refer to Table 3.3) and the Type 1 (distal inversion) had a higher proportion of inhibitor positive patients, the frequency of inhibitor development among the three types of int22 mutations did not differ significantly (refer to Table 3.3). All three types of the int22 mutation results in a severe disruption of the FVIII protein and this probably explains the similar incidence of inhibitor development observed for each type. Unfortunately there were no reported international data available to compare these

findings to.

4.2.2 Ethnicity and the frequency of inhibitor development

A significant association was shown to exist between ethnic background and inhibitor development in the cohort studied with 72% of inhibitor positive probands reported to be black although an almost equivalent number of black and white probands were present. This supports the higher prevalence of inhibitor development in African-Americans compared to white patients observed by Astermark et al. (2001) and Viel et al. (2009). The results generated in the present study are therefore strongly suggestive that black patients are at higher risk of developing inhibitors.

4.2.3 Int22 mutation, ethnicity and the frequency of inhibitor development

There was a three-fold increase in inhibitor development observed in black int22 positive probands compared to white int22 positive probands (refer to Figure 3.7). These results revealed a significant association that exists between ethnicity and inhibitor development within a genetically homogenous group of patients. Likewise, there was a two-fold increase in inhibitor development between the int22 negative black and white probands (refer to Figure 3.8).

There was also an observed two-fold increase in inhibitor development in the int22 positive black probands compared to the int22 negative black probands (refer to Table 3.1). Hence in the present study, black int22 positive probands were reported to have a statistically significant

higher prevalence of inhibitor development which is suggestive that not only does ethnicity predispose patients to inhibitors but so does having a severe *F8* gene defect such as the int22 mutation.

These findings are similar to the results observed in The Malmo International Brother Study (Astermark et al., 2001), where a significant association between inhibitor development and ethnicity was reported. This international study showed that the int22 positive African-American patients have a higher prevalence of inhibitor development compared to int22 positive and negative White-American patients and int22 negative African-American patients.

Although the results from the present and international studies support the significant role that *F8* gene mutation contributes to inhibitor development the findings are also suggestive that other genetic factors may be responsible for inhibitor development.

4.2.4 *F8* gene haplotype and the frequency of inhibitor development

As mentioned previously, in South Africa, pdFVIII therapy is used predominantly for the treatment of HA patients and donors are mainly white individuals (Mahlangu & Gilham, 2008). As elucidated by Viel et al. (2009), white individuals have either the H1 or H2 haplotype therefore suggesting that a possible explanation for inhibitor development could be mismatched replacement therapy. Only black patients were reported to have the H3 haplotype as established by Viel et al. (2009) as well as in the recently published study by Miller et al. (2012). The frequency of inhibitor development patients with the H3, H4, H5 and H6 haplotypes was therefore expected to be higher in the present study.

We found that the H1 haplotype was the most common haplotype in the cohort followed by

the H2 haplotype and the probands within the H1/H2 haplogroup had a lower frequency of inhibitor development compared to the H3/H5 haplogroup. An important observation was that black probands with the H1 and H2 haplotypes had a higher prevalence of inhibitors than the white probands with the same haplotype. This difference in the frequency of inhibitor development among the white and black probands within the H1/H2 haplogroup was shown to be of statistical significance (p=0.04), (refer to section 3.2.5) and suggests that, despite having the same H1 or H2 haplotype and receiving FVIII replacement therapy containing either the H1 or H2 haplotype, black patients are at a higher risk of inhibitor development. These findings do not support the hypothesis that *F8* gene haplotype mismatch therapy is the only factor that predisposes black patients to inhibitor development (Viel et al., 2009), (refer to section 1.2.1.2).

The prevalence of inhibitor development in the H3/H5 haplogroup that consisted of black probands only, was expected to be higher than that of the H1/H2 haplogroup. On the whole, there was a 38% frequency of inhibitor development in the H3/H5 haplogroup while 24% of probands were inhibitor positive in the H1/H2 haplogroup. Although this result is indicative to some degree that *F8* gene haplotype mismatch may be the causative factor, the number of probands in the H3/H5 haplogroup were very small (8/52) and the H1/H2 haplogroup had a much larger number of probands (44/52).

Although international studies (Viel et al., 2009) hypothesised that *F8* gene haplotype may be a predisposing factor to inhibitor development, the present study does not fully support this finding since both black and white HA patients had the H1 and H2 haplotypes and the black patients had a higher prevalence of inhibitors for both haplotypes. Although, the hypothesis of

F8 gene haplotype mismatch therapy may be true for the rarer H3 and H4 haplotypes.

FVIII replacement therapy may play a role in predisposing black patients to inhibitors as the majority of plasma derived FVIII replacement therapy in South Africa is derived from white individuals, however, *F8* gene haplotype mismatch replacement therapy may not account for inhibitor development as established in the present study. This finding is suggestive of genetic factors other than *F8* gene haplotype being responsible for predisposing HA patients to inhibitor development such as variations or polymorphisms in different immune response genes which modify the risk of developing FVIII inhibitors in HA patients as postulated by Lozier, et al. (2011).

Ultimately, comparative analysis of the genetic factors and inhibitor development in the South African cohort of HA patients revealed that black int22 positive HA patients had the highest frequency of inhibitor development irrespective of their *F8* gene haplotype and this finding was of statistical significance.

Therefore, the present study supports the significant contribution that ethnicity and intron 22 mutation status have in predisposing HA patients to inhibitor development in the South African cohort patients.

4.3 LIMITATIONS OF THE STUDY

- The lack of knowledge on patients that may have had *F8* gene analysis at other local or international laboratories or patients who have not had diagnostic testing is a limiting factor of this study as it is uncertain as to whether the study sample is representative of the HA patients at a national level in South Africa.

- The lack of data on affected family members prevented an intra-familial correlation being performed to ascertain if other genetic factors are important in inhibitor development.

- There may also be bias toward severe HA patients as well as inhibitor positive patients as they seek health care more often than mild and moderate HA patients and inhibitor negative patients.

- The small study sample size was a limiting factor for the following reasons:

  - Lack of follow up of many HA patients by attending physicians therefore data on inhibitors was not available.
  - Lack of and difficulty in obtaining data on inhibitor status
  - Inadequate quantity and poor quality of the DNA specimens meant that mutation data was only available on smaller group of patients
  - Indian and Coloured patients needed to be excluded due to few patients being present on the molecular laboratory database.

4.4 RECOMMENDATIONS

In light of the limitations experienced and the results generated in the present study, the following recommendations can be made:

- An updated national HA registry is recommended so that data on patients affected with HA can be easily accessed by medical professionals throughout South Africa who are involved in the care of these patients. This would also aid further research to be performed on a representative South African cohort of HA patients on a national basis.

- Detailed studies on other postulated predisposing genetic factors such as immune response genes (HLA/MHC) is recommended on a large cohort.

- As the present study was performed on a small cohort of HA patients, a larger multi-centred and multiethnic study is required to confirm the results of this study.

CHAPTER 5: CONCLUSION

In this small HA cohort comprising an almost equivalent number of black and white patients, black patients had a higher frequency of the int22 mutation and inhibitor development. Black and white patients' positive for the int22 mutation also had a higher incidence of inhibitor development than the int22 negative patients. These results support a significant association between inhibitor development and ethnicity, as well as, inhibitor development and *F8* gene mutation type, suggesting that ethnicity and *F8* gene mutation type are important genetic factors in inhibitor development.

F8 gene haplotype analysis revealed that an association does exist between haplotype and inhibitor development, therefore, indicative that inhibitor development may be related to haplotype mismatch therapy. The findings in this study were consistent with data published from larger cohort studies; however, they need to be confirmed in a larger multi-centred cohort study due to the limited statistical power of this study. However, this does not explain the difference in inhibitor development between different ethnic groups within each haplotype suggesting that there are other important genetic factors predisposing to inhibitor development.

Overall, ethnicity and *F8* gene mutation type are important genetic factors that predispose to inhibitor development in the South African HA cohort. *F8* gene haplotype may contribute to inhibitor development but other genetic factors need to be identified and studied.

These results have important implications for the type of FVIII replacement therapy used in management of South African HA patients. If *F8* gene haplotype mismatch is a risk factor that may influence the development of inhibitors to FVIII in HA patients, the use of recombinant FVIII replacement products will need to be strongly considered and motivated for, in place of plasma derived FVIII products. Better insight into the factors predisposing HA patients to inhibitor development allows for more cost effective and efficient care which is proven to improve the physical and mental quality of life for patients with HA.

Future studies involving larger multi-centred and multiethnic HA patient cohorts are required to confirm the results of this study. The haplotypes of all HA patients should be analysed irrespective of their mutation type with the aim of understanding the influence of genetic factors, such as *F8* gene haplotype, on inhibitor development in South African HA patients. Further research with regard to the influence of genetic factors on inhibitor development may enable the development of improved FVIII replacement therapy to meet the needs of all HA patients in turn reducing the cost of care of patients with FVIII inhibitors ultimately improving their quality of life.

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APPENDIX A: ETHICS CLEARANCE CERTIFICATE

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Anneline Lochan

CLEARANCE CERTIFICATE	**M10248**
PROJECT	Genetic Factors Influencing Inhibitor Development in a Cohort of South African Haemophillia A Patients
INVESTIGATORS	Dr Anneline Lochan.
DEPARTMENT	School of Pathology/NHLS
DATE CONSIDERED	26/02/2010
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 26/02/2010 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Prof A Krause

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

APPENDIX B: DATA COLLECTION SHEET

Patient Code	Ethnicity	Severity	Inhibitors	Intron 22 inversion status	*F8* gene haplotype - inversion 22 positive patients