CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.0 Introduction

Haemostasis is the ability of the body to stop bleeding when faced with a haemostatic challenge. This mechanism has two primary functions which are; to confine circulating blood to the vascular network and to arrest bleeding from injured vessels. This dual function is disturbed in patients with haemophilia, as they may bleed spontaneously at sites where people without haemophilia do not bleed and may also bleed for an abnormally long time after trauma and other haemostatic challenges. (Rick, Walsh & Key., 2003). The haemostatic system and its ability to respond is dependant on three critical components, which are platelets, blood vessels and clotting factors (Bowen, 2002).

Factor VIII (FVIII) is a protein cofactor with no enzyme activity, while factor IX (FIX) is a serine protease, which requires FVIII as a cofactor. Upon activation, FVIII and F IX form an active tenase complex, which activates factor X (FX) (Fig 1). FVIII and FIX are crucial for the accelerated formation of active FX (FXa). This results in approximately fifty fold more efficient conversion of FX into FXa (Bowen, 2002).
Succeeding stages of the cascade culminate in the deposition of fibrin, which is the structural polymer of a blood clot. This results in the cessation of bleeding. A deficiency or dysfunction of either FVIII or FIX compromises the activation of FX.

This results in the disturbance of the whole coagulation cascade therefore fibrin deposition is either inefficient or non-existent. The fundamental biochemical lesion underlying haemophilia is a disturbance of the activity of the tenase complex, brought about either by a deficiency of coagulation FVIII cofactor activity (haemophilia A) or coagulation FIX enzyme activity (haemophilia B) (Bowen, 2002).

The similarity in clinical phenotype of haemophilia A and B patients is therefore not surprising as both evolve from the disturbance of FX activation, which is an essential step in fibrin formation.

In haemophilia there is usually an inverse relationship between the level of clotting factor, and the clinical phenotypic presentation or haemophilia disease severity. Theoretically there should be a negative correlation between the clotting factor level and severity of bleeding. However in clinical practice at the Charlotte Maxeke Johannesburg Academic Hospital Haemophilia Comprehensive Care Centre (CMJAH-HCCC), it has been observed that some biochemically mild haemophiliacs behave like clinically severe haemophiliacs and vice versa. Several prospective studies that looked at phenotypic heterogeneity in severe haemophilia have documented that some 3-10% of patients with severe haemophilia rarely bled, do not suffer joint bleeds, have no or minimal arthropathy and did not need any prophylactic treatment (Molho, et al., 2000; Aledort , et al., 1994). Despite the overall observation that there is a large clinical heterogeneity among patients, very limited data is available on how to identify patients at different bleeding risks.

This discrepancy was hypothesized in the current study to be due to a number of epigenetic factors such as inflammation, cytokine profiles, inhibitor status of patients and polymorphisms in immune response genes. The distinction of phenotypes carries an imperative amount of implications for both clinical practice and science; therefore it is a very fundamental subject for study.

1.1 Haemostasis

Haemostasis is the system of reactions that enable the body to act in response to a haemostatic challenge. Not only does this system prevent blood loss from injured vessels, it also prevents the inappropriate cessation of flow. Haemostasis depends upon interactions between the vessel wall, platelets and clotting factors. The coagulation system has two primary aims when stopping bleeding in the event of a haemostatic challenge. These are to confine circulating blood to the vascular bed and to arrest bleeding from damaged vessels; therefore haemostasis is the consequence of a balanced interaction of cellular and molecular components responsible for an intact circulation (Rick, et al; 2003).

The coagulation system comprises of a succession of soluble zymogen proteins known as coagulation factors, which are designated by roman numerals in order of discovery and not of reaction sequence. Upon proteolytic cleavage and activation, each coagulation factor is able to activate one or more of the subsequent components

of the coagulation cascade. Active coagulation factors are designated by the suffix 'a' (Bowen, 2002).

Two pathways of activation i.e. the intrinsic and extrinsic pathways are identified. The extrinsic pathway, where coagulation is initiated by the interaction of FVII and tissue factor (TF) is the main physiological mechanism in vivo (Fig 1) (Bowen, 2002).

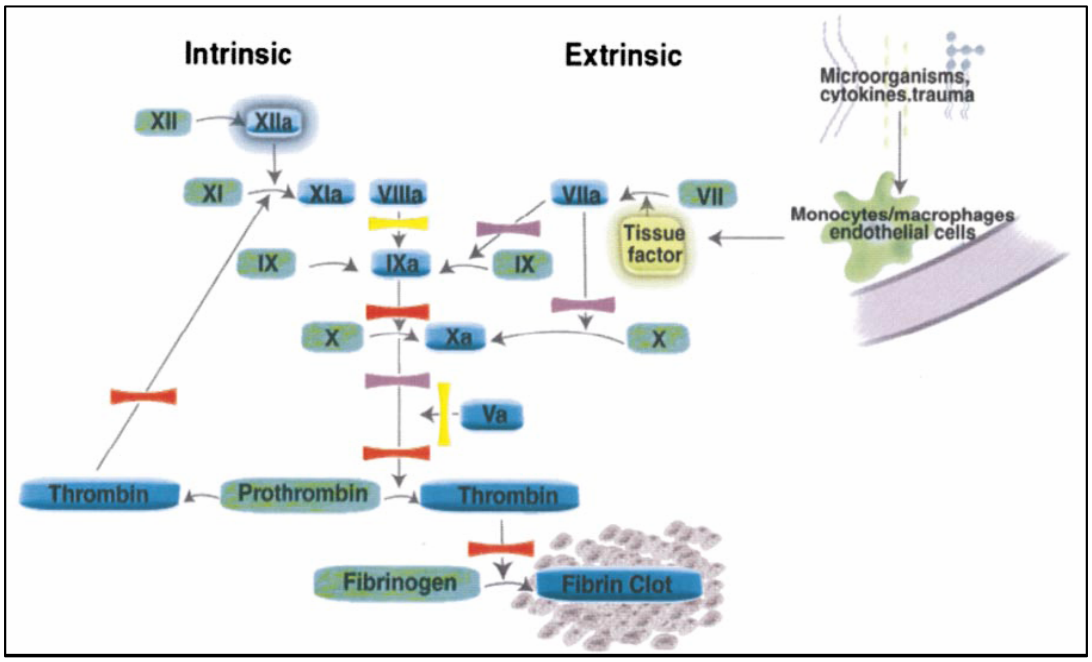

Figure 1. The normal haemostatic mechanisms with clotting factors of the intrinsic and extrinsic pathways of the coagulation cascade. Inhibitor effects- Purple concaved rectangle=TFPI (tissue factor pathway inhibitor), Red concaved rectangle=ATIII (anti-thrombin III), Yellow concaved rectangle= Protein C and Protein S (Bowen, 2002).

TF is a trans-membrane protein expressed by monocytes, organ capsules, gastrointestinal and respiratory tracts, the brain, epidermis and renal glomeruli. Furthermore, it is expressed during damage to endothelial cells. Clotting factors are synthesized by the liver, and Factor V (FV) is also produced by endothelial cells and platelets (Bowen, 2002).

When a blood vessel has been damaged, TF is released into the circulation by the injured endothelial cells and forms a complex with FVIIa (TF-FVIIa complex). This complex activates FX. FVIIIa and FIXa are crucial for the accelerated formation of FXa. This acceleration results in approximately fifty fold more efficient conversion of FX to FXa. FXa then forms a complex with FVa on the surface of activated platelets thereby converting inactive pro-thrombin to active thrombin. This in turn converts fibrinogen to the fibrin monomer, which polymerizes and is cross-linked by FXIII to form a stable fibrin clot (Bowen, 2002).

The coagulation process is down-regulated by inhibitors such as anti-thrombin (AT), protein C, protein S and Tissue factor pathway inhibitor (TFPI). Defects in these inhibitors and the more common mutation in the FV gene (FV Leiden) often lead to a prothrombotic status (Van den Berg, De Groot & Fischer., 2007).

A disorder that affects any of the individual coagulation factors may disrupt the entire cascade. Haemophilia is the most common of the inherited bleeding disorders. It is

characterized by inadequate production of clotting factor VIII (Haemophilia A) and clotting factor IX (Haemophilia B). Haemophilia C is the third type of haemophilia which is due to a deficiency in FXI. This condition has a high prevalence in Ashkenazi Jews and uncommon in the general population of SA and was therefore not part of the current study (Mahlangu & Gilham., 2008).

1.2 Haemophilia A & B

Haemophilia A is also known as FVIII deficiency or Classic haemophilia. It is the most common of the two haemophilias and affects approximately 1 in 10 000 male births. This represents approximately 85% of all haemophilia cases. Aberrations of the FIX gene result in the reduction of FIX giving rise to haemophilia B also known as Christmas disease. Although less common, this disorder is indistinguishable from Haemophilia A clinically and on screening tests. Their diagnosis is confirmed by specific factor assays (White & Rosendaal., 2003).

The FVIII and FIX genes are localized on the X-chromosome; therefore haemophilia is an X-linked recessive bleeding disorder due to a defective FVIII or FIX gene. This defect results in either a decreased or an absent circulating level of functional FVIII or FIX proteins (White, et al., 2003).

Females who inherit a defective gene are obligate carriers while males who inherit an altered or missing gene are affected. On pedigree grounds, all daughters of

haemophiliacs are obligate carriers and sisters have a 50% chance of being carriers. If the carrier has a son, he has a 50% chance of having haemophilia (Josephson & Abshire., 2006).

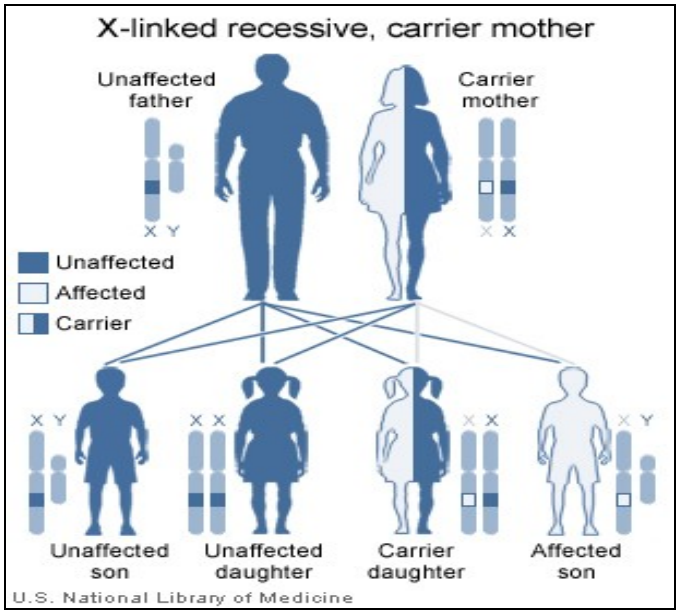

Fig 2 – A family tree of a haemophilic family showing x-linked pattern of inheritance of the disease (Josephson, et al., 2006).

1.3 Epidemiology of haemophilia

1.3.1 United States

The annual incidence of haemophilia A in Europe and North America is approximately 1 case per 5000 male births. It is the most common X-linked genetic disease and the second most common clotting factor deficiency after von Willebrand disease (VWD). The incidence of haemophilia B is estimated to be approximately 1

case per 30,000 male births. In the United States, the prevalence of haemophilia A is 20.6 cases per 100,000 male individuals, with 60% of those having severe disease. The prevalence of haemophilia B is 5.3 cases per 100,000 male individuals, with 44% of those having severe disease (Aronson, 1988).

1.3.2 International

The worldwide incidence of haemophilia A is approximately 1 case per 5000-10 000 male individuals, with approximately one third of affected individuals not having a family history. Haemophilia B occurs in 1 case per 35,000 male individuals and represents approximately 15-20% of all patients with haemophilia. The prevalence of haemophilia A varies with the reporting country, with a range of 5.4-14.5 cases per 100,000 male individuals. The prevalence of haemophilia B varies from 0.9-3.2 cases per 100,000 male individuals (Jones P & Ratnoff O., 1991, Chorba, Holman, Strine., 1994). The true incidence and prevalence in South Africa is currently unknown, but likely to be similar to the world-wide figures.

1.3.3 Mortality/Morbidity

Before the widespread use of replacement therapy, patients with severe haemophilia had a shortened lifespan and a significantly diminished quality of life largely as a

result of pain and disability due to haemophilic arthropathy. Home therapy for haemarthroses became possible with factor concentrates. Prophylactic therapies with lyophilized concentrates that eliminate bleeding episodes help prevent joint deterioration, especially when instituted early in life (age 1-2 years). Life expectancy in haemophilia patients had increased from 11 years before the 1960s for haemophilia patients who were severely affected to older than 50-60 years by the early 1980s in most developed countries (Vlot, Mauser- Bunschoten, Zarkova, et al., 2000).

1.3.4 Race

Haemophilia is observed in all ethnic and racial groups and does not have a predilection for a specific age or geographic distribution. Ethnic differences in FIX polymorphisms are important because they provide linkage data when identifying carriers, particularly when the mutation is unknown or for identification of de novo mutations (Quadros, Ghosh & Shetty., 2007).

A common G10430A mutation (Gly 60 Ser) in the factor IX gene was described in the moderate and mild haemophilia B in the majority of the Gujarati population (http://ublib.buffalo.edu/libraries/projects/cases/hemo.htm., accessed 12 April 2008).

1.3.5 Sex

Both forms of haemophilia are sex-linked coagulation disorders because they are inherited as X-linked traits; therefore, the disease primarily affects male individuals and females are obligate carriers. Carrier females usually do not have bleeding manifestations. Lyonized females (those with unequal inactivation of FVIII or FIX alleles and with hemizygosity of all or part of the X chromosome) may be symptomatic (Venkateswaran, Wilimas, Jones & Nuss., 1998).

Female patients may have clinical bleeding due to haemophilia A or B if 1 of 3 conditions is present: (1) extreme lyonization, (2) hemizygosity for the haemophilia gene (father with hemophilia and mother who is a carrier), or (3) Turner syndrome associated with the affected haemophilia gene. Mild haemophilia may be more common in girls than previously recognized. In 1 study, 5 of 55 patients with mild haemophilia (factor levels 6-40%) were girls (Venkateswaran, et al., 1998).

1.3.6 Age

Haemophilia B can be detected prenatally by measuring FIX activity in foetal blood samples obtained at 20 weeks of gestation by foetoscopy, but the presence of maternal FIX in amniotic fluid may preclude accurate foetal FIX assessment. In addition, the procedure carries a high risk of complications, with a risk of foetal death of up to 6%. Detection of haemophilia B by linkage studies or gene mutation analysis

(when the defect is known) can be performed by chorionic villus sampling at 12 weeks of gestation or by amniocentesis from 16-20 weeks, with complication rates of up to 2.0% (Pollmann, Richter, Ringkamp & Jürgens., 1999).

Postnatal evaluation is triggered by a history of bleeding, which can start immediately after birth (e.g., intracranial bleeding) or may be delayed in those with mild haemophilia. Oral bleeding starts with teething and cuts and abrasions to the lips, tongue, and frenulum, followed by joint and muscle bleeding with the start of ambulation. In a single-center study, the age at which bleeding starts was found to vary. Approximately 44% bled within the first year, while others did not experience their first bleeding episode until age 4 years. Recurrent episodes of joint bleeding usually started approximately 6 months after the first bleeding episode; half the patients had their first bleeding episode by age 1.22 years, while the mean age for the first joint bleed was 1.91 years. These data support the concept that primary prophylaxis need not begin at the same age in all patients (Pollmann, et al., 1999).

1.4 Brief History of Haemophilia

The first record of haemophilia in the Talmud Jewish holy text, states that males did not have to be circumcised if two brothers had already died from the procedure (Rick, et al., 2003). During the 12th century, the Arab physician Albucasis wrote of a family whose males died of bleeding after minor injuries. Thereafter Dr John Conrad Otto, a

Philadelphia physician wrote a report entitled “A haemorrhagic disposition existing in certain families.” He identified that the disorder was hereditary and affected males and rarely females. He was able to map out the disease back to a woman who had settled near Plymouth in 1720. The term haemophilia was first used in a description of the condition written by Hopff at the University of Zurich in 1828. Pavlosky, a doctor from Buenos Aires, did a lab test that demonstrated that haemophilia A and B are not the same. He achieved this by transferring the blood of one haemophiliac to another, and found that it corrected the clotting problem. (Ingram, 1980).

Haemophilia figured prominently in the history of European royalty and thus is sometimes known as "the royal disease". Queen Victoria passed the mutation to her son Leopold who at the age of 31 died of a cerebral haemorrhage after falling and hitting his head. His daughter Alice, born the previous year, who became Princess of Teck, had a haemophilic son Rupert Viscount Trematon born in 1907, who died at 21, also of a cerebral heamorrhage (Aronson, 1973; Ingram, 1980; Meacham & Pedersen., 1995).

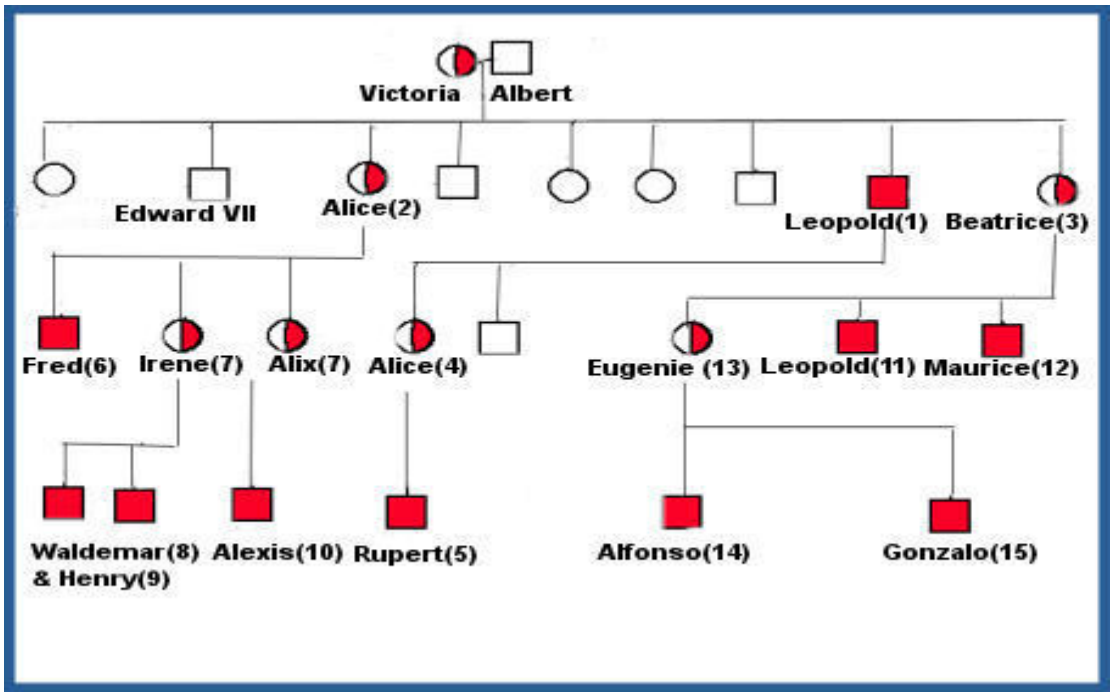

Fig 3. The British Royal family haemophilia pedigree .

Half filled oval = Carrier Female, Empty oval = unaffected Female.

Empty rectangle = Unaffected Male, Full rectangle = Affected Male.

http://www.gfmer.ch/Medical_education_En/PGC_RH_2005/pdf/Genetic_epidemiology.pdf

(accessed on 20 April 2008)

Two of Queen Victoria's daughters also proved to be carriers, transmitting the disorder to three of her grandsons and six of her great-grandsons thereby spreading the disorder to various royals across the continent, including the royal families of Spain, Germany, and Russia (Aronson, 1973; Ingram, 1980; Meacham & Pedersen., 1995).

The condition is not known among any of the Queen's antecedents, so it is supposed that a mutation occurred at spermatogenesis in her father, Edward, Duke of Kent, a

mischance made more likely by the fact that he was in his fifties when she was conceived (Aronson, 1973; Ingram, 1980; Meacham & Pedersen., 1995).

1.5 Classification of Haemophilia and Clinical Phenotype.

In haemophilia there is usually an inverse relationship between the level of clotting factor, and the clinical phenotypic presentation or haemophilia disease severity (White, et al., 2001). This negative correlation is shown in Table 1 below.

Table 1- Relationship between clotting factor level and clinical phenotype in haemophilia†.

CLOTTING FACTOR LEVEL*%	**CLINICAL SEVERITY OF HAEMOPHILIA.**
‹ 1IU	SEVERE: Frequent and spontaneous musculoskeletal and internal bleeding.
2-5IU	MODERATE: Less frequent, spontaneous or induced bleeding following minor injury or trauma.
6-40IU	MILD: Bleeding following major haemostatic challenges such as trauma, surgery and dental extraction.
* IU=inhibitor unit. 1U/ml of clotting is equivalent to 100% factor activity. U/ml is the amount of factor activity in 1ml of average normal plasma.	

† Adapted from White et al 2001.

Theoretically there should be a negative correlation between the clotting factor level and severity of bleeding (White, et al., 2001). However in clinical practice it has been observed at the CMJAH-HCCC, that some biochemically mild haemophiliacs present clinically with severe phenotypes and vice versa.

Several prospective studies that looked at phenotypic heterogeneity in severe haemophilia have documented that some 3-10% of patients with severe haemophilia rarely bled, do not suffer joint bleeds, have no or minimal arthropathy and did not need any prophylactic treatment (Molho, et al., 2000, Aledort , et al., 1994). On the contrary, 12% of mild and moderate patients in the Netherlands are frequent bleeders (Plug, Van der Bom, Peters, et al., 2004). Other interesting studies have suggested that a combination of prothrombotic mutations in a patient with haemophilia would change his phenotype and compensate for the low FVIII/FIX levels and result in more efficient thrombin generation and clot formation (Nihols, Amano, Cacheris, et al., 1996; Escuriola-Ettingshausen, Halimeh, Kurnik, et al., 2001).

The pharmacokinetics of prophylactic FVIII have also been noted as a parameter that could influence the phenotype in patients receiving prophylactic treatment. The half-life of FVIII infused is in the range of 8-12 hours. (Escuriola-Ettingshausen, et al.,2001; Fijnvandraat, Peters & Cate., 1995). Factors that influence the inter-individual variances in FVIII pharmacokinetics have been shown to be the Von Willebrand antigen level and the ABO blood group (Fijnvandraat, et al., 1995, Vlot, Mauser- Bunschoten, Zarkova, et al., 2000). Patients with blood group O as present in

50% of the population were found to have a 30% lower Von Willebrand antigen level and a significantly decreased FVIII half-life (Fijnvandraat, et al., 1995, Vlot, et al., 2000). Although these studies are very interesting, they do not provide conclusive or absolute explanations of observed phenotype-biochemical differences.

Despite the overall observation that there is a large clinical heterogeneity among patients, very limited and fragmentary data is available on how to identify patients at different bleeding risks.

This inconsistency was hypothesized in this study to be due to a number of epigenetic factors such as inflammation, cytokine profiles, polymorphisms in immune response genes as well as the inhibitor status of the patient, which were thus explored. The distinction of phenotypes carries an imperative amount of implications for both clinical practice and science therefore it is a very fundamental subject for study.

Individuals with severe haemophilia have less than 1% clotting factor and their clinical course is dominated by spontaneous painful haemarthroses in large joints and muscle haematomas. These usually occur without apparent trauma and most commonly affect the knees, elbows, hips and ankles (Mahlangu, et al., 2008).
Although haemophilia is a systemic haemorrhagic disorder, haemophilic bleeds transpire in comparatively limited sites (Table 2 - below).

Table 2 - Sites of bleeding in Haemophilia (Mahlangu, et al., 2008**)**

Non –life or limb threatening bleeds.	**Life or organ- threatening bleeds**
Joints	Intracranial
Muscles	Muscle compartment
Easy bruising	Neck/throat
Mucosal bleeding (epistaxis,gingival)	Massive gastrointestinal
Gastrointestinal tract	Genitourinary

Severe hemophiliacs may have one or two bleeds each week. Recurrent joint bleeds lead to synovial hypertrophy, destruction of cartilage and secondary osteoarthrosis. Muscle haematomas may occur in any muscle, but most commonly in the calf and psoas muscles. Although muscle bleeds are less common than skeletal bleeds, a single episode can result in severe lasting damage if not properly treated. A large psoas bleed may extend to press on the femoral nerve. (Mahlangu, et al., 2008). Although less common, spontaneous intracranial haemorrhage occurs more frequently in severe haemophiliacs and is a prominent cause of death (Stein & Duthie., 1981).

Patients with moderate haemophilia normally present with occasional spontaneous musculoskeletal bleeding caused by mild trauma or surgery and those with the mild form of the disorder experience haemorrhage following more major trauma or surgery.

Mild and moderate haemophiliacs may escape diagnosis until adulthood, whereas severe haemophilia is usually diagnosed within the first two years of life (Van den Berg, et al., 2007).

1.6 Therapy and its Complications

Although there is no cure for haemophilia, it can be controlled with regular injections of the deficient clotting factor. In the West, there are common standards of therapy that fall into one of two categories, i.e. prophylaxis or on demand treatment. Prophylaxis involves the infusion of clotting factor on a regular program so as to maintain sufficient levels of clotting, to prevent spontaneous bleeding episodes. On-demand treatment involves treating bleeding episodes once they occur. (Aronson., 1988).

As a direct result of the contamination of the blood supply in the late 1970s and early/mid 1980s when concentrates were first virally inactivated with heat or chemicals, many patients became infected with HIV and hepatitis viruses (Aronson, 1988).

Since the advent of pathogen-safe clotting factor replacement therapy, the development of polyclonal antibodies (inhibitors) against the genetically deficient clotting factor has become the most significant therapeutic complication in haemophilia. An inhibitor is a polyclonal inhibitory immunoglobulin G antibody

directed against FVIII or FIX. The majority of these antibodies belong to the immunoglobulin (Ig)G4 subtype. (Berntorp, Shapiro, Astermark, et al., 1981; Darby, Keeling & Spooner., 2004).

Since their earliest recognition in the 1940s, the patho-biology and treatment of FVIII inhibitors has been an area of intense activity for the scientific and clinical haemophilia communities (Darby, et al., 2004). Inhibitors develop in 20-30% of patients with haemophilia A, and only 5% of patients with haemophilia B (Berntorp, et al., 1981). Inhibitor development has been found to be influenced at large by the type of mutation. Genotyping therefore provides a significant prediction of the inhibitor risk for approximately 20% of patients with severe haemophilia, while in the remaining 80% this power is limited (Berntorp, et al., 2006, Oldenburg, et al., 2006). Other factors such as presence of inflammatory states as well as polymorphisms in immune response gene expressions have been suggested but remain unproven. These will be analyzed as they may be as significant as the specific factor gene mutation.

1.7 Immune Response and Inhibitor Development

The CD4+ T cells play the principal role in the humoral immune response to protein antigens like FVIII and FIX (Fig 4).

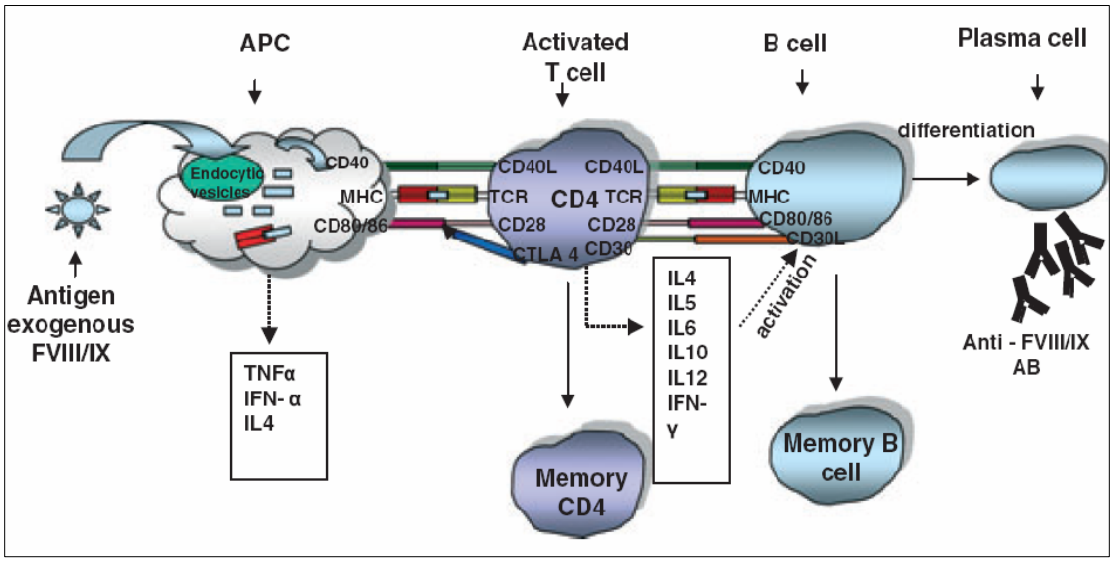

Figure 4. Schematic model of the immune response to exogenous FVIII/FIX protein (Oldenburg & Pavlova., 2006).

Non-haemophilic individuals are naturally tolerant to FVIII and FIX because their FVIII/FIX-reactive T cells have been deleted during the process of central tolerance induction in the thymus. However, in haemophiliacs with no FVIII/FIX, T cells with T cell receptors that recognize FVIII/FIX peptides may be present. The ability to mount a productive immune response against infused factor relates in part, to the

presence of these FVIII cognate CD4+ T cells and the optimal MHC class II receptors to bind to and display FVIII peptides on the surface of antigen-presenting cells (APCs) (fig. 5). (Darby, et al., 2004; Saint-Remy, Lacroix-Desmazes & Oldenburg., 2004).

The immune system of some haemophilia patients recognizes infused factor as foreign and therefore mounts an immune response to eradicate it. The infused factor is taken up by antigen-presenting cells, and then undergoes proteolytic degradation into peptide fragments. These linear peptide sequences are then presented on the surface of the antigen-presenting cell in association with MHC class-II molecules. This complex (MHC-FVIII/FIX) is then recognized by the T-cell receptor of a CD4+ cell specific for the factor. A second co-stimulatory signal is instigated by means of interactions with CD80/60 and CD28 which stimulates the secretion of cytokines. Surface molecules such as CD30, CD40 and CD40L are then upregulated and they interact with corresponding proteins on B-cells. This culminates in B-cell proliferation, differentiation and the subsequent induction of FVIII/FIX antibody production (Fig 5). (Gringeri, Monzini, Tagariello, et al., 2006; Hoots., 2006).

Indirect verification of the role that CD4+ cells play in anti-FVIII antibody synthesis came from the observation of a spontaneous loss of high responder inhibitors in severe haemophilia patients in conjunction with an HIV-associated decline in CD4+ counts (Bray, Kroner & Arkin., 1993). More recently, prevention of inhibitor

synthesis in a murine haemophilia model by blockade of co-stimulatory signals has provided direct verification that CD4+ cells are indeed essential for the development of an anti-FVIII antibody response (Qian, Collins, Sharpe & Hoyer.,2000).

It is not difficult to understand how a haemophilia patient with an intact immune system can direct an immune response to eliminate infused factor as the body recognizes it as foreign, but what remains puzzling is the fact that not all haemophiliacs develop such responses. Why some haemophilia patients develop inhibitors while others do not and whether it may be possible to predict their development, are two major issues that the current study attempted to resolve.

Patients who develop inhibitors have a much severe disease prognosis and their treatment options, costs of treatment as well as potential complications differ adversely to patients without inhibitors (Darby, et al., 2004).

Patients with persistent inhibitors are classified according to the definitions of the Factor VIII/IX Subcommittee of the International Society of Thrombosis and Haemostasis as high or low responder patients. High responder patients are identified by peak inhibitor titres of ›5 Bethesda units (BU) ml $^{-1}$ and low responders are patients with inhibitor titres ≤ 5 BU ml $^{-1}$. (Gringeri, et al., 2006).

1.8 Risk Factors for Inhibitor Development

A number of prognostic factors, both genetic and environmental, have been suggested, and some clinicians currently base their management decisions on these reported risk factors. However, the evidence to support them is sometimes drawn from retrospective studies in small numbers of patients, and the inhibitor incidence rates associated with these treatment variables have sometimes differed markedly from one report to another. Although it is widely accepted that the driving force of inhibitor formation is the presentation of an immunologically altered FVIII/FIX antigen to the patient's immune system, the pathogenesis of inhibitor formation is only partially understood (Berntorp, et al., 2006, Oldenburg, et al., 2006).

1.8.1 Genetic Risk Factors

1.8.1.1 Severity of haemophilia

Severity of haemophilia has been known for many years to represent a major risk factor for inhibitor development particularly in severe haemophilia A. In patients with a severe form of the disease, the inhibitor risk is about 25–30%, while in mild to moderate haemophilia A only 5% of the patients develop inhibitors. (Oldenburg, El-Maarri & Schwaab R., 2002).

1.8.1.2 Race and ethnicity.

Race and ethnicity has been found in several studies to be a highly significant risk factor for inhibitor development (Addiego, Kasper & Abildgaard., 1994; Lusher, Arkin & Hurst., 1997; Gruppo, Chen, Schroth & Bray., 1998; Astermark, Berntorp, White & Kroner., 2001). These studies reported corresponding data that documented the incidence of inhibitors in African-American and Latino patients to be twice that of Caucasian patients.

Scharrer, et al (1999), clearly demonstrated inhibitor incidence in severe haemophilia patients of African origin was twice that in Caucasians (51.9% vs. 25.8%). Similar data were reported by Astermark, et al (2001) in the Malmo International Brother Study (MIBS).

1.8.1.3 Family history of inhibitors.

The frequency of inhibitor development amongst members of the same family has also been found to be an important determinant. In the MIBS, a relative risk of 3.2 for formation of an inhibitor was observed for patients whose older brother had an inhibitor. Astermark, et al (2001), observed in this study of 388 families, that 48% of patients developed inhibitors in families with a positive inhibitor history, compared with 15% in families with no history of inhibitors. A comparable study reported a higher incidence of inhibitor formation in haemophilia siblings (50%), compared with

that observed in more extended haemophilic relatives (9%) (Gill, 1999). This could be due to genetic determinants such as immune response genes which will be explored in the current study, or from environmental factors that might be shared more by siblings than the extended family.

1.8.1.4 Type of factor mutation.

It has been repetitively demonstrated that, the mutation in the FVIII/FIX gene, underlying the haemophilia is a vital determinant of inhibitor development (Goodeve, et al., 2000; Oldenburg, et al., 2004; Oldenburg, et al., 2006). Mutations associated with the absence of a gene product, such as large multi-domain deletions or nonsense mutations, confer a high, approximately 80% risk for inhibitor production and mutations associated with the presence of a gene product (even very low amounts of the protein) confer a low risk for inhibitor production (Oldenburg, et al., 2006).

However, in reality, the situation is not so clear cut. Among patients with identical mutations, some may produce inhibitors and others may not (Goodeve, et al., 2000). There is a lack of substantial evidence as to why only 20% of patients with the same severe molecular defect, i.e. the intron 22 inversion, develop inhibitors while 80% do not. In addition, it is currently not understood why haemophilia A patients with different types of null mutations exhibit different risks for inhibitor development i.e. 88% for large multidomain deletions vs. 25% for single-domain deletions vs. 20% for

intron 22 inversion, although all null mutations result in the inability to synthesize FVIII (Goodeve, et al., 2000; Oldenburg, et al., 2004; Oldenburg, et al., 2006).

Clearly other factors are implicated. The possibility that these may include the genotype at the MHC locus has been investigated in patients with severe haemophilia A, with and without the intron 22 inversion. Only a weak association between human MHC (HLA) class II genotype and the development of inhibitor antibodies against factor VIII was obtained (Hay, Ollier & Pepper., 1997; Oldenburg, Picard & Schwaab., 1997).

1.8.2 Environmental Risk Factors

Monozygotic twins should, if inhibitor development is purely genetically determined, phenotypically behave the same. However, discordant twins have been described, indicating that non-genetic factors influence the immune response (Astermark, et al., 2001). The most debated non-genetic factors to date are age at start of treatment, the impact of immune system challenges, the type of factor concentrate used, and the mode of administration.

The patho-physiological mechanisms behind the nature of immune responses are not known, and there are currently no systems in place to predict which immune response will be encountered in individual patients. The ongoing challenge is to fully discover and characterize the underlying variables and to apply that information to prevent

inhibitor formation, thus overcoming this most serious complication of haemophilia treatment.

1.9 FVIII and FIX Genes and Proteins.

Over the last decade there has been a dramatic increase in the understanding of the pathology of haemophilia in molecular terms, at the levels of nucleic acid sequence and to a much lesser extent, protein structure. By 1983 FVIII and FIX had been purified to homogeneity (Rotblat, William, Daniel, et al., 1983) and shortly afterwards the genes were successfully cloned (Gitschier, Rotblat, William, et al., 1984). The FVIII and FIX genes code for FVIII and FIX respectively and are both located on the X-chromosome. There is a considerable genetic distance of approximately 0.5 cM between the two genes (Rotblat, et al., 1983; Gitschier, et al., 1984).

1.9.1 The FVIII gene

The FVIII gene is located at the most distal band of the long arm of the X-chromosome at Xq28 (the long (q) arm of the X chromosome at position 28). More precisely, the FVIII gene is located from base pair 153,717,259 to base pair 153,904,191 on the X chromosome (Kaufman & Antonarakis., 2007).

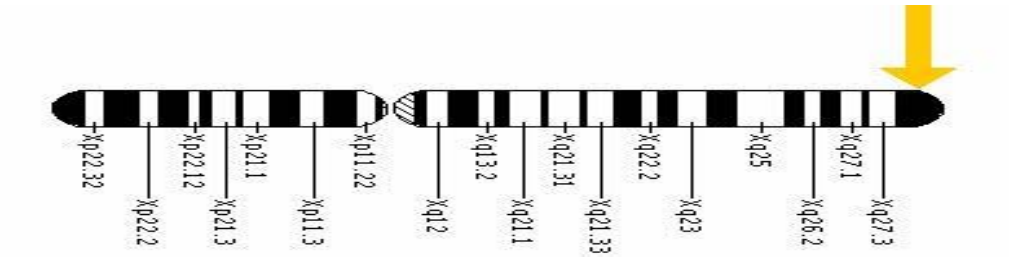

Figure 5. Location of the FVIII gene on the long (q) arm of the X chromosome at position 28. http://ghr.nlm.nih.gov/handbook/F8 (accessed 14 May 2008)

1.9.2 The FIX gene

The FIX gene is significantly smaller and structurally simpler than the FVIII gene. FIX is coded by a gene close to the gene for FVIII near the tip of the long arm of the X chromosome more towards the centromere at Xq27.2 (long (q) arm of the X chromosome between positions 27.1 and 27.2). More precisely, the FIX gene is located from base pair 138,440,560 to base pair 138,473,282 on the X chromosome (Hirowasa & Shinsaku., 1990). It was discovered after a young boy named Stephen Christmas was found to be lacking this exact factor, leading to hemophilia, in 1952 (http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=2158 (accessed 14 May 2008).

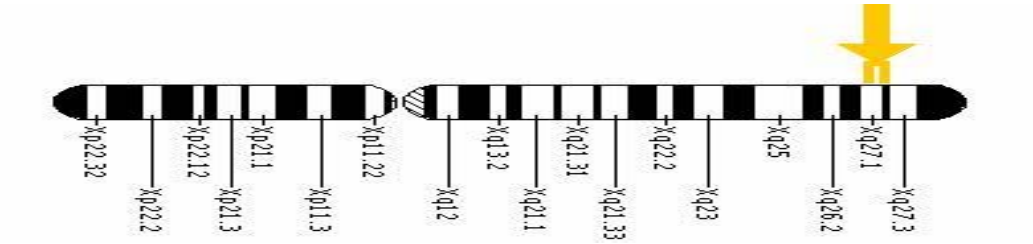

Figure 6. Location of the FIX gene on the long (q) arm of the X chromosome between positions 27.1 and 27.2. http://ghr.nlm.nih.gov/handbook/F9 (accessed 14 May 2008)

1.9.3 FVIII Protein Structure and Activation.

FVIII is a large glycoprotein synthesized as a precursor of 2,332 amino acids, which comprises three distinct types of domains, arranged in the order A1, A2, B, A3, C1, C2 (Lavigne-Lissalde, Schved, Granier & Villard., 2005).

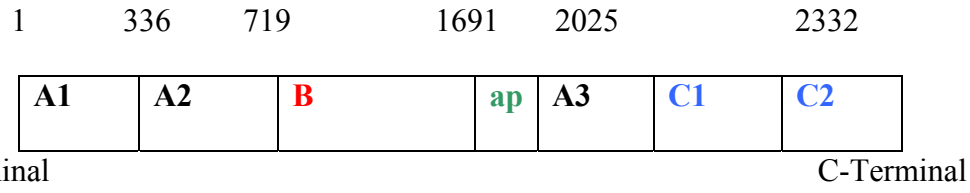

Figure 7. The Factor VIII protein molecular stucture comprising a mature peptide of 2332 amino acids (total length, 2351 amino acids). A1-3, B, C1, and C2 represent domains assigned according to homologies within the protein (the boundaries are somewhat arbitrary) (Bowen DJ., 2002).

Originally defined based on intron–exon boundaries, these domains are now generally defined by reference to thrombin cleavage sites. The A1, A2, and B domains

comprise the FVIII heavy chain, while the light chain consists of the A3, C1, and C2 domains.

In addition, two acidic amino acid regions (a1 and a2) flank the A2 domain and an acidic activation peptide (ap) is located at the amino terminal end of the A3 domain. Thrombin activates FVIII by proteolytic cleavage between the A1 and A2 domains, the A2 and B domains, and the ap and the A3 domain, resulting in an activated heterotrimer consisting of a heavy and a light chain. Thereafter, FVIII associates with von Willebrand factor (VWF) in heterodimers of a heavy (A1-a1-A2-a2) and a light (a3-A3-C1-C2) chain associated by a metal ion interaction. FVIII circulates in the bloodstream in an inactive form bound to VWF. In response to injury, FVIII is activated and dissociates from VWF. (Bowen, 2002).

1.9.4 FIX Protein Structure and Activation.

This protein is synthesized by hepatocytes as a precursor protein then it undergoes extensive post-translational modification to become the fully gamma-carboxylated mature zymogen that is secreted into the blood. The FIX precursor is a glycoprotein consisting of a signaling peptide at the amino (NH_2) terminal end, which directs the protein to the endoplasmic reticulum in the liver. This is followed by the pre-pro leader sequence recognized by the gamma-glutamyl carboxylase, which is responsible for the post-translational modification (carboxylation) of the glutamic acid residues (Gla) in the NH_2-terminal portion of the molecule, an amino terminal

carboxyglutamic acid (Gla) region which is characteristic of all vitamin K dependent proteins, followed by two epidermal growth factor (EGF)-like modules, an activation peptide (ap) and a serine protease or catalytic domain which contains the enzymatic activity (Lavigne-Lissalde G, Schved JF, Granier C & Villard S., 2005). The signaling peptide and the pre-pro leader sequence are both removed before the protein is secreted into the circulation resulting in single chain plasma FIX (Hirowasa., et al., 1990).

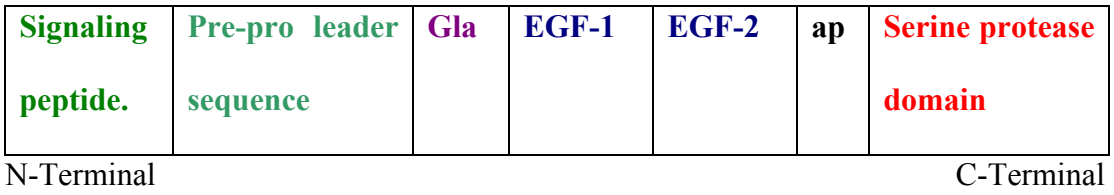

Figure 8. Structure of the FIX precursor protein (Bowen DJ., 2002).

This protein is approximately 34kb in length and contains only 8 exons, the largest of which is 1945bp. The Gla domain is responsible for Ca^{2+} binding, which is necessary for the binding of FIX to phospholipid membranes so FIXa can express its full pro-coagulant activity (Hirowasa , et al., 1990).

FIX is present in a concentration of 4-5 µg/ml with a half-life of approximately 18-24 hours. A 3-fold variation in the activity of FIX in plasma is normal (Hirowasa , et al., 1990).

1.10 Characteristics and Actions of FVIII and FIX Inhibitors

The characteristics and features of inhibitory antibodies may have connotations to the immune response from which they occur. Inhibitory antibodies that bind to the particular regions of the factor molecule inactivate the factor by changing its protein conformation (Christophe, Lenting & Cherel., 2001).

1.10.1 Characteristics of FVIII & FIX inhibitors

The population of inhibitory antibodies formed in patients with haemophilia A and B after exposure to exogenous FVIII and FIX consists of polyclonal IgG antibodies. The majority of these antibodies belong to the immunoglobulin (Ig)G4 subtype, although this IgG type only accounts for a few percent of the IgG fraction in normal plasma (Christophe, Lenting & Cherel., 2001).

The patho-physiologic mechanisms explaining and determining the type of immune responses are not known, and it is not possible to predict which immune response will be encountered in individual patients. However, a better understanding of this process may provide new treatment options, as patients with low levels of the inhibitor will experience a haemostatic effect of regular replacement therapy as long as saturating doses of the factor are given.

1.10.2 Actions of FVIII Inhibitors.

The main inhibitory action of anti-factor VIII antibodies involves steric hinderance of the interaction with both the enzyme FIXa and the substrate FX (Lollar, 2004). In addition, antibodies binding to the C-terminal segment of the molecule prevent it from binding to phospholipids, and antibodies directed towards both the heavy and the light chains may compete with the normal protective binding of VWF, thereby causing an enhanced clearance of FVIII from the plasma. Although the entire FVIII molecule potentially may serve as a target for antibodies, early experiments indicated that the major binding areas were localized to the A2 domain and the light chain. (Scandella, DeGraaf, Mattingly, et al., 1988; Scandella, Mattingly, de Graaf & Fulcher., 1989).

The use of recombinant hybrid human/porcine FVIII molecules has allowed a detailed characterization of the antibody-binding region within the A2 domain and found it to involve residues Arg484 to Ile508 (Healey, Lubin & Nakai., 1995). Experiments with monoclonal antibodies and synthetic peptides have identified additional inhibitory epitopes in the heavy chain, i.e. in the acidic areas flanking the A2 domain (a1 and a2), the binding to which seems to counter-act the proteolytic cleavage by thrombin and FXa generation, as well as the binding of the VWF (Fig. 9) (Raut, Villard & Grailly., 2003).

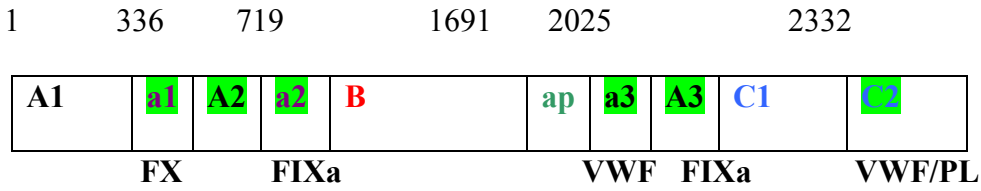

Fig. 9. Schematic model showing the domain structure of factor VIII (FVIII) and the localization of the main binding epitopes of FVIII antibodies highlighted (Bowen DJ., 2002).

1.10.3 Actions of FIX Inhibitors.

Antibodies to FIX are rarely encountered and typically seen in less than 5% of the patients. Consequently, the recognition sites and functional implications are not as well characterized as for anti-factor VIII antibodies. However, by using FX chimeric molecules consisting of various modules of FIX, Christophe, Lenting & Cherel (2001), were able to localize the main binding epitopes to the Gla region and the serine protease domain (Fig. 11). Some patients had antibodies directed against both areas, but no antibodies were found to recognize the EGF like modules. They could also not identify antibodies towards the activation peptide, although antibodies directed towards this region have previously been described using synthetic peptide.

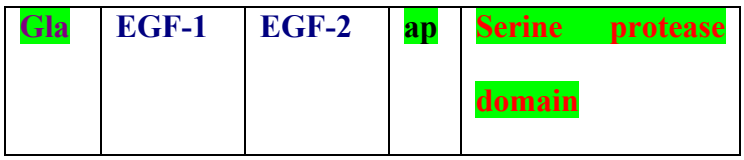

Figure. 10. Schematic model showing the domain structure of factor IX (FIX) and the main binding areas of inhibitory FIX antibodies highlighted (Bowen DJ., 2002).

1.11 Cytokines

The cytokine family consists mainly of smaller water soluble proteins and glycoproteins with a mass of 8-30 kDa. Cytokines are a group of proteins and peptides that function in organisms as signaling compounds between cells. These chemical signals are similar to neurotransmitters and hormones, but while hormones are released from specific organs into the blood and neurotransmitters are released by nerves, cytokines are released by many types of cells. Cytokine is a general name; other names include monokine (cytokines made by monoocytes), lymphokine (cytokines made by lymphocytes), chemokine (cytokines with chemotactic activities), and interleukin (cytokines made by leukocytes and acting on other leukocytes).

Cytokines have a central role in the immune system and are involved in a variety of immunological, inflammatory and infectious diseases (Balkwill.,2001; Oppenheim., 2002).

1.11.1 Cytokine Effects

Each cytokine binds to a specific receptor on the target cell surface. Subsequent cascades of intracellular signaling then alter the cell's functions. This may include the upregulation or downregulation of several genes and their transcription factors, thereby promoting the production of other cytokines, increasing surface receptors and the downstream signals activated by receptor binding. Cytokines are characterized by

functional pleiotropy, short in vivo half lives and redundancy, which highly complicate their isolation and characterization, making scientific studies investigating cytokines in haemophilia very fragmentary (Oppenheim, 2002).

Generalization of functions is not possible with cytokines but these functions can be safely grouped as autocrine, if the cytokine acts on the cell that secretes it, paracrine if the action is restricted to the immediate vicinity of the cytokine's secretion, and endocrine if the cytokine is carried by blood to distant body areas to affect different tissues (Oppenheim, 2002).

The largest group of cytokines stimulates immune cell proliferation and differentiation. This group includes Interleukin 1 (IL-1), which activates T cells; IL-2, which stimulates proliferation of antigen-activated T and B cells; IL-4, IL-5, and IL-6, which stimulate proliferation and differentiation of B cells; IFN-γ, which activates macrophages; and IL-3, IL-7 and Granulocyte Monocyte Colony-Stimulating Factor (GM-CSF), which stimulate haematopoiesis. (Balkwill,2001; Oppenheim, 2002).

Other groups of cytokines include interferons and chemokines. Interferons (IFN-α and IFN-β) inhibit virus replication in infected cells, while IFN- γ stimulates antigen-presenting cell MHC expression. Chemokines attract leukocytes to infection sites (Balkwill,2001).

Helper T cells have two important functions i.e. to stimulate cellular immunity and inflammation and to stimulate B cells to produce antibody. Two functionally distinct subsets of T cells secrete cytokines which promote these different activities. T Helper 1 (Th1) cells produce IL-2, IFN-γ, and TNF-α, which activate T cells and macrophages to stimulate cellular immunity and inflammation. Th1 cells also secrete IL-3 and GM-CSF to stimulate the bone marrow to produce more leukocytes. T Helper 2 (Th2) cells secrete IL-4, IL-5, IL-6, and IL-10, which stimulate antibody production by B cells. (Hoots, 2006).

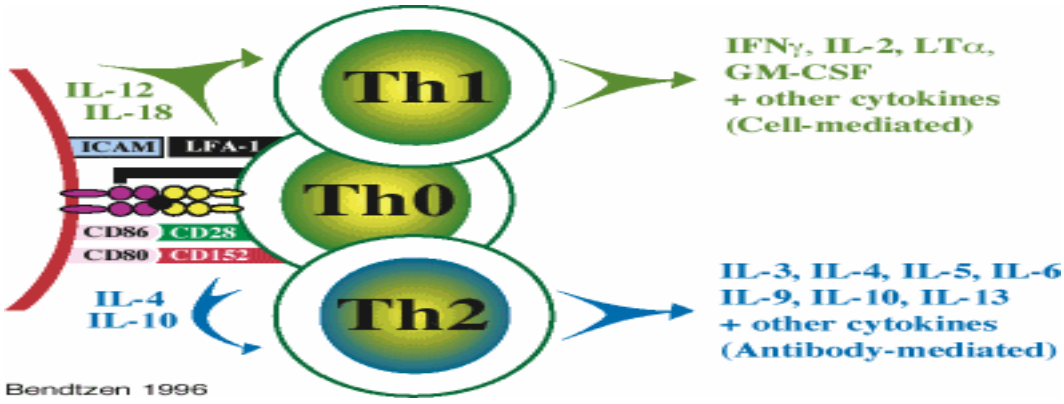

Figure 11. Cytokines control the immune response by influencing and changing the balance of Th1 and Th2 cells. This in turn has a direct effect on what type of immune response will occur. When the Th1 cells outnumber the Th2 cells, cell-mediated immunity is induced, including the activation of cytotoxic T cells and the delayed-type hypersensitivity reaction. When Th2 cells predominate, the humoral arm of the immune response is targeted. (Hoots, 2006).

T cells are initially activated as Th0 cells, which produce IL-2, IL-4 and IFN-γ. The nearby cytokine environment then influences differentiation into Th1 or Th2 cells. IL-4 stimulates Th2 activity and suppresses Th1 activity, while IL-12 promotes Th1 activities. Th1 and Th2 cytokines are antagonistic in activity. Th1 cytokine IFN- γ inhibits proliferation of Th2 cells, while IFN-γ and IL-2 stimulate B cells to secrete IgG_2 and inhibit secretion of IgG_1 and IgE. Th2 cytokine IL-10 inhibits Th1 secretion of IFN- γ and IL-2; it also suppresses Class II MHC expression and production of bacterial killing molecules and inflammatory cytokines by macrophages. IL-4 stimulates B cells to secrete IgE and IgG_1. The balance between Th1 and Th2 activity may steer the immune response in the direction of cell-mediated or humoral immunity (Balkwill,2001; Oppenheim, 2002).

The selected cytokines by virtue of their potential involvement in anti- FVIII/FIX antibody production, and inflammatory response perpetuation, which were analyzed in this study, are the Th1 cytokines IL-1β, IL-2, IFN-γ, and TNF-α and Th2 cytokine IL-4, IL-6 and IL-10.

TNF-α and IL-1β have been found to be up-regulators of tissue factor expression. This promotes coagulation as tissue factor plays a central role in the extrinsic pathway of coagulation (Van den Berg, et al., 2007). These cytokines were analyzed and compared to the patient's phenotypic presentations as this could rationalize the clinically mild presentations in biochemically severe patients.

Inflammation of the synovial membrane caused by the recurrent presence of blood has been shown to result in the production of pro-inflammatory cytokines such as IL-1, IL-6, and TNFα in haemophilic patients (Roosendaal, Vianen, Wenting, et al., 1998), therefore these cytokines were analyzed in the patients to determine if the presence of inflammatory states has any relation to the inhibitor development as well as phenotypic presentations of the patients. These pleiotrophic cytokines, IL-1 and IL-6 are also promoters of antibody production by supporting the growth and differentiation of B-cells which then produce antibodies (Chapel & Mansel., 1999).

Knowledge about the specific T cell mechanisms responsible for inhibitor development is very limited, but the inhibitor antibodies have been found in most investigations to be of the IgG isotype (Hirowasa, et al., 1990), therefore the presence of cytokines such as the Th2 cell derived IL-4, IL-6, and IL-10 which activates B-lymphocytes resulting in the up-regulation of antibody production was anticipated in patients who develop inhibitors.

Their short half life, low plasma concentrations, pleiotropy, and redundancy all complicate the isolation and characterization of cytokines and this has resulted in a paucity and fragmentation of cytokine studies in haemophilia.

1.12 Polymorphisms in Immune Response Genes

Cytokines are more or less directly involved in the antibody mediated immune response and therefore have the potential to be determinants for the immune response.

IL-10 is an important anti-inflammatory cytokine, and it exerts a broad spectrum of activities. IL-10 enhances the in vitro production of all types of immunoglobulins by peripheral blood mononuclear cells (PBMCs) in patients with systemic lupus erythematosus (SLE), and the serum concentration of IL-10 is correlated with disease activity in these patients (Llorente L, Zou W & Levy Y., 1995). In addition, a 134-bp variant of a CA repeat microsatellite in the promoter region of the IL10 gene is associated with autoantibody concentrations in SLE, myasthenia gravis, and Wegener granulomatosis and with the concentration of the monoclonal immunoglobulin in multiple myeloma (Huang, Zhou, Xia, et al 1999, Zhou, Giscombe, Huang & Lefvert.,2002).

Polymorphisms in immune response genes have been suggested to be involved in determining inhibitor risk. A strong association ($P<0.001$) between an allele with 134bp in one of the CA repeat microsattelites of the IL-10 gene, and inhibitor development was found in the MIBS (Astermark, Oldenburg, Pavlova, et al., 2006), but no associations were found between inhibitor development and the IL-1β

restriction length polymorphisms and the single nucleotide polymorphisms in the promoter regions of IL-4 genes (Astermark, et al., 2006).

-1173

CTGCCCCG**GT** **CCTTCCCCAG** **GTAGAGCAAC** **ACTCC**TCGCC TGGCTCCCCT TACCTTCTAC **ACACACACAC ACACACACAC ACACACACAC** AAATCCAAGA **CAACACTACT AAGGCTTCTT TGGGA**AGGGG

-1083

Figure 12. The portion of the IL-10 gene promoter region showing the CA repeat microsattelites (red). The blue highlights are the forward and reverse primer compliments used in the current study (Eskdale, Kube, Tesch & Gallagher., 1997).

A preceding study reported a higher frequency of inhibitors and the IL-10 gene CA repeat microsattelites in African Americans compared to Caucasian people (Astermark, et al., 2001). In the present study, we evaluated whether the 134bp variant of a CA repeat microsattelite in the promoter region of the IL10 gene is associated with inhibitor development as well as the existence of ethnic variations in our study population. Alleles were named after the length of PCR products.

Polymorphisms in the IL-10 gene of patients with and without inhibitors, may lead to an understanding of the immuno-biology of FVIII and FIX inhibitors and ultimately guide the therapeutic strategy aimed at suppressing inhibitor formation.

1.13 Aim of the Present Study

The underlying mechanism and determinants of inhibitor formation are not fully understood, and a large amount of the data on immune responses against FVIII and FIX is from animal models. Studies investigating cytokines in haemophilia are very limited and fragmentary, and the classification of haemophilia patients according to their factor activity levels has been observed to be inconsistent. Finding the associations between factor activity, clinical phenotype, cytokine profiles and polymorphisms in immune response genes of haemophilia A and B patients with and without inhibitors, may give more insight into the pathophysiology of haemophilia, improve the understanding of the pathogenic mechanisms that underlie inhibitor development, and facilitate new diagnostic and therapeutic strategies for haemophilia.

CHAPTER 2: MATERIALS AND METHODS

2.1. Study Population

Haemophilia A and B patients with and without inhibitors were studied. Forty (40) patients from the CMJAH-HCCC were randomly enrolled in the study. Both inhibitor and non-inhibitor patients were included. Attempts were made to get an equal number of patients who are frequent bleeders and those who are infrequent bleeders. Frequent bleeders were defined as those patients with 2 or more bleeding episodes per month on three consecutive months. Bleeding frequency was be evaluated on the patient's bleeding charts.

2.1.2 Inclusion Criteria

-Participants had to sign an informed consent form to participate in the study.

-Congenital haemophilia A and B patients were selected.

2.1.3 Exclusion Criteria

-Patients who had not signed consent forms.

-HIV positive patients.

-Patients with renal diseases as diagnosed by creatinine levels >130mg/dL.

-Diabetic patients as diagnosed by fasting glucose levels > 7mmol/L.

-Hypertensive patients as diagnosed by blood pressure levels of ≥140/90 mmHg.

-Obese patients as diagnosed by a BMI ≥29.5kg/m^2

This information was obtained from the CMJAH-HCCC database.

Patients with HIV, renal diseases, diabetes, hypertension, and obesity would have been sources of bias, and confounding variables as they have been scientifically proven to have increased cytokine levels (Blake & Ridker., 2004).

2.2 Study Design

Cross-sectional study.

2.3 Methodology Outline

- Potential participants were randomly selected from the CMJAH-HCCC records based on the inclusion and exclusion criteria review of clinical history.
- Eligible participants were provided with a written participant information leaflet, and requested to sign a written consent form.
- The haemophilia clinic staff took venous fasting blood samples from patients as they came for their routine clinic visits.
- FVIII and FIX activity levels of all patients were measured using the Dade Behring Sysmex CA-7000 coagulation analyzer, and information on each patient's bleeding episodes, was obtained from the CMJAH-HCCC database.
- The inhibitor status of all patients was evaluated using the Bethesda inhibitor assay after being exposed to factor.

- IL-1β, IL-6 and TNFα were analyzed in all patients using ELISA kit methods.
- IL-2, IL-4, IL-10 and IFN-γ were quantified by the Cytometric Bead Array method.
- DNA was extracted using the Nucleon BACC3 kit from Amersham Biosciences.
- Polymorphisms in immune response gene of the Th2 cytokine IL-10 were analyzed using PCR.
- The Statistica Release 8 statistics package was used for statistical analysis.

2.4 Blood Collection and Storage.

Blood was collected by the CMJAH-HCCC staff, in the morning between 8:00 and 9:00 after an overnight fast (10-12hours). For cytokine studies, venous blood samples were collected into a yellow top vacutainer tube with no anticoagulant. This blood sample was centrifuged at 3 500rpm for 20 minutes and the resultant serum was aliquoted into clean eppendorf tubes and stored at -70^0C until use. For genetic studies, venous blood was collected in EDTA vacutainer tubes (purple top) and centrifuged at 2000 rpm for 5 minutes. A buffy coat was transferred into a clean eppendorf tube and stored at -70 degrees Celsius until DNA extraction.

2.5 Quantitative Measure of Inhibitors

The Bethesda method used in the Main Haematology Laboratory was used for the determination of the inhibitor status of all patients.

Factor VIII inhibitors are time dependant. Thus factor VIII was added to plasma containing an inhibitor and the mixture was incubated. Factor VIII was progressively neutralized by the inhibitor. The amount of factor VIII added and the duration of incubation were standardized. The strength of the inhibitor was measured in units according to how much of the added factor VIII was destroyed.

In the Bethesda assay, 1Bethesda Unit (BU) was defined as the amount of inhibitor, which will neutralize 50% of factor VIII in 2 hours at 37^0C. Dilutions of test serum were incubated with an equal volume of the normal serum pool at 37^0C. The normal serum pool was taken to present 1 unit of factor VIII/IX. Dilutions of control normal plasma containing no inhibitor were treated in the same way.

At the end of the incubation period the residual factor VIII/IX was assayed and the inhibitor strength was calculated from a graph of residual factor VIII/IX activity versus inhibitor units.

2.6 Clotting Factor Activity Determination

The Dade Behring Sysmex CA-7000 coagulation analyzer was used for factor activity determination. This instrument has the capacity to provide direct haemostasis measurements and calculated parameters, by using the clotting method, chromogenic principle and the Immunoassay method. The clotting method was used to determine the factor activity levels.

The clotting method uses the principle of optical detection (light scattering) and therefore detects the change in turbidity with clot formation. The factor deficient plasma was reconstituted with 1ml of distilled water and left to stand 15 minutes. This was then placed in position F of the Marburg, Dade Behring Sysmex CA-7000 analyzer. Thromborel S used for extrinsic pathway factors was also reconstituted with 10ml distilled water and incubated to reach 37^0C, mixed and placed in position R1-R4. APTT Actin FSL used for the extrinsic pathway factors was placed in position R5-R7. Calcium chloride was placed in position R1-R4 and Owren`s buffer was placed in the buffer table. The sample and reagents were mixed by centrifugal force. The light ray was directed to the measuring cuvette of the rotor by means of an optic fiber system and the scattered light was read at a wavelength of 600nm by means of a solid state detector. A curve was generated which plotted time against the amount of scattered light and expressed in percentages.

2.7 Cytokine Quantification

2.7.1 IL-1β, IL-6 and TNF-α Quantification

Specific BioSource Enzyme Amplified Sensitivity Immunoassays Enzyme Linked Immuno Sorbent Assay (ELISA) were used for the determination of individual cytokines. The ELISA kits were first optimized using normal serum prior to analysis of participant samples.

The BioSource ELISAs were performed on microtiter plates. The assays use monoclonal antibodies (MAbs) directed against distinct epitopes of cytokines. Calibrators and samples reacted with the capture monoclonal antibody (MAb 1) coated on microtiter well and with a monoclonal antibody (MAb 2) labelled with horseradish peroxidase (HRP).

After an incubation period that allowed the formation of a sandwich: coated MAb 1 – human cytokine – MAb 2 – HRP, the microtiterplates were washed to remove unbound enzyme labelled antibody. Bound enzyme labelled antibodies were measured through a chromogenic reaction.

Chromogenic solution (TMB) was added and incubated. The reaction was stopped with the addition of Stop Solution and the microtiterplate was then read at the appropriate wavelength. The amount of substrate turnover was determined colourimetrically on an ELISA reader, by measuring the absorbance, which was proportional to the cytokine concentration.

A calibration curve was plotted and the cytokine concentration in samples was determined by interpolation from the calibration curve.

2.7.2 IL-2, IL-4, IL-10 and IFN-γ Quantification

The multiplexed cytometric bead array (CBA) system from BD Bioscience was used for the quantification of the above cytokines. This technology combines flow cytometry and ELISA. Flow cytometry is an analysis tool that enables us to measure multi-parametric physical characteristics of single cells or particles in solution. Multiplexing is the simultaneous assay of many analytes in a single sample. The BD™ CBA system employs a series of particles with discrete fluorescence intensities to simultaneously detect multiple soluble analytes. It is combined with flow cytometry to create a powerful multiplexed assay.

Test samples were diluted using the assay diluent and transferred to tubes containing mixed capture beads and phycoerythrin (PE) detection reagent. PE absorbs light at 532nm (excitation) and emits light whose fluorescence intensity is equal to the amount of captured antibody. Fifty micro-liters (50µl) of this mixture was added to 50µl of human Th1/Th2 PE detection reagent and 50µl of the resulting solution incubated for 3 hours at room temperature in the dark. One milliliter of wash buffer was then added and the assay tubes were centrifuged at 200xg for 5 minutes. The supernatant was discarded and the pellets were resuspended in wash buffer. Samples were then read in a BD FACSArray Bioanalyzer. This analyzer has laser light at

532nm that the fluorochrome PE absorbs and then releases by vibration and heat dissipation. This emitted light was directed by filters to the appropriate optical detectors and then converted to proportional digital signals.

2.8 DNA Extraction

DNA was extracted using the Nucleon BACC3 from Amersham Biosciences.

Cell Preparation

Blood was collected in EDTA tubes and 10ml was transferred into 50ml polypropylene centrifuge tubes.

Cell Lysis

Four times the volume of blood (40ml) of reagent A was added to the blood sample to separate the cells and break down the cell and nuclear membranes. The tubes were then rotary mixed for 4 minutes at room temperature then centrifuged at 1300g for 4 minutes. The supernatant was discarded without disturbing the pellet.

To the pellets, 2.0ml of Reagent B (detergent) was added to remove the lipid membranes. This was vortexed briefly to re-suspend the pellets. The suspension was then transferred to a screw-capped polypropylene micro-centrifuge tube**.**

Deproteinisation

500µL of the enzyme sodium perchlorate was added to remove proteins from the DNA, and mixed by inverting at least 7 times. The sodium also aids in the precipitation of dissolved DNA.

DNA Extraction

2mL of chloroform was then added and also mixed by inverting 7 times to emulsify the phases. The chloroform ensures that the proteins remain in an organic phase and can be drawn off carefully. 300µL of Nucleon Resin was added and without re-mixing the phases, centrifuged at 1300g for 3 minutes.

DNA Precipitation

Without disturbing the Nucleon Resin layer (brown in colour), the upper phase was transferred to a 5ml tube. When the dissolved salty DNA comes into contact with ethanol, it precipitates. Two volumes of cold absolute ethanol were added and then inverted several times until the DNA was precipitated.

DNA Washing

This was then centrifuged at top speed for 5 minutes to pellet the DNA. The supernatant was then discarded and 1ml of cold 70% ethanol was added and mixed several times by inversion. This was re-centrifuged and the supernatant discarded. The pellet was air dried for 10 minutes to ensure total removal of the ethanol. The DNA was re-dissolved in distilled water and placed on a rotary mixer for 2 hours.

2.8.2 DNA Quantification and Purity Check.

The absorbance of suspended DNA was measured at 260nm and 280nm in an appropriately callibrated Nanodrop Spectrophotometer. The ratio of DNA absorbance at 260/280 was calculated and used as a measure of DNA purity. The DNA was stored at -20^0C until further analysis.

2.9 PCR Master Mixture Preparation

The Roche Expand High Fidelity PCR System was used for the Polymerase Chain Reaction (PCR). The PCR master mixture comprised of the reagent shown in table 2.1 below.

Table 2.1 Master mixture components

Master mixture components	**Volume (µl)**	**Final concentration**
Distilled water	18.5 (up to 50µl)	-
Reaction Buffer 5x with 7.5 Mm $MgCl_2$	10	1x
dNTP mixture	1	0.2mM
Forward primer	5	10pmol
Reverse primer	5	10pmol
Enzyme blend	0.5	2.5 U
DNA	10	
Total volume	50	

Table 2.2 IL-1O G primer properties

Primer name	**Primer sequence**	**Annealing temperature**
IL-10-1	5`GTCCTTCCCCAGGTAGAGCAACACTCC3`	65^0C
IL-10-2	5`CTCCCAAAGAAGCCTTAGTAGTGTTG3`	65^0C

This mixture was then put in the thermal cycler with the following settings.

Table 2.3 DNA Amplification .

Temperature (^{0}C)	**Time**		**Step**
95	5 minutes	1x	Initial denaturation
94	30 seconds	30x	DNA denaturation
65	1 minute		Annealing
72	1 minute		DNA elongation
72	5 minutes	1x	Final extension
4	Until removed from cycler		Cooling

2.10 DNA Agarose Gel Fractionation

A 2% gel was prepared with 3 g agarose powder and 150ml TBE buffer. 7.5 µl of Ethidium bromide was added. 25 µl of DNA was mixed with 5 µl of loading dye and loaded on the gel. A 50 base pair ladder was loaded in lane 1 as a DNA size marker. This was then run at 120 volts for 60 minutes. The gel was then viewed and photographed under ultra violet light. Alleles of the targeted region of the IL-10 gene promoter were named after the length of PCR products.

2.10 Statistical Analysis

The Statistica Release 8 statistics package was used for statistical analysis. The normally distributed parameters were expressed as Means (95% Confidence Intervals), and the abnormally distributed data was log transformed and presented as Medians (Interquartal Ranges). The mean value of each item between groups was compared using the student t test. Pearson Correlation was performed and the probability value (p value) <0.05 was defined as significant.

From the 40 patients enrolled, haemophilia A and B patients were tested as one group as the only distinguishing factor between the two is the specific factor deficiency. The fundamental biochemical lesion underlying haemophilia is an insufficiency of the

activity of the tenase complex. It is therefore not surprising that the two disorders are clinically similar.

The mild and moderate patients were analyzed as one group as their phenotypic presentations have been observed in the CMJAH-HCCC to be indistinguishable and they are handled the same therapeutically. The groups compared were therefore Mild & Moderate vs. Severe; Inhibitor vs. Non-inhibitor; Frequent vs. Non- frequent bleeders.

2.10.1 Categorising codes used for statistical analysis

Table 2.4 Variable codes

Variable	**Codes**	
	1	**2**
Sex	Male	Female
Race	Black	White
Bleeding Frequency	Frequent bleeder	Infrequent bleeder
Biochemical Classification	Mild/Moderate	Severe
Inhibitors	Non-inhibitors	Inhibitors

Table 2.5 Grouping of participants according to clinical phenotypes and biochemical classifications.

Codes	**Description**
MFB	Mild/Moderate Frequent Bleeder
MNFB	Mild Non-Frequent Bleeder
SFB	Severe Frequent Bleeder
SIFB	Severe In-Frequent Bleeder
ISFB	Inhibitor Severe Frequent Bleeder

CHAPTER 3: RESULTS

Evaluation of distribution showed that all cytokine quantification variables were not normally distributed in all groups. Due to this lack of normality, all zero values were changed to 0.01 and all variables were log transformed for statistical analysis. An example of a log transformed variable is shown below (Fig 3.1.1 and Fig 3.1.2). Results were reported as Median (Interquartal Range).

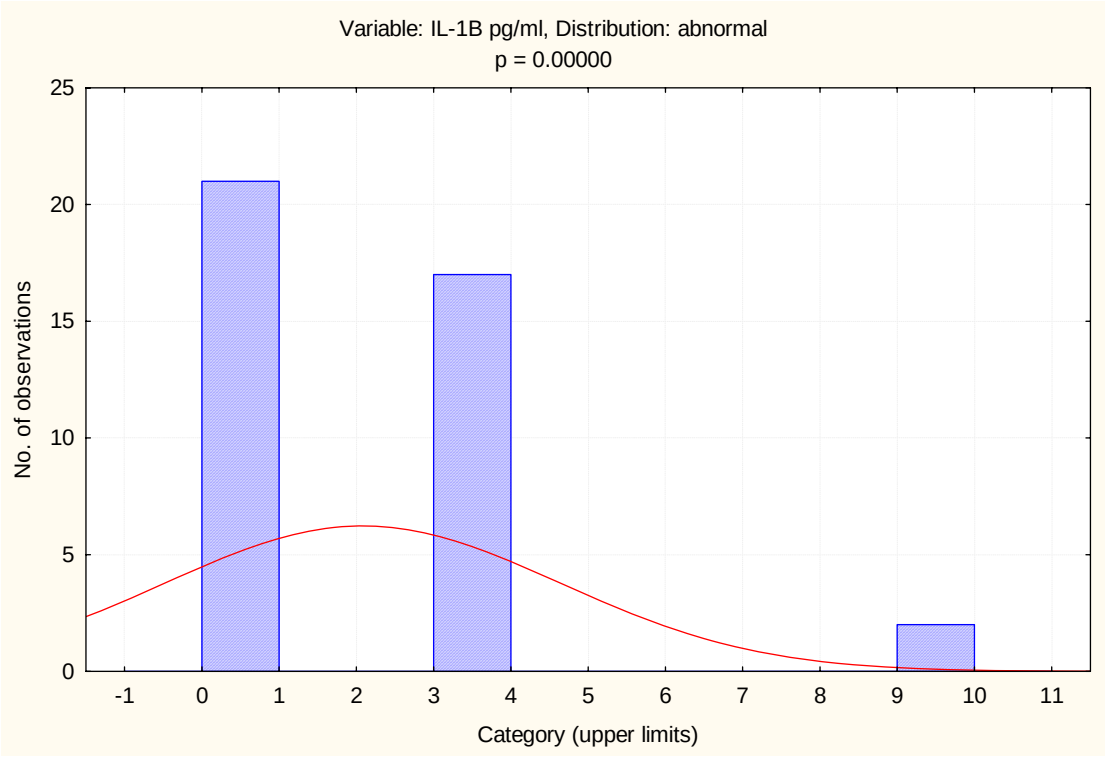

Figure 3.1.1 Histogram showing the abnormal distribution of IL-1β quantification. The graph is not bell-shaped, symmetric or uni-modal and the p=0.0000. A normal distribution shows a bell shaped graph (Gaussian) and a p value that is more than 0.2.

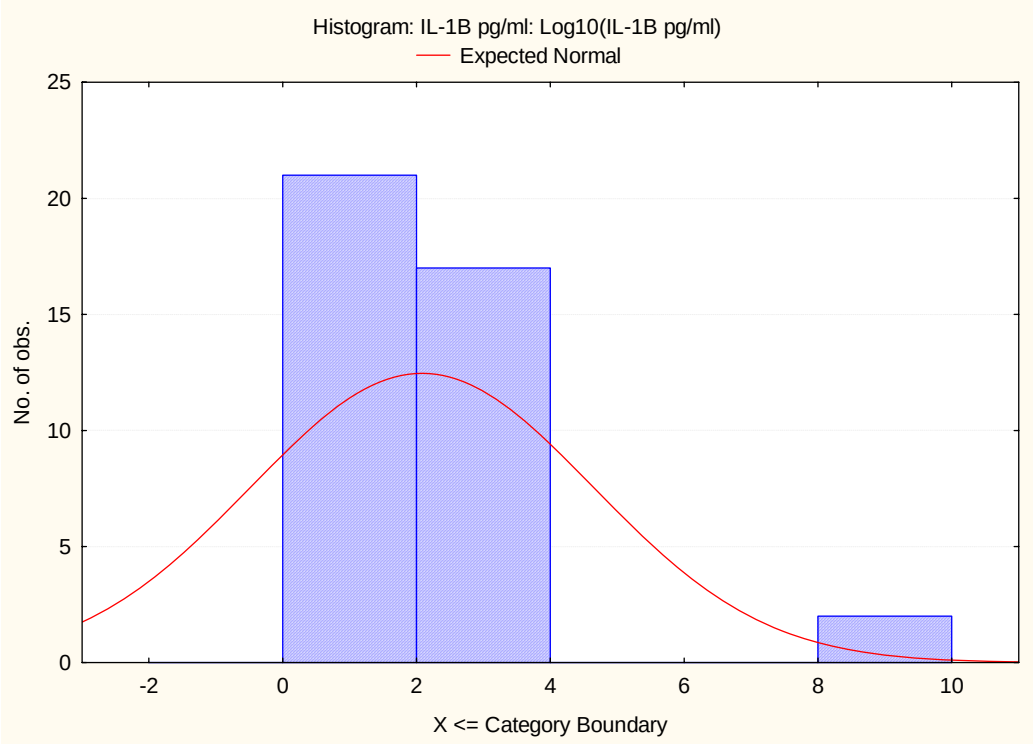

Figure 3.1.2 Histogram showing a log transformed distribution of IL-1β.

Comparison was computed using the independent sample t-test. Correlation studies were computed using the Bivariate-Pearson`s test. Linear regression analysis was performed on all groups with the aim to find independent predictors of Bleeding Frequency and Biochemical Classification.

Participant names in the study were replaced with participant numbers to maintain confidentiality. Eighty potential participants were recruited to the study. Forty (40) of these participants did not meet the eligibility criteria and were therefore excluded from the study (fig 3.1 below). Results of 40 subjects consisting of 35 men and 5 women between the ages of 2-83 were analyzed and reported here.

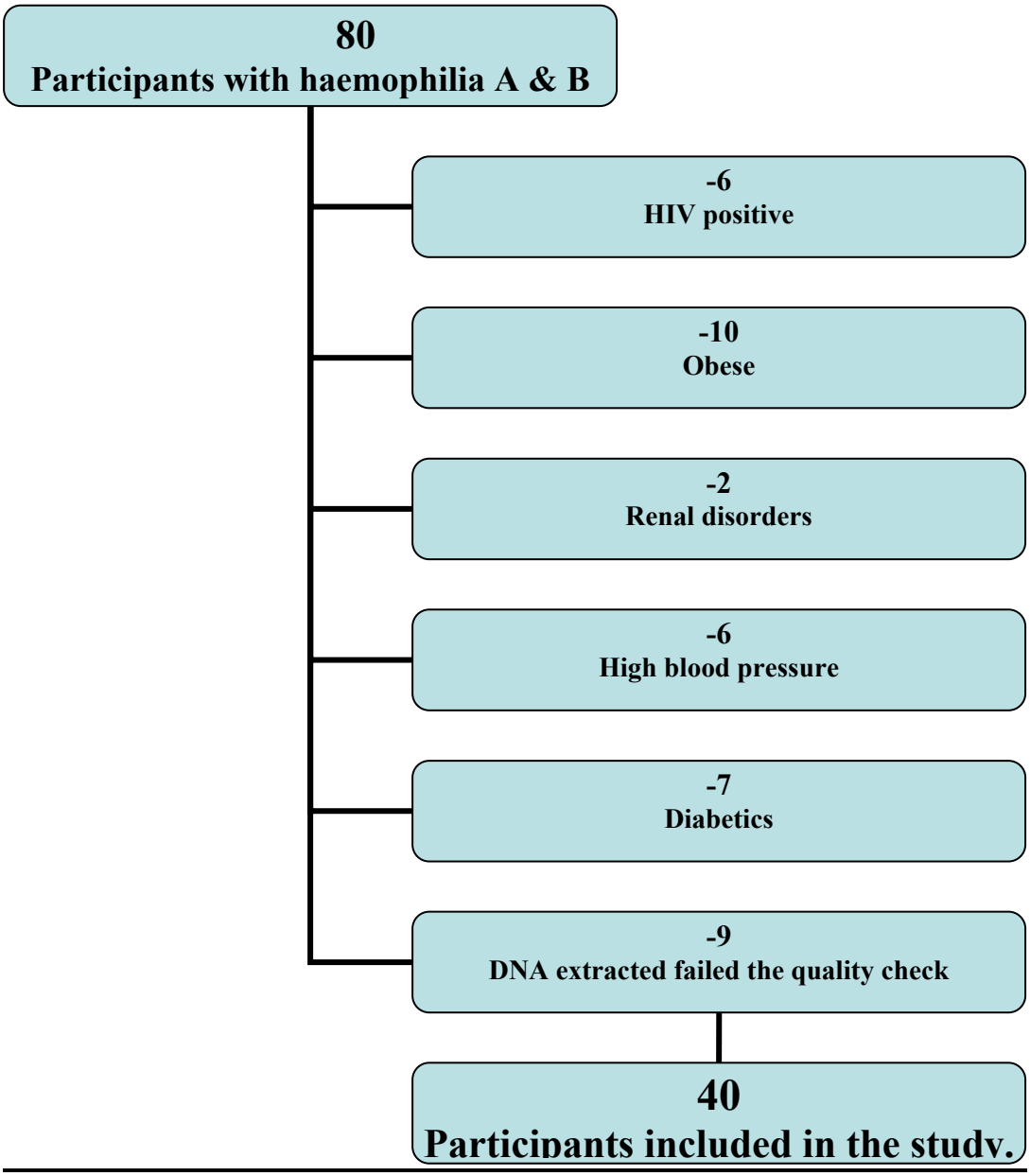

Figure 3.2 Study population selection.

3.1 Demographics of the study population

Table 3.1 Demographics of the study population

Participant number	Race	Age (years)	Gender
MFB1	Black	25	Male
MFB2	Black	35	Male
MFB3	White	56	Male
MFB4	White	25	Male
MFB5	White	50	Male
MFB6	White	40	Male
MFB7	White	8	Male
MFB8	White	6	Male
MNFB1	Black	28	Male
MNFB2	White	45	Male
MNFB3	White	83	Male
MNFB4	White	2	Male
MNFB5	White	56	Male
MNFB6	White	43	Male
MNFB7	White	37	Male
MNFB8	White	42	Male
SFB-1	White	50	Male
SFB-13	Black	31	Male
SFB-7	Black	45	Male
SFB-8	White	66	Male
SFB-9	Black	27	Male
SFB-10	Black	16	Male
SFB-11	White	50	Female
SFB-12	Black	11	Female
SIFB-1	White	69	Male
SIFB-2	Black	29	Male
SIFB-3	White	55	Female
SIFB-4	Black	41	Female
SIFB-5	Black	22	Male
SIFB-6	White	20	Male
SIFB-7	White	30	Male
ISFB1	Black	24	Male
ISFB2	White	32	Male
ISFB3	Black	18	Female
ISFB4	Black	30	Male
ISFB5	Black	26	Male
ISFB6	White	42	Male
ISFB7	Black	39	Male
ISFB8	Black	23	Male
ISFB9	Black	28	Male

Table 3.1 shows the demographics of the study population. The mean age of the 40 patients included in this study was 35.13 years with a range from 2 to 83 years. There was a very strong predominance of males comprising 35 patients (87.5%), and only 5 females (12.5 %) in the study. There were 18 black subjects (45%) and 22 white subjects (55%).

3.2 Quantitative Measure of Inhibitors

Inhibitors were detected in 10 (25%) of the study population as shown in table 3.2 below.

Table 3.2 Participants with inhibitors

Participant numbers	Race	Inhibitor titres (BU)	Responder status (High or Low responder)
ISFB1	Black	20	High responder
ISFB2	White	3.42	Low responder
ISFB3	Black	21.28	High responder
ISFB4	Black	108	High responder
ISFB5	Black	104	High responder
ISFB6	White	1.52	Low responder
ISFB7	Black	527	High responder
ISFB8	Black	20.48	High responder
ISFB9	Black	1000	High responder

Participants with inhibitors were classified according to the definitions of the Factor VIII/IX Subcommittee of the International Society of Thrombosis and Haemostasis as either high or low responder patients. High responder patients were identified by peak inhibitor titres of ›5 BU mL^{-1} and low responders were participants with inhibitor titres ≤ 5 BU mL^{-1} (Qian , Collins ., 2000).

Amongst the 9 patients with inhibitors 7 (77%) were black and 2 were white. All the black patients showed high inhibitor titres ranging from 20 to 1000 BU and were all high responders. The 2 white patients showed low inhibiter titres of 1.52 and 3.42 BU and were low responders.

3.3 Factor Activity, Biochemical Classification and Bleeding Frequency.

Patients were classified as mild / moderate (M) or severe (S) according to their factor activity levels. Severe was defined as <1% factor level, moderate as 1-5% and mild as 5-50% (White, et al., 2001).
Table 3.3 Factor Activity, Biochemical Classification and Bleeding Frequency.

Participant number	Factor activity levels	Classification	Bleeding Frequency
MFB1	4.5%	M	FB
MFB2	3.3%	M	FB
MFB3	30,6%	M	FB
MFB4	28,7%	M	FB
MFB5	4.2%	M	FB
MFB6	4,0%	M	FB
MFB7	7.8%	M	FB
MFB8	11.5%	M	FB
MIFB1	12%	M	IFB
MIFB2	40%	M	IFB
MIFB3	45%	M	IFB
MIFB4	38%	M	IFB
MIFB5	26,8%	M	IFB
MIFB6	12,8%	M	IFB
MIFB7	30,9%	M	IFB
MIFB8	3.7%	M	IFB
SFB-1	<1%	S	FB
SFB-13	<1%	S	FB
SFB-7	<1%	S	FB
SFB-8	<1%	S	FB
SFB-9	<1%	S	FB
SFB-10	<1%	S	FB
SFB-11	<1%	S	FB
SFB-12	<1%	S	FB
SIFB-1	<1%	S	IFB
SIFB-2	<1%	S	IFB
SIFB-3	<1%	S	IFB
SIFB-4	<1%	S	IFB
SIFB-5	<1%	S	IFB
SIFB-6	<1%	S	IFB
SIFB-7	<1%	S	IFB
ISFB1	<1%	S	FB
ISFB2	<1%	S	FB
ISFB3	<1%	S	FB
ISFB4	<1%	S	FB
ISFB5	<1%	S	FB
ISFB6	<1%	S	FB
ISFB7	<1%	S	FB
ISFB8	<1%	S	FB
ISFB9	<1%	S	FB

Frequent bleeders (FB) were defined as those patients with 2 or more bleeding episodes per month on three consecutive months.

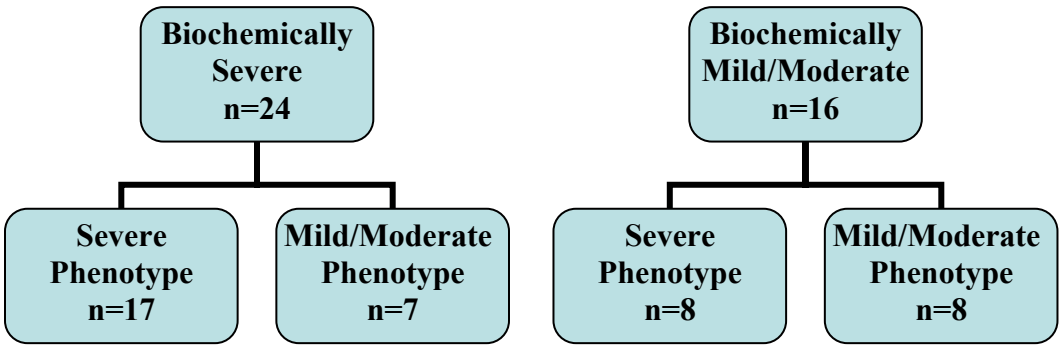

Figure 3.3 Stratification of participants according to severity of bleeding.

Twenty nine percent (29%) of the biochemically severe haemophilia patients (factor level<1%) were infrequent bleeders (IFB) and 50% of the biochemically mild/moderate (factor levels of 1-50%) haemophilia patients were frequent bleeders (FB).

3.4 IL-1β, IL-6 and TNF-α Quantification

Table 3.4 IL-1β, IL-6 and TNF-α Quantification. Amplified Sensitivity ELISA.

Participant numbers	Classification	Bleeding Frequency	IL-1B pg/ml	IL-6 pg/ml	TNF-α pg/ml
MFB1	M	FB	3.75	57.5	11
MFB2	M	FB	3.75	57.5	83.5
MFB3	M	FB	3.75	51.5	83
MFB4	M	FB	3.75	57.5	79
MFB5	M	FB	3.75	57.5	102
MFB6	M	FB	3.75	51.5	93
MFB7	M	FB	0,01	51.5	88
MFB8	M	FB	0,01	57.5	97.5
MIFB1	M	IFB	9.75	103	96
MIFB2	M	IFB	3.75	57.5	105
MIFB3	M	IFB	0,01	68	106
MIFB4	M	IFB	3.75	34.5	109
MIFB5	M	IFB	3.75	34.5	109
MIFB6	M	IFB	0,01	34.5	93
MIFB7	M	IFB	0,01	57.5	508
MIFB8	M	IFB	3.75	57.5	111
SFB-1	S	FB	3.75	57.5	107
SFB-13	S	FB	0,01	34.5	109
SFB-7	S	FB	0,01	34.5	103
SFB-8	S	FB	0,01	34.5	105
SFB-9	S	FB	0,01	68	107
SFB-10	S	FB	0,01	34.5	95
SFB-11	S	FB	0,01	34.5	106
SFB-12	S	FB	3.75	34.5	108
SIFB-1	S	IFB	3.75	34.5	99
SIFB-2	S	IFB	0,01	593	106
SIFB-3	S	IFB	0,01	417	98
SIFB-4	S	IFB	0,01	417	106.5
SIFB-5	S	IFB	0,01	417	103
SIFB-6	S	IFB	0,01	417	104
SIFB-7	S	IFB	0,01	593	110
ISFB1	S	FB	3.75	593	115
ISFB2	S	FB	3.75	593	247
ISFB3	S	FB	0,01	593	205
ISFB4	S	FB	0,01	593	202
ISFB5	S	FB	3.75	593	120
ISFB6	S	FB	0,01	417	114
ISFB7	S	FB	3.75	417	113
ISFB8	S	FB	0,01	417	107
ISFB9	S	FB	9.75	417	102

Table 3.4.1. Cytokine levels of Mild/Moderate (M) compared to Severe (S) haemophiliacs.

Variables	Clinical Phenotype	N	Median (Interquartal Range)	p-value
IL-1β	M	16	3.73(0.01-3.75)	0.007*
	S	15	0.01(0.01-0.01)	
IL-6	M	16	57.50(51.5-57.5)	0.009*
	S	15	57.5(34.5-417.0)	
TNF-α	M	16	96.75(85.75-107.5)	0.65
	S	15	106(103.0-107.0)	

Severe haemophilia patients had significantly higher (p=0.009) levels of IL-6 than the Mild/Moderate group. IL-1β was found to be significantly higher (p=0.007) in the Mild/Moderate group than in the Severe group.

Table 3.4.2. Cytokine levels of Frequent (FB) compared to Infrequent Bleeders (IFB).

Variables	Frequent/Infrequent Bleeders	N	Median (Interquartal Range)	p-value
IL-1β	FB	25	3.75(0.01-3.75)	0.5
	IFB	15	0.01(0.01-3.75)	
IL-6	FB	25	57.5(51.5-417.0)	0.8
	IFB	15	68.00(34.5-417.0)	
TNF-α	FB	25	106.0(95.0-113.0)	0.4
	IFB	15	106.0(99.0-109.0)	

No significant differences were found between cytokine profiles of frequent and infrequent bleeders.

Table 3.4.3 Inhibitor (I) vs. Non-Inhibitor (NI) patients.

Variables	Inhibitors/Non-Inhibitors	N	Median(Interquartal Range)	p-value
IL-1β	I	9	3.75(0.01-3.75)	0.5
	NI	31	0.01(0.01-3.75)	
IL-6	I	9	593.00(417.0-593.0)	<0.002*
	NI	31	57.5 (34.50-68.00)	
TNF-α	I	9	115.00(113.00-202.00)	0.03*
	NI	31	104.00(95.00-107.00)	

IL-6 and TNF-α were found to be significantly higher ($p<0.0002$ and $p=0.03$ respectively) in inhibitor haemophiliacs than in haemophiliacs without inhibitors. The IL-1β levels were also slightly higher, but this difference was statistically not significant ($p=0.5$).

Interestingly, there were no statistically significant differences in cytokine profiles of haemophiliacs who had conflicting phenotypic features and those with theoretically rational phenotypes.

Table 3.4.4 Low Responder vs. High Responder patients.

Participant numbers	Race	Inhibitor titres (BU)	Responder status (High or Low responder)	IL-1B pg/ml	IL-6 pg/ml	TNF-a pg/ml
ISFB1	Black	20	High responder	3.75	593	115
ISFB2	White	3.42	Low responder	3.75	593	247
ISFB3	Black	21.28	High responder	0.01	593	205
ISFB4	Black	108	High responder	0.01	593	202
ISFB5	Black	104	High responder	3.75	593	120
ISFB6	White	1.52	Low responder	0.01	417	114
ISFB7	Black	527	High responder	3.75	417	113
ISFB8	Black	20.48	High responder	0.01	417	107
ISFB9	Black	1000	High responder	9.75	417	102

Due to the fact that there were only 2 Low Responders compared to 7 High Responders, statistical analysis was not done. Observational analysis showed that there are no differences in the cytokine profiles of Low and High Responders.

3.5 IL-2, IL-4, IL-10 and IFN-γ Quantification (CBA)

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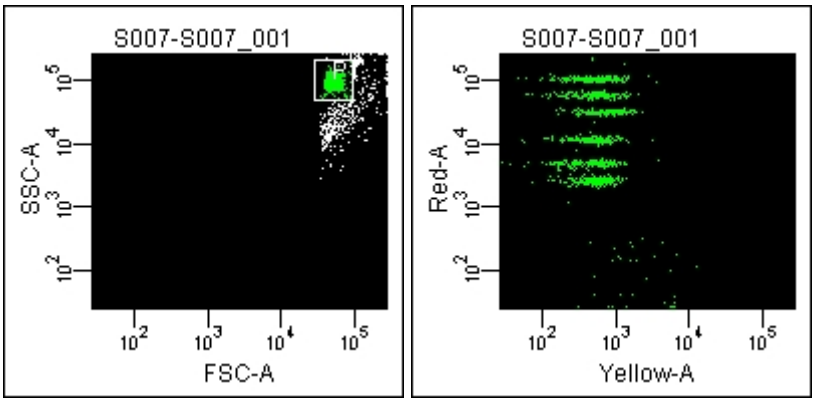

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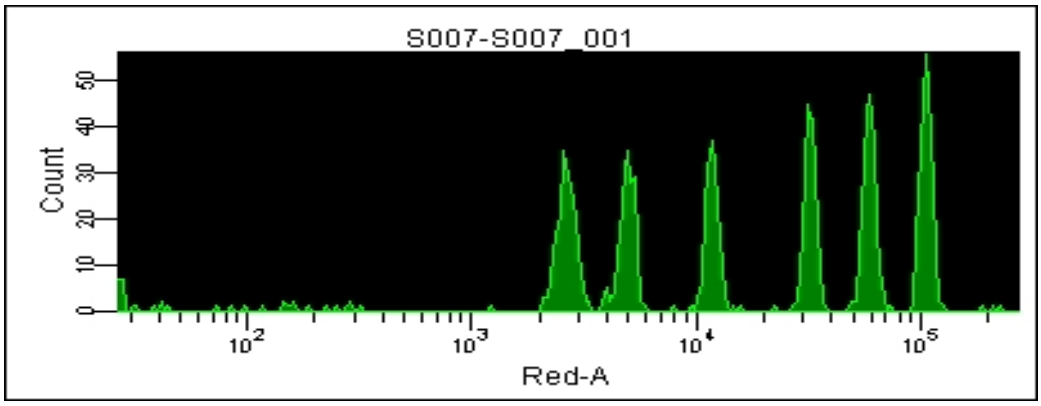

Figure 3.5.1 CBA Data Acquisition on the FACS Array instrument from BD Biosciences.

1) The gate P1 was placed around the bead population and adjusted well. The threshold was set on the forward scatter in order to eliminate debris.
2) The cytokine concentration (labeled with PE) was detected on the yellow channel. The cytokines represented by the bead populations from top to bottom are, -: IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α.
3) The bead peaks were all distinguished on the Red histogram.

Table 3.5 IL-2, IL-4, IL-10 and IFN-γ Quantification (CBA)

Participant number	Classification	Bleeding Frequency	IL-2 pg/ml	IL-4 pg/ml	IL-10 pg/ml	IFN-γ pg/ml
MFB1	M	FB	0.01	0.01	0.01	0.01
MFB2	M	FB	0.01	0.01	0.01	0.01
MFB3	M	FB	7.96	15	0.01	0.01
MFB4	M	FB	22	25	13	19
MFB5	M	FB	23	25	0	0.01
MFB6	M	FB	6.43	15	13	0.01
MFB7	M	FB	11.49	20	0.01	20
MFB8	M	FB	0.01	0.01	0.01	0.01
MIFB1	M	IFB	4.38	6	0.01	0.01
MIFB2	M	IFB	0.01	0.01	0.01	0.01
MIFB3	M	IFB	0.01	0.01	0.01	0.01
MIFB4	M	IFB	0.01	0.01	0.01	0.01
MIFB5	M	IFB	0.01	0.01	0.01	0.01
MIFB6	M	IFB	0.01	0.01	0.01	0.01
MIFB7	M	IFB	0.01	0.01	0.01	0.01
MIFB8	M	IFB	5.79	6	0.01	0.01
SFB-1	S	FB	0.01	0.01	0.01	0.01
SFB-13	S	FB	0.01	0.01	0.01	0.01
SFB-7	S	FB	3.55	3	0.01	0.01
SFB-8	S	FB	3.98	4	0.01	6.56
SFB-9	S	FB	0.01	3	0.01	0.01
SFB-10	S	FB	0.01	0.01	0.01	0.01
SFB-11	S	FB	0.01	0.01	0.01	0.01
SFB-12	S	FB	0.01	0.01	0.01	0.01
SIFB-1	S	IFB	0.01	0.01	0.01	0.01
SIFB-2	S	IFB	0.01	0.01	0.01	0.01
SIFB-3	S	IFB	4.75	4	0.01	0.01
SIFB-4	S	IFB	0.01	0.01	0.01	0
SIFB-5	S	IFB	0.01	0.01	0.01	4.83
SIFB-6	S	IFB	4.75	4	0.01	0.01
SIFB-7	S	IFB	6.43	8	0.01	0.01
ISFB1	S	FB	0.01	0.01	0.01	0.01
ISFB2	S	FB	0.01	6	0.01	16
ISFB3	S	FB	5.11	25	0.01	6.88
ISFB4	S	FB	10.32	0.01	0.01	0.01
ISFB5	S	FB	0.01	0.01	0.01	0.01
ISFB6	S	FB	0.01	0.01	0.01	0.01
ISFB7	S	FB	5.45	5	0.01	0.01
ISFB8	S	FB	0.01	0.01	0.01	0.01
ISFB9	S	FB	0.01	0.01	0.01	0.01

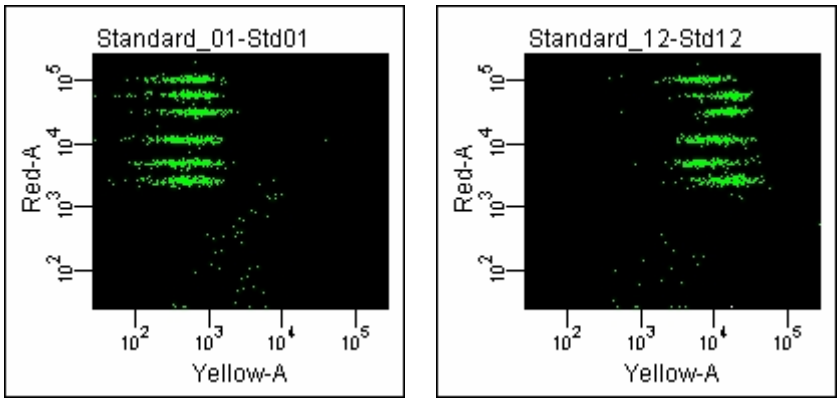

Figure 3.5.2 Cytometric Bead Array standards. Standard one has the lowest cytokine concentrations (bead populations cluster towards the left), and standard 12 has the highest cytokine concentrations (bead populations move towards the right).

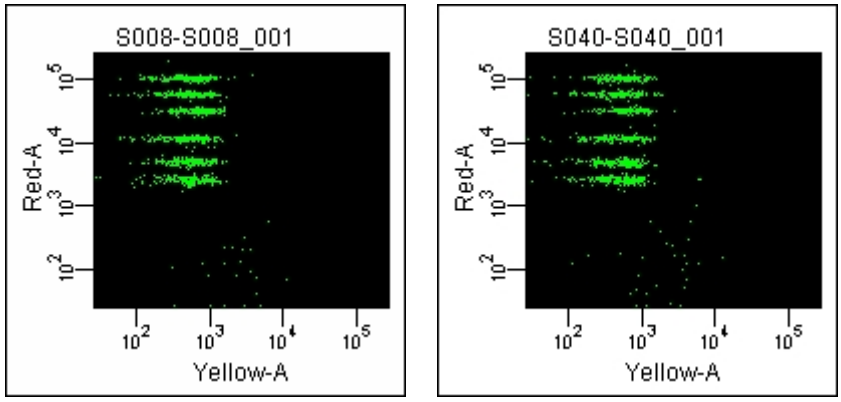

Figure 3.5.3. Subject MFB-8 (mild frequent bleeder) and subject ISFB-9 (severe frequent bleeder with inhibitors) showing low cytokine concentrations. Very low cytokine levels were detected with the CBA technology in all subjects.

Table 3.5.1. Cytokine levels of Mild/Moderate (M) compared to Severe (S) haemophiliacs.

Variables	Clinical Phenotype	N	Median (Interquartal Range)	p-value
IL-2	M	16	0.01(0.01-7.19)	0.10
	S	15	0.01(0.01-3.98)	
IL-4	M	16	0.01(0.01-15.00)	0.04*
	S	15	0.01(0.01-4.00)	
IL-10	M	16	0.01(0.01-0.01)	0.17
	S	15	0.01(0.01-0.01)	
IFNγ	M	16	0.01(0.01-0.01)	0.39
	S	15	0.01(0.01-0.01)	

Mild/Moderate haemophilia patients had significantly higher (p=0.04) levels of IL-4 than the Severe group. There were no significant differences in the levels of IL-2, IL-10, and IFNγ between the Mild/Moderate and Severe group.

Table 3.5.2. Cytokine levels of Frequent (FB) compared to Infrequent Bleeders (IFB).

Variables	Frequent/Infrequent Bleeders	N	Median (Interqurtal Range)	p-value
IL-2	FB	25	0.01(0.01-5.4)	0.21
	IFB	15	0.01(0.01-4.75)	
IL-4	FB	25	0.01(0.01-6.00)	0.10
	IFB	15	0.01(0.01-4.00)	
IL-10	FB	25	0.01(0.01-0.01)	0.27
	IFB	15	0.01(0.01-0.01)	
IFNγ	FB	25	0.01(0.01-0.01)	0.31
	IFB	15	0.01(0.01-0.01)	

No significant differences were found between cytokine profiles of frequent and infrequent bleeders.

Table 3.5.3. Inhibitor (I) vs. Non-Inhibitor (NI) haemophiliacs.

Variables	Inhibitors/Non-Inhibitors	N	Median(Interquartal Range)	p-value
IL-2	I	9	0.01(0.01-5.11)	0,62
	NI	31	0.01(0.01-4.75)	
IL-4	I	9	0.01(0.01-5.00)	0.87
	NI	31	0.01(0.01-6.00)	
IL-10	I	9	0.01(0.01-0.01)	0.44
	NI	31	0.01(0.01-0.01)	
IFNγ	I	9	0.01(0.01-0.01)	0.77
	NI	31	0.01(0.01-0.01)	

No significant differences were found between cytokine profiles of patients with inhibitors and patients without inhibitors.

TABLE 3.6 PEARSON CORRELATION COEFFICIENTS OF ALL VARIABLES IN THE STUDY POPULATION.

VARIABLES	CLASS	RACE	AGE	GENDER	BLEED FREQUENCY	INHIBITORS	IL-1B pg/ml	IL-6 pg/ml	TNF-a pg/ml	IL-2 pg/ml	IL-4 pg/ml	IL-10 pg/ml	IFN-g pg/ml
CLASS	1.00												
RACE	-0.47*	1.00											
AGE	-0.14	0.39*	1.00										
GENDER	0.18	-0.11	-0.00	1.00									
BLEED FREQUENCY	-0.36*	0.29	0.22	0.02	1.00								
INHIBITORS	0.82*	-0.36*	-0.19	-0.02	-0.42*	1.00							
IL-1B pg/ml	-0.10	-0.09	-0.02	-0.20	-0.06	0.14	1.00						
IL-6 pg/ml	0.76*	-0.35*	-0.23	0.14	0.01	0.71*	-0.11	1.00					
TNF-a pg/ml	0.13	0.11	-0.02	0.03	0.13	0.21	-0.17	0.17	1.00				
IL-2pg/ml	-0.23	0.25	0.01	-0.08	-0.20	-0.08	0.08	-0.07	-0.08	1.00			
IL-4pg/ml	-0.20	0.24	-0.07	0.07	-0.26	-0.03	0.08	-0.04	-0.03	0.83*	1.00		
IL-10pg/ml	-0.25	0.21	-0.03	-0.09	-0.18	-0.12	0.15	-0.17	-0.11	0.47*	0.48*	1.00	
IFN-g pg/ml	-0.04	0.26	-0.15	-0.05	-0.16	0.05	-0.04	-0.00	0.06	0.41*	0.53*	0.34*	1.00

* Correlation is significant at the P value of < 0.05 level (2-tailed).

There was a negative correlation (r=-0.36) between the biochemical classification and bleeding frequency of the study population. Biochemical classification correlated positively with inhibitors (r=0.82) and IL-6 (r=0.76).

There was a negative correlation (r=-0.42) between bleeding frequency and inhibitors.

There was also a negative correlation between inhibitors and race (r=-0.36).

3.7 LINEAR REGRESSION ANALYSIS

Table 3.7.1 Dependent Variable: Bleeding Frequency

	STANDARDISED COEFFICIENTS	p-value
	Beta	
Inhibitors	0.95 (**)	0.01
IL-1β	0.142	0.29
Il-2	-0.04	0.85
Il-4	-0.31	0.17
Il-6	0.886 (**)	<0.002
IL-10	-0.11	0.41
TNF-α	0.182	0.15
IFN-γ	0.058	0.68

(**) - Significant beta p<0.05

Inhibitors and IL-6 were found to be significant predictors of bleeding frequency.

Table 3.7.2 Dependent Variable: Biochemical Classification

	STANDARDISED COEFFICIENTS	p-value
	Beta	
Inhibitors	-0.25	0.33
IL-1β	-0.16	0.26
Il-2	-0.06	0.78
Il-4	-0.28	0.26
Il-6	0.71 (**)	0.004
IL-10	-0.08	0.56
TNF-α	-0.04	0.77
IFN-γ	0.07	0.65

(**) - Significant beta p<0.05

IL-6 was the only significant predictor of biochemical classification.

Table 3.7.3 Dependent Variable: Inhibitors

	STANDARDISED COEFFICIENTS	p-value
	Beta	
IL-1β	0.21 (**)	0.02
Il-2	-0.60	0.70
Il-4	-0.13	0.44
Il-6	0.77 (**)	<0.002
IL-10	-0.07	0.48
TNF-α	0.16	0.07
IFN-γ	0.07	0.50

(**) - Significant beta p<0.05

IL-1β and IL-6 were the only significant predictors of the inhibitor status of haemophilia patients.

3.8 DNA quantification and purity check

Table 3.6 DNA quantification and purity check

Participant number	Absorbance at 260nm	Absorbance at 280nm	Purity check (Ratio 260/280)	DNA concentration µg/µl
ISFB-7	0.437	0.247	1.77	80.9
ISFB-1	0.360	0.185	1.94	100.1
ISFB-8	0.688	0.400	1.72	98.4
ISFB-9	0.265	0.138	1.92	78.2
SFB-5	0.554	0.284	1.95	77.6
MNFB-6	0.549	0.288	1.90	94.1
Control-1	0.280	0.150	1.86	90.5
Control-2	0.320	0.170	1.88	87.6

DNA extraction was performed on 4 haemophilia patients with inhibitors, 2 haemophilia patients without inhibitors and 2 healthy controls.

The absorbance of 10 microlitres of DNA suspended in elution buffer was measured at two wavelengths. The measurement at 260nm, measured the nucleic acid absorbance and the measurement at 280 nm measured the protein contamination. The ratio 260/280 represented the nucleic acid purity. The acceptable ratio was in the range 1.7 to 1.9.

The DNA was of high quality, showed minimal degradation and RNA contamination and therefore suitable for subsequent analysis.

3.9 DNA agarose gel electrophoresis

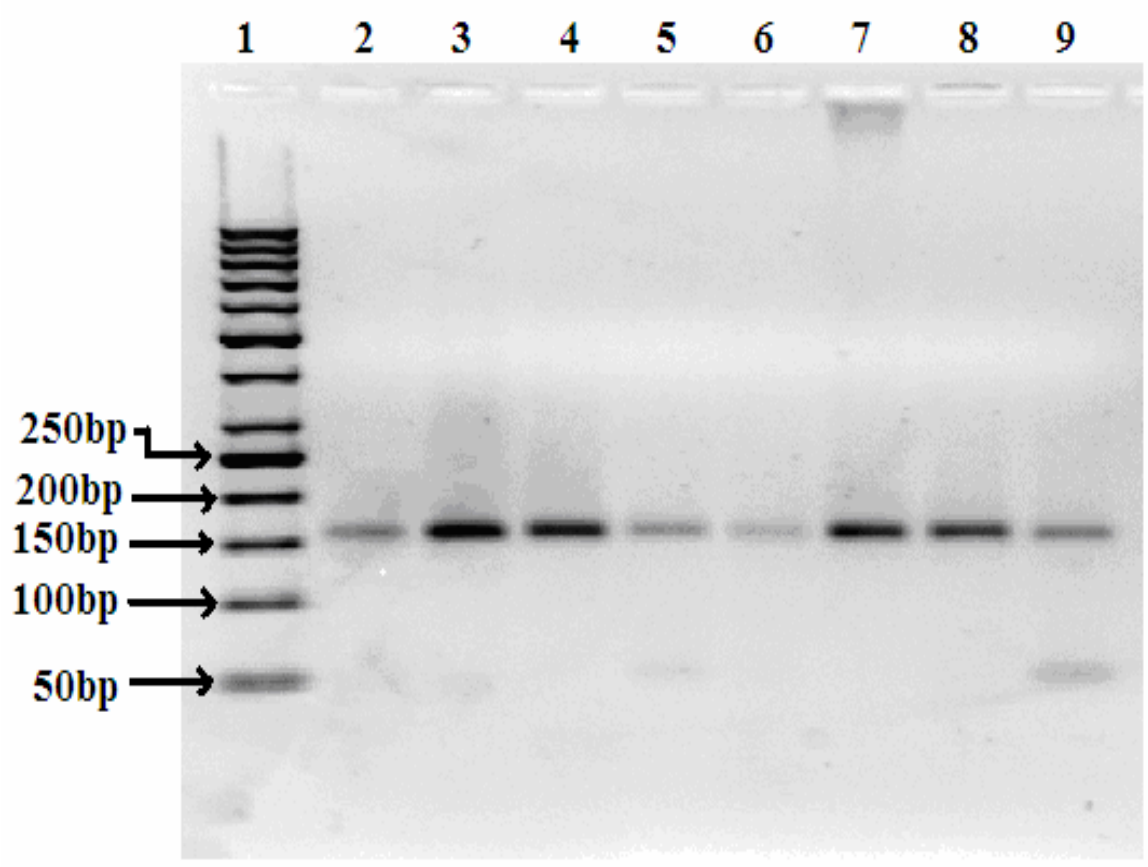

Figure 3.7 Agarose gel electrophoresis of PCR (IL-10 promoter region). Twenty five micro liters of PCR product was run in 2% agarose gel. Lane 1 is the 50 base pair ladder DNA molecular marker (Roche Diagnostics). Lanes 2-5 are haemophilia patients with inhibitors. Lanes 2 and 3 are white patients and lanes 4 and 5 are black patient. Lanes 6 and 7 are haemophilia patients without inhibitors. Lanes 8 and 9 are healthy controls.

The PCR picture shows a 150 bp allele in patients with and without inhibitors as well as the healthy controls. The 150 bp allele was also observed in both black and white subjects.

CHAPTER 4 DISCUSSION & CONCLUSION

4.1 Discrepancies in the theoretic classification of haemophilia patients.

In clinical practice, at the Charlotte Maxeke Johannesburg Academic Hospital Haemophilia Comprehensive Care Centre, we observed that some biochemically mild haemophiliacs present with severe clinical phenotypes and vice versa. A number of factors could be responsible for this discrepancy and these include inhibitor development, physical activity of haemophiliacs, intrinsic genetic factors, and cytokine profiles of frequent bleeders compared to infrequent bleeders. These theoretical possibilities have been poorly documented in literature and the underlying reasons for the biochemical-phenotypic discrepancy have not have not yet been established.

Several prospective studies that looked at phenotypic heterogeneity in severe haemophilia have documented that some 3-10% of patients with severe haemophilia rarely bled, do not suffer joint bleeds, have no or minimal arthropathy and did not need any prophylactic treatment (Molho, et al., 2000; Aledort , et al., 1994). On the contrary, 12% of mild and moderate patients in the Netherlands have very frequent bleeds (Plug, Van der Bom, Peters, et al., 2004). Other interesting studies have suggested that a combination of prothrombotic mutations in a patient with haemophilia would change his phenotype and compensate for the low FVIII/FIX levels and result in more efficient thrombin generation and clot formation (Nihols, Amano, Cacheris, et al., 1996; Escuriola-Ettingshausen, Halimeh, Kurnik, et al., 2001).

Despite the overall observation that there is a large clinical heterogeneity among patients, very limited and fragmentary data is available on how to identify patients at different bleeding risks.

The present study population has shown a significant discrepancy in the theoretic classification of haemophilia patients. Twenty nine percent (29%) of the biochemically severe haemophilia patients (factor level<1%) were infrequent bleeders and 50% of the biochemically mild/moderate (factor levels of 1-50%) haemophilia patients were frequent bleeders (fig 3.3).

Pearson correlation of all study variables further demonstrated this discrepancy (table 3.6). There was a negative correlation (r=-0.36) between the biochemical classification and bleeding frequency of the study population.

4.2 Cytokine analysis in haemophilia.

Cytokines have a short in vitro half life, low plasma concentrations, are pleiotropic, and redundant. These characteristics have complicated the isolation and characterization of cytokines and this has resulted in a paucity and fragmentation of the limited number of cytokine studies in haemophilia.

A recent study showed that the inflammation of the synovial membrane caused by the recurrent presence of blood resulted in the production of pro-inflammatory cytokines

such as IL-1, IL-6, and TNF-α in haemophilia patients (Roosendaal, Vianen, Wenting, et al., 1998). These pleiotrophic cytokines, IL-1 and IL-6 are also promoters of antibody production by supporting the growth and differentiation of B-cells which then produce antibodies (Chapel & Mansel., 1999).

Another study suggested that TNF-α and IL-1β are up-regulators of tissue factor expression and therefore promoted coagulation as tissue factor plays a central role in the extrinsic pathway of coagulation (Van den Berg, et al., 2007).

The largest group of cytokines has been found to stimulate immune cell proliferation and differentiation. This group includes IL-1, which activates T cells; IL-2, which stimulates proliferation of antigen-activated T and B cells; IL-4, IL-6, and IL-10, which stimulate proliferation and differentiation of B cells and IFN-γ, which activates macrophages. (Balkwill, 2001; Oppenheim, 2002).

Knowledge about the specific T cell mechanisms responsible for inhibitor development is very limited, but the inhibitor antibodies have been found in most investigations to be of the IgG isotype (Lavigne-Lissalde, et.al., 2005), therefore the presence of cytokines such as the Th2 cell derived IL-4, IL-6, and IL-10 which activates B-lymphocytes resulting in the up-regulation of antibody production was anticipated in patients who develop inhibitors.

The candidate cytokines chosen in this study, for their potential in influencing anti-FVIII/FIX antibody production, and inflammatory response perpetuation, were the TH1 cytokines IL-1β, IL-2, IFN-γ, and TNF-α and TH2 cytokine IL-4, IL-6 and IL-10.

4.2.1 Cytokines and disease severity

The results of the current study showed that severe haemophilia patients had significantly higher (p=0.009) levels of IL-6 than the Mild/Moderate group. In this study population, all patients with inhibitors were also severe. IL-6 in this group could be responsible for the differentiation of activated plasma cells into B-cells as well as antibody secretion. In patients without inhibitors, IL-6 could be released by monocytes, macrophages, Th2 cells and stromal cells to target various cells for acute phase responses. Pearson correlation also showed that biochemical classification correlated positively with IL-6 (r=0.76) (1=Mild/Moderate; 2=Severe), and the linear regression analysis results showed corresponding results as IL-6 was the only significant predictor of biochemical classification (table 3.7.2).

IL-1β was found to be significantly higher (p=0.007) in the Mild/Moderate group than in the Severe group (table 3.4.1). The majority of Mild/Moderate patients in the current study were frequent bleeders. IL-1β in these patients could be propagated by the continuous presence of blood in the joints which perpetuates the production of inflammatory cytokines.

Mild/Moderate haemophilia patients had significantly higher (p=0.04) levels of IL-4 than the Severe group. This cytokine could be responsible for the proliferation of cytotoxic T cells in Mild/Moderate group therefore propagating the inflammatory response and thus increasing the bleeding frequency of these patients.

There were no significant differences in the levels of IL-2, IL-10, and IFNγ between the Mild/Moderate and Severe group (table 3.5.1).

4.2.2 Cytokines and bleeding frequency

No significant differences were found between cytokine profiles of frequent and infrequent bleeders (table 3.4.2 and table 3.5.2), but the Pearson correlation results showed IL-6 to be a significant predictor of bleeding frequency (table 3.7.1).

4.2.3 Cytokines and inhibitor development

IL-6 and TNF-α were found to be significantly higher ($p<0.0002$ and $p=0.03$ respectively) in patients with inhibitor than in haemophilia patients without inhibitors. IL-6 in patients who develop inhibitors could be responsible for the differentiation of activated plasma cells into B-cells as well as antibody secretion. TNF-α could be responsible for increasing the expression of inflammatory cytokines in these patients as the patients with inhibitors were all severe haemophiliacs.

No significant differences were found in the levels of IL-2, IL-4, IL-10, and IFNγ between patients with inhibitors and patients without inhibitors (table 3.5.3).
There were also no differences in the cytokine profiles of Low and High Responders (table 3.4.4). This suggests that the cytokine levels did not determine the inhibitor titres of these patients.

IL-6 and IL-1β were the only significant predictors of the inhibitor status of haemophilia patients (table 3.7.3).

Interestingly, there were no statistically significant differences in cytokine profiles of haemophiliacs who had conflicting phenotypic features and those with theoretically rational phenotypes.

4.3 Haemophilia severity and the risk of inhibitor development.

Severity of haemophilia has been known for many years to represent a major factor for inhibitor development particularly in severe haemophilia A. In patients with a severe form of the disease, the inhibitor risk is about 25–30%, while in mild to moderate haemophilia A only 5% of the patients develop inhibitors. (Oldenburg, El-Maarri & Schwaab R., 2002).

Statistical analysis of the current study showed congruent results as biochemical classification correlated positively with inhibitors (r=0.82). In fact all patients with inhibitors in this study were severe patients. The negative correlation (r=-0.42) between bleeding frequency and inhibitors further exhibited the existence of discrepancies in the theoretic classification of haemophilia patients. This was again shown by the linear regression analysis results, which showed inhibitor development as a significant predictor of bleeding frequency (table 3.7.1)

4.4 Race as a risk factor for inhibitor development

Race and ethnicity have been found in several studies to be a highly significant risk factor for inhibitor development (Addiego, et.al., 1994; Lusher, et,al., 1997; Gruppo, et.al., 1998; Astermark, et.al., 2001). These studies reported corresponding data that documented the incidence of inhibitors in African-American and Latino patients to be twice that of Caucasian patients. Scharrer et al. (1999) clearly demonstrated inhibitor incidence in severe haemophilia patients of African origin was twice that in Caucasians (51.9% vs. 25.8%). Similar data were reported by Astermark et al (2001), in the MIBS study.

The current study also found a negative correlation between inhibitors and race (r=-0.36) (black=1, white=2) (table 3.6). Out of the 9 patients with inhibitors in this study, only 2 were white and 7 were black.

4.5 Polymorphisms in the IL-10 gene promoter region.

A 134-bp–long variant of a CA repeat microsattelite in the promoter region of the IL10 gene has been shown to be associated with autoantibody concentrations in SLE, myasthenia gravis, and Wegener granulomatosis and with the concentration of the monoclonal immunoglobulin in multiple myeloma (Zhou, et.al.,2002; Huang, et al 1999).

A strong association ($P<0.001$) between an allele with 134bp in one of the CA repeat microsattelites of the IL-10 gene and inhibitor development was also found in the Malmo¨ International Brother Study (MIBS) (Astermark, et al., 2006).

A preceding study reported a higher frequency of inhibitors and the IL-10 gene CA repeat microsattelites in African Americans compared to Caucasian people (Astermark, et al., 2001).

In the current study, there was a higher frequency of inhibitors in the black patients compared to the white patients, but a 150 bp allele was observed in patients with and without inhibitors as well as the healthy controls. The 150 bp allele was also observed in both black and white subjects (fig 3.7). More studies in larger populations have to be conducted in the African population to determine if there are polymorphisms in the other cytokine genes, which are associated with inhibitor development in haemophilia.

4.6 CONCLUSION

The current study aimed to find the associations between coagulation factor levels, clinical phenotype, cytokine profiles and polymorphisms in immune response genes of haemophilia A and B patients with and without inhibitors.

Large phenotypic heterogeneity exists in haemophilia patients. 29% of the studied severe patients rarely bled and 50% of the studied mild/moderate patients bled frequently.

The pro-inflammatory cytokines IL-6 and IL-1β together with IL-4 may be involved in determining the biochemical severity of haemophilia. IL-6 was found to be a significant predictor of bleeding frequency and none of the other cytokines studied seem to be involved in determining bleeding frequency or clinical phenotype. The study results also suggest that IL-6 and IL-1β may be involved in the production of antibodies against infused factor in patients with inhibitors.

Severity of haemophilia and race were found to be significant risk factors for inhibitor development. Although there was a higher frequency of inhibitors in the black patients compared to the white patients, a 150 bp allele of the highly polymorphic IL-10 promoter region was observed in patients with and without inhibitors as well as the healthy controls. The 150 bp allele was also observed in both black and white subjects. This suggests that, polymorphisms in this gene do not influence inhibitor development in this population.

The confirmation of these findings in a larger haemophilia population is highly recommended.

4.7 RECOMMENDATIONS

There is a need for more extensive and more rigorous prospective research in a fully genotyped population, to clarify the associations between coagulation factor levels, clinical phenotype, cytokine profiles and polymorphisms in immune response genes of haemophilia A and B patients with and without inhibitors since this cross sectional study did not have enough statistical power to demonstrate such associations.

The same technique or techniques of equal sensitivity must be used for cytokine quantization, as the results of the cytokines analyzed with the amplified sensitivity ELISA kits could not be compared to the cytokine levels measured by the Cytometric Bead Array (CBA) technology. The ELISA kits were able to measure very minute cytokine levels whereas the CBA technology was not sensitive enough to measure such amounts.

Furthermore, supplementary studies with larger study groups have to be conducted in the African population to determine if there are polymorphisms in the other immune response genes that are associated with inhibitor development in haemophilia.

APPENDIX

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Nldovu

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M070616**
PROJECT	Cytokine Analysis in Haemophilia
INVESTIGATOR	Ms N Nldovu
DEPARTMENT	Molecular Medicine & Haema
DATE CONSIDERED	07.06.29
DECISION OF THE COMMITTEE*	APPROVED UNCONDITIONALLY Part of M070616, Dr J Mhlangu

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

DATE 07.07.12 **CHAIRPERSON**.....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr J Mahlangu

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 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

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