ASSOCIATION BETWEEN COAGULATION FACTOR LEVELS, CYTOKINE PROFILES, CLINICAL MANIFESTATIONS AND GENOTYPIC FEATURES IN FACTOR X DEFICIENCY

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fulfillment of the requirements for the degree

of

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DECLARATION

I, Cyprian Mcwayizeni Thwala declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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…….day of…….2010

ABSTRACT

Factor X deficiency is a rare bleeding disorder with an incidence of one in a million in the general population. Patients with the severe form of factor X deficiency suffer from serious bleeds occurring mainly into the joints and the muscle. In the two factor X deficient families currently looked after at the Haemophilia Comprehensive Care Centre, there are definite differences in the bleeding tendencies between and within family members. We hypothesize the differences in genetic mutations and the influence of cytokines to be responsible for these bleeding variabilities. These factors were explored in our study.

The study population included a total of fourteen members of the two families with factor X deficiency. Blood for factor X measurement, cytokine studies and genetic studies was collected in the Haemophilia Comprehensive Care Centre of the Charlotte Maxeke Johannesburg Academic Hospital. Each blood was processed according to the test to be performed. Factor X activity levels were measured using the factor X assay, and the information on each patient's bleeding episodes was obtained from the Haemophiliac Clinic database. Cytokines were analyzed in all patients using the ELISA kits from Biosource. Factor X gene was amplified using PCR and sequenced with Spectrumedix SCE 2410.

For cytokine studies, high levels of IL-1beta and TNF-alpha were observed in frequent bleeding patients compared to infrequent bleeders. These cytokines are known to be involved in acute inflammatory process leading to cellular infiltrate and joint swelling. This results in synovitis and the creation of massive joint bleeding. The low levels of IL-1beta and TNF-alpha detected in infrequent bleeding patients appear to be related to the high levels of IL-1Ra and IL-10. These anti-inflammatory cytokines are known to inhibit the inflammatory synovitis and lessen the severity of joint bleeding.

For genetic studies, differences were observed between the amino acid sequence of the three frequent bleeding patients and the consensus. In addition, a novel mutation Cys350Phe was detected in two of these patients. This mutation is characterized by very low factor X levels which sometimes are not detectable in circulation. The substituted cystine is known to cause defect in the substrate binding, leading to the lost of enzyme activity. From these findings we have concluded that the origin of the heterogeneity of bleeding in factor X deficiency is multifactorial, cytokines and genetic mutations seems to have a role in determining the clinical manifestations of the factor X deficient patients.

DEDICATION

To my Mom

Rose Thwala

"*Be blessed with essence of hope"*

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LIST OF ABBREVIATIONS

aPTT…….....Activated partial thromboplastin time

Arg……...Arginine

Asp…….……Aspartate

Bp…… ….Base pair

Cys…….….Cystine

DNA ……..……Deoxyribonucleic acid

ELISA…….....Enzyme-Linked ImmunoSorbent Assay

EGF……Epidermal growth factor-like regions

FIX…….....Factor IX

FX……Factor X

FXD……..…..Inherited factor X deficiency

FLS……..Fibroblast-like synoviocytes

Gla…….....Glutamic acid

Gly……....Glycine

Glu……....Glutamine

His……Histamine

HIV……...Human immunodeficiency virus

IFN-γ……Interferon gamma

IL-1beta……Interleukin 1 beta

IL-1Ra……...Interleukin 1 Receptor antibody

IL-2……...Interleukin 2

IL-4…….....Interleukin 4

IL-10…….Interleukin 10

IL-12……..…Interleukin 12

IL-13…….....Interleukin 13

IL-18…..……....Interleukin 18

IL-32…….....Interleukin 32

Ile……..Isoleucine

Kb…..……Kilobases

Lys……Lysine

MMPs……...Matrix metalloproteinases

Met……...Methionine

m RNA ……Messenger ribonucleic acid

MNC……..…Mononuclear cells

NO…….…….….Nitric oxide

NF-kB …….Nuclear factor kappa B

Prol……..Proline

PCC ……Prothrombin complex concentrates

PCR……...……..Polymarase chain reaction

PT……Prothrombin time

RBC……Red blood cells

RA……..…….Rheumatoid arthritis

Ser……..….Serine

Thr……..Threonine

TNF-alpha……..Tumor necrosis factor alpha

Val……..Valanine

VEGF…….. …….Vascular endothelial growth factor

LIST OF SYMBOLS

^{o}C ……..Degree Celsius

γ…….Gamma

IU/dl…….....International units per dl-alphatocopherylacetate

μ mole ……..Micro mole

μl……Micro litre

ng/ml…….....Nano gram/millitre

pg/ml..……..….Pico gram/millitre

p mole……..……..…Pico mole

CHAPTER I

1.1 INTRODUCTION

Coagulation factor X, is a vitamin K-dependent serine protease which plays a crucial role in blood clotting as the first protein in the common pathway of coagulation cascade (Figure 1). Factor X (FX) is also known as the Stuart-Prower factor after the index cases in which this deficiency was first described (Hougie and Barrow 1957; Telfer and Denson 1956). Factor X deficiency results in a life long bleeding diathesis characterized by bleeding into joints, mucous membranes and other sites after haemostatic challenges. This deficiency could be a consequence of the factor X genetic mutations, or acquired causes resulting in quantitative or qualitative abnormalities of the factor X protein (Uprichard and Perry 2002).

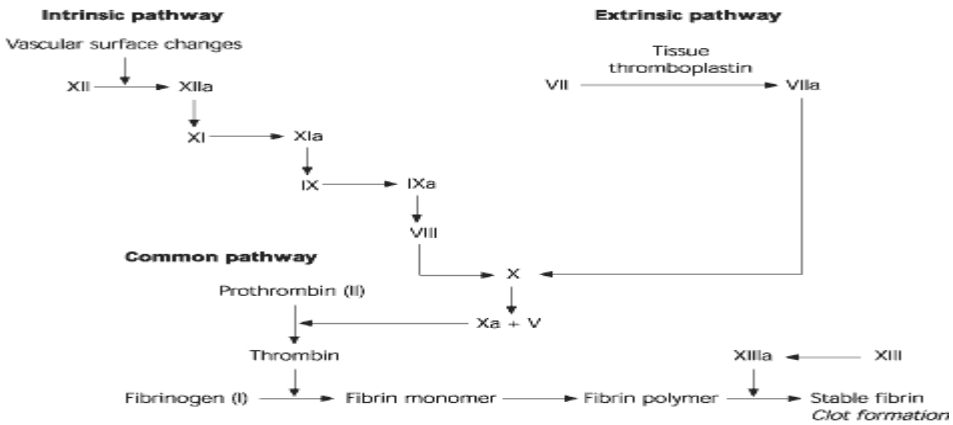

Figure 1. Schematic showing the coagulation cascade leading to fibrin formation. A deficiency or dysfunction of coagulation factor X compromises both the intrinsic and extrinsic pathways of coagulation (Bowen 2002).

In factor X deficiency, the clinical phenotype correlates poorly with the laboratory phenotype and factor X coagulation activity (Sucker et al., 2006). Therefore, factor X cannot be unambiguously used as the basis for the classification of clinical severity. In previous studies, the clinical phenotype of factor X deficiency was generally described only as severe, mild or asymptomatic depending on bleeding symptoms (Sucker et al., 2006; Takabe et al., 2004).

In the two factor X deficient families currently looked after at the Haemophilia Comprehensive Care Centre of the Charlotte Maxeke Johannesburg Academic Hospital, there are definite differences in the bleeding tendencies between and within families. A number of factors could be plausible reasons for these phenotypic discrepancies, and these include different genetic mutations in the factor X gene, different factor X protein levels or the influence of other epigenetic factors such as cytokines.

The recurring joint bleeding in factor X deficiency result in the development of joint destruction known as haemophilic arthropathy (Bae et al., 2009; Meenakshi et al., 2008). The bleeding is caused by synovitis which is characterized by neo-vascularization and villous formation inside the joint. This condition sets up a vicious cycle of bleeding causing synovitis resulting in more bleeding (Bae et al., 2009; Meenakshi et al., 2008).

After the onset of active synovitis synovial bleeding may not be related only to clotting factor X deficiency but, also to pre-existing vascular damage and inflammation (Ehrenforth et al., 1992; Meenakshi et al., 2008). There is evidence to suggest that iron may play a central role in the development and sustenance of synovitis. This involved the release of cytokines which were suggested to perpetuate this condition and lead into massive joint bleeding (Meenakshi et al., 2008; Suchitra 2008).

In human in *vitro* and in animal in *vivo* models, it was shown that a limited number of short-term joint haemorrhages could induce lasting changes to cartilage matrix turnover (Valentine et al., 2007). This eventually led to cartilage destruction. The current opinion is that chronic synovitis is the main factor for progressive cartilage destruction as a result of joint bleeds (Roosendaal and Lafeber 2003).

It was reported that the initial and irreversible changes in cartilage matrix metabolism were independent of synovial inflammation (Hakobyan et al., 2004). The primary adverse changes in cartilage were found to be induced by the combination of mononuclear cells (MNC) plus red blood cells (RBC) (Hakobyan et al., 2004). However, the mechanism played by MNC plus RBC in the pathophysiology of blood-induced cartilage damage is still unclear (Hooiveld et al., 2003; Wen et al., 2002; Hoots 2007).

Currently, it is known that the blood MNC in the joint cavity produce interleukin -1beta (IL-1beta) and tumor necrosis factor-alpha (TNF-alpha) after a joint bleed (Lotz 2001). The production of these cytokines was found to be independent of an inflammatory response (Hoots 2007; Lotz 2001). The concurrent presence of RBC was suggested to play a role in RBC-derived toxicity. It was argued that haemoglobin iron may result in oxidative stress, which may cause damage to chondrocytes (Dunn et al., 2005; Harris et al., 2006). This was based on the fact that iron can act as a catalyst for the Fenton reaction in which hydroxyl radicals are formed from hydrogen peroxide. Hydrogen peroxide produced by chondrocytes was found to be increased after stimulation with IL-1beta and TNF-alpha. This has been suggested to have an adverse effect on cartilage matrix turnover (Donadel 2006; Dunn et al., 2005; Harris et al., 2006).

The cytokine balance and receptor antagonists might be determinants not only for initiation and progression, but also for the clinical expression of synovitis (Meenakshi et al., 2008). It was reported that during synovitis, a complex inflammatory response was developed. It was found that there was a local imbalance between the expression of interleukin -1beta (IL-1beta), tumor necrosis factor -alpha (TNF-alpha) and interleukin -1receptor antibody (IL-1Ra). This was associated with joint destruction and the clinical outcome of bleeding (Meenakshi et al., 2008; Saski et al., 2007; Suchitra 2008).

Association of pro-inflammatory and anti-inflammatory cytokines have not been studied in factor X deficiency. Literature on this association is therefore scanty. To date, there are no published studies in the mainstream journals exploring the cytokine levels as a possible explanation for the phenotypic discrepancy in patients with factor X deficiency. We hypothesized that, in these two families of factor X deficient individuals, reasons for their variation in bleeding phenotype could be multifactorial with genetic, protein levels as well as cytokines possibly contributing.

1.2 Aim of the study

The aim of this study is to establish if there are any associations between coagulation factor X activity levels, bleeding frequencies, genotypic features and cytokine profiles in factor X deficient patients.

1.3 Objectives

- To determine the clotting factor X activity and phenotypic presentations in factor X deficiency.
- To determine the cytokine profiles (IL-1beta, TNF-alpha, IL-10, IL-18, and IL-1Ra) of factor X deficiency.
- To sequence the factor X gene in factor X deficiency.
- To establish if there are any associations between coagulation factor X activity levels, clinical manifestations, genotypic features and cytokine profiles in factor X deficiency.

1.4. LITERATURE REVIEW

1.4.1 Factor X gene

Factor X is encoded by a gene, which has 8 exons located on chromosome 13. Each exon encodes a specific peptide of the factor X preproprotein (Figure 2). Exon I encodes the signal peptide, exon II encodes the propeptide and glutamic acid (Gla) domain, exon III encodes the aromatic amino acid stack, exons IV and V each code for the epidermal growth factor-like regions (EGF), exon VI encodes the activation domain, and exons VII and VIII encode the catalytic domain (Millar et al., 2000). The causative molecular defects of factor X deficiency are heterogeneous. To date, approximately 87 factor X mutations have been described. The majority of these mutations are missense with a few splicing frameshift and large deletions (ISTH factor X database, 2009).

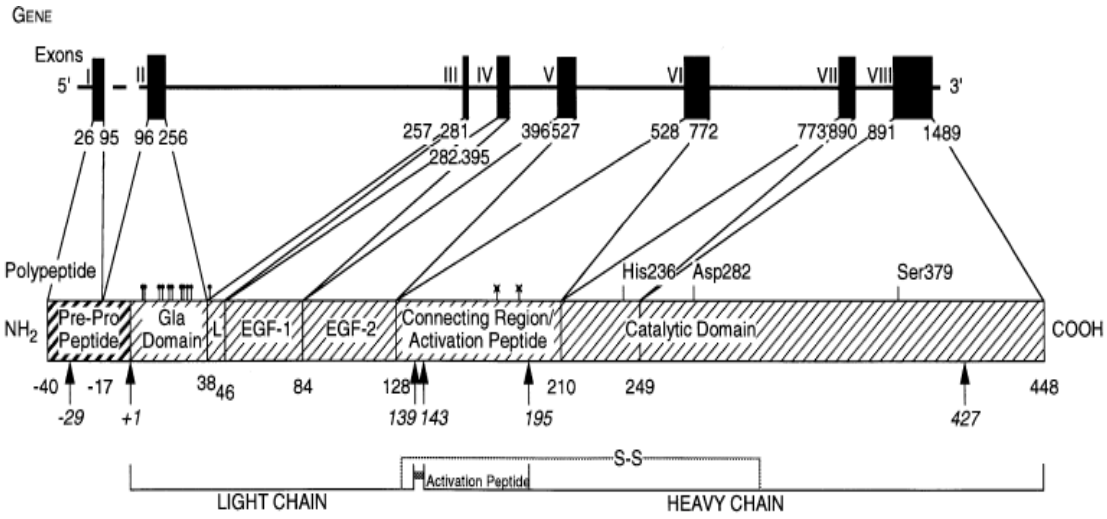

Figure 2. Schematic representation of organization of the human factor X gene and its encoded polypeptide. The upper part of the illustration represents the factor X gene with the eight exons shown by filled boxes. The positions of the first and last nucleotides of each exon are shown. The lower part of the illustration shows the polypeptide structure and the various functional domains encoded. (Residue +1 is the first amino acid of the mature protein). Residue – 29 indicates the site of probable cleavage by signal peptidase. The light chain is encoded by residues + 1 to 139 and the heavy chain by residues 143 to 448. The connecting tripeptide is located between residues 140 and 142 and the activation peptide residues within the heavy chain at residues 143-195. Activation of factor X occurs through cleavage at Arg 194-Ile195. Residue 427 shows the site of cleavage of factor X that generates factor Xa. His 236, Asp 282 and Ser 379 are the residues which constitute the catalytic triad (Divi et al., 2002).

1.4.2 Factor X protein

Factor X is a product of hepatocytes. Before its secretion, this protein undergoes proteolytic processing in the endoplasmic reticulum. This produces the circulating factor X as a two-chain polypeptide linked with a disulphide bond. Factor X is activated by a complex of negatively charged phospholipids, factor IXa and factor VIIIa or by membrane-bound complex of factor VIIa and tissue factor. Factor X can also be activated by a component of Russell's viper venom. In each case, activation of factor X is accomplished by proteolytic cleavage and subsequent removal of the activation peptide (Mizuno et al., 2001).

Once it is activated, factor X with its cofactor (factor V) catalyses the conversion of prothrombin to thrombin. This reaction requires the negatively charged phospholipids and calcium ions. A deficiency or dysfunction of either factor V or factor X compromises the conversion of prothrombin, and the ensuing steps of the coagulation cascade resulting in inefficient or non-existent fibrin deposition. Therefore, the fundamental biochemical lesion underlying the factor X deficiency is the failure to generate thrombin as a result of a deficiency of coagulation factor X. Levels of 10-20 IU/dl is sufficient to stop bleeding (Hsiao-Ling et al., 2001).

1.4.3 Factor X deficiency

Inherited factor X deficiency (FXD) is a very rare recessive coagulation disorder. Patients with factor X deficiency present with bleeding disorders of variable magnitude. Dewerchin et al. (2000) showed that FX-/- knockout mice die in utero or days after birth, indicating that the complete absence of factor X is incompatible with life. This explains the extremely low incidence of homozygous FXD (1:1 000 000) in the general population (Dewerchin et al., 2000). The prevalence of FXD is much greater among the populations in which consanguineous marriage is common (for example most middle countries such as Iran). In such populations, the prevalence of heterozygous FXD is about 1: 500, but this is usually asymptomatic (Masood et al., 2005).

Factor X deficiency is suspected when there is a prolonged activated partial thromboplastin time (aPTT) and prothrombin time (PT). The diagnosis of factor X deficiency is confirmed by measuring factor X levels, which can be done either by clotting or immunological assay. (Masood et al., 2005; McMullen et al., 1983). Inherited factor X deficiency must be differentiated from various acquired causes such as systemic amyloidosis. Factor X deficiency that occurs in this disorder can result from selective binding of factor X to the amyloid fibrils. This can be erroneously recognized as the presence of an inhibitor (Masood et al., 2005; Perry 1997).

1.4.4 Genetics of factor X deficiency

Congenital deficiencies of coagulation factor X usually arises from missense mutations. In such mutations, the substitution of one nucleotide for another results in the translation of a different amino acid from that of the wild type protein. If the nucleotide substitution results in an amino acid which does not significantly alter the protein structure or function, then the individual will not demonstrate a clinical deficiency. However, if the new residue does alter the structure and the function of the protein, then deficiency will follow and the individual will exhibit a bleeding disorder (Perry 1997).

A number of restriction fragment length polymorphisms within the factor X gene have been identified. Such polymorphisms are of potential use in tracking mutant alleles in families with factor X deficiency (Cooper and Tuddenham 1997; ISTH factor X database, 2009). Most of the deletion mutations results in impaired protein synthesis or in synthesis of unstable or dysfunctional proteins. These variants may affect factor X function in several ways. Activation through the tissue factor pathway may be affected when the mutations are located in the Gla domain. This is detected in Glu7Gly (St. Louis II) (Rudolph et al., 1996), Glu14Lys (Vorarlberg) (Herrmann et al., 2001) and Ser379Lys (Watzke et al., 1990). These mutations are predicted to result in a reduced affinity for calcium which plays an important role in activation of factor X.

Activation through factor IXa is affected by Thr318Met (Roma) (Marchettgi et al., 1995), Arg139Cys (Wenatchee I) (Kim et al., 1995) and Arg139Ser (Kurayochi) (Lijima et al., 2001). It was shown that these mutations prevent the removal of the activation peptide (Lijima et al., 2001). This then results in dysfunctional protein which is rapidly degraded in plasma. Activation of factor X through Russell's viper venom is almost intact in the Pro343Ser (Friuli) (James et al., 1991). This mutation was predicted to form a new hydrogen bond between Ser343 and Thr318. This affects the tertiary structure of the region and its catalytic potential (James et al., 1991).

Missense mutations may also affect synthesis or secretion, thus producing dysfunctional factor X (Racchi et al., 1993; Millar et al., 2000).This is detected with Gly-20Arg (Santo Domingo) (Racchi et al., 1993), Thr-2Met and the Val298Met (Stuart mutation)(Millar et al., 2000). In these mutations factor X is synthesized normally and translocated into the endoplasmic reticulum, but not processed by the signal peptidase. It was argued that these mutations lead to a complete loss of translation of FX molecule, because the codon GTC cannot initiate translation (Miyata et al., 1998).

The clinical manifestations of FXD may also be influenced by epigenetic factors. This arises from family studies that show a discrepancy in bleeding between siblings with same genetic mutation (Kram et al., 2007; Van Den Berg et al., 2007). These authors have pointed out the role of genetically determined characteristics of the immune response, fibrinolysis and pharmacokinetics as factors that might explain the discrepancy of bleeding. These factors are yet to be explored (Musacchio et al., 2009; Kram et al., 2007; Van Den Berg et al., 2007).

1.4.5 Clinical manifestations of factor X deficiency

Patients with FXD may present with a bleeding tendency at any age, although the more severely affected ones present early in life with umbilical-stump bleeding. Haemathroses, severe post operative haemorrhage and central nervous system haemorrhage have also been reported (Laffan and Manning 2001). More unusual bleedings are intestinal bleeding, urinary tract bleeding and soft tissue bleeding. In individuals homozygous for moderate or mild deficiencies of factor X, bleeding is less common usually occurring only after trauma or after surgery. Such patients are reported to experience easy bruising as the only clinical manifestation (Laffan and Manning 2001).

The presence of chronic synovitis in joints of FXD patients results from recurrent joint bleeds (Wiedel 2002). This is an inflammatory condition characterized by villous formation and markedly increased vascularisation. This state leads into pannus formation and destructive arthritis which marks the onset of arthropathy. The chronic synovitis leads into maximum limitation of motion. After the onset of active synovitis synovial bleeding may not be related only to clotting factor X deficiency but, also to pre-existing vascular damage and inflammation which is often difficult to control clinically (Figure 3)(Fei-Qiu et al., 2002; Wiedel 2002).

The acute bleeding episode initiates the onset of a series of recurrent joint bleedings. This creates a circle of bleeding→synovitis→bleeding (Fei-Qiu et al., 2002). However, the risk factors for the progression of these joint bleeds are not well defined. There is evidence to suggest that iron may play a central role in the pathogenetic process causing changes at the molecular level. This involves the production of cytokines which were suggested to perpetuate the chronic inflammatory state (Meenakshi et al., 2008; Roosendaal and Lafeber 2003; and Suchitra 2008).

More studies are needed to try to understand the mechanisms involved in the pathogenesis of these bleeding series. This may provide insight into the discrepancy of bleeding in both haemophiliacs and factor X deficient patients. Management of chronic arthropathy remains a problem for most coagulation disorders. Such data might be used to facilitate the development of monoclonal antibodies to inhibit all the components that uphold the process (Suchitra 2008).

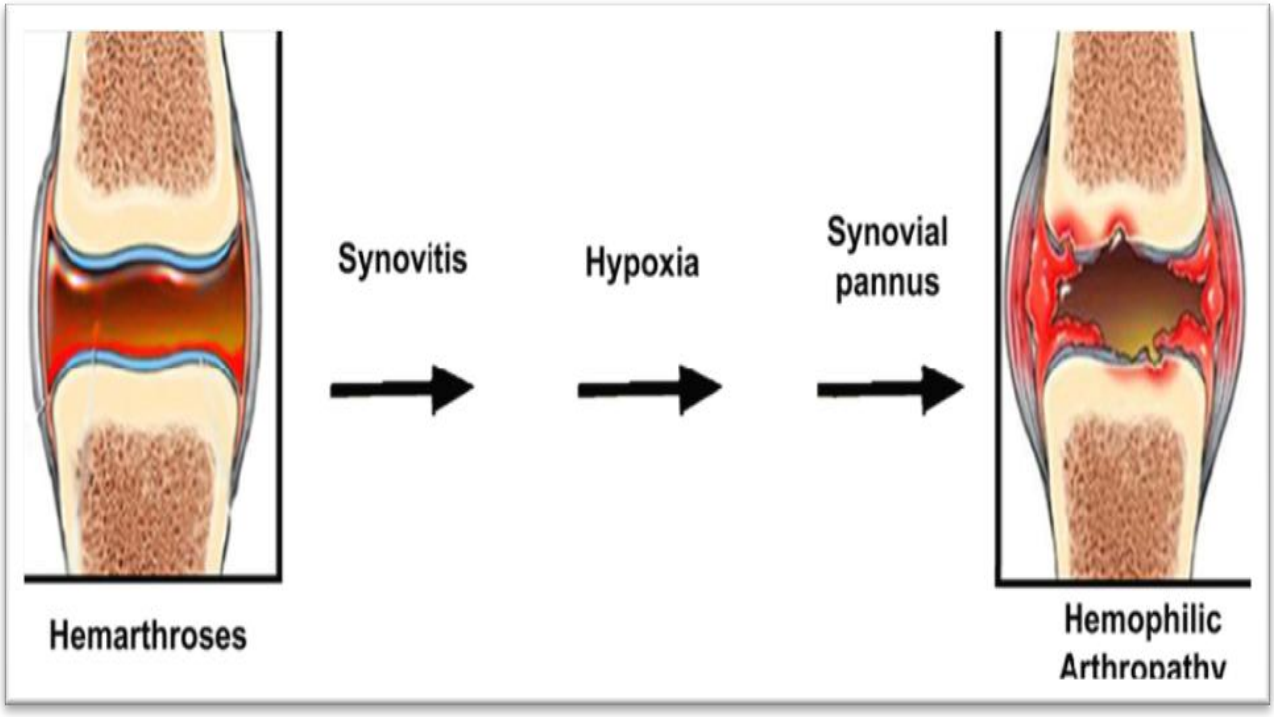

Figure 3. **Schematic representation of synovial changes in hemophilic joint disease. A hemophilic joint depicting hemarthroses leading to the development of synovitis. The red lining depicts synovial hyperplasia and hypertrophy progressing to cartilage erosion and arthropathy (Suchitra 2008).**

1.4.6 Inflammatory cytokines and synovial bleeding

Traditionally it is thought that recurrent joint bleeds stimulates synovial proliferation and the release of hydrolytic enzymes (Suchitra2008). These enzymes along with elevated levels of cytokines help in maintaining the inflammatory response in the synovium. Hence, synovitis is thought to be degenerative and inflammatory in nature (Berntorp et al., 2003; Fischer et al., 2002).

Bleeding into the joint space exposes synovial cells to blood and all its components including iron (Feldman et al., 2006; Fischer et al., 2003). Suchitra (2008) studied a central role for iron in the development of hemophilic synovitis. Iron deposits were observed in the cytoplasm of the lining synovial cells along with neo-visualization. It was further observed that when iron loaded tissues were cultured in *vitro* they synthesized more pro-inflammatory cytokines such as interleukin-18 (IL-18), IL-1beta and TNF-alpha compared to normal appearing synovial tissue (Harris et al., 2006; Hilberg et al., 2003; Suchitra 2008).

Haemophiliacs with synovitis were also found to exhibit a fivefold increase in 12 circulating angiogenesis-related cytokines compared to individuals with a bleeding disorder without joint disease (Beeton et al., 2000; Harris et al., 2006; Suchitra 2008). Likewise, plasma vascular endothelial growth factor (VEGF) and matrix metalloproteinases were significantly elevated in those subjects compared to the non-joint disease groups (Beeton et al., 2000; Harris et al., 2006; Suchitra 2008).

Some aspects of haemophilic synovitis remain unclear. Firstly, mediators participating in cartilage breakdown are uncertain. Secondly, the way in which iron enters chondrocytes is not yet explained (Suchitra 2008). An understanding of these mechanisms may provide insight into the pathogenesis of these bleeding series. In both rheumatoid and haemophilic arthritis microbleeding is well recorded (Harris et al., 2006). The synovial iron deposition was found to correlate closely with the extent of joint destruction and was associated with poor prognosis (Suchitra 2008).

A possible sequence of events in haemophilic arthritis may be caused by intra-articular bleeding (Fischer et al., 2002). This provokes a nonspecific inflammatory response and cause macrophage to accumulate around synovial iron deposits (Suchitra 2008). The macrophages then release cytokines, and stimulate production of latent matrix metalloproteinases (Suchitra 2008).With repeated joint bleeding, the synovium proliferates and start producing a cycle of synovitis →bleedings →synovitis (Suchitra 2008).

Currently, most synovitis studies are reporting IL-1beta and TNF-alpha as cytokines that causes an increase in the synthesis of nitric oxide (NO) and matrix metalloproteinases (MMPs) (De Jager et al., 2007; Del Porto et al., 2007; Lerner 2006; Suchitra 2008). The NO produced in the inflamed joint was found to contribute to the peri-articular bone-loss, leading into massive pain and joint bleeding (De Jager et al., 2007; Del Porto et al., 2007; Lerner 2006; Suchitra 2008). The matrix metalloproteinases (MMPs) were found to be enzymes that break down collagen from the cartilage (Edwards 2005). Other authors have reported the MMPs to be involved in angiogenesis and in recruitment of some pro-inflammatory cytokines that contribute in excessive destruction of cartilage (Clavel and Bessis 2007; Folkman and Shing 1992).

Direct evidence was observed for the involvement of angiogenesis in the development and sustenance of haemophilic synovitis (Harris et al., 2006; Suchitra 2008). Histological sections of synovial tissue from patients with haemophilic synovitis were found to be highly vascularized and contained a high number of inflammatory cells, predominantly macrophages. These sections were co-localized with secreted pro-angiogenic cytokines including vascular endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF-alpha), interleukin-1beta (IL-1beta) and interleukin-18 (IL-18) (Harris et al., 2006; Hilberg et al., 2003; Ovlisen et al., 2009).

The elevated levels of interleukin-18 (IL-18), TNF-alpha and VEGF were found to be dominant activators of angiogenesis. This condition resulted in the formation of premature blood vessels that leaks into the joint and perpetuates the synovitis and joint bleeding (Figure 4) (Clavel and Bessis 2007; Dai et al., 2004; Suchitra 2008). In the study by Dai et al. (2004), IL-18 was found to promote synovitis through a mechanism shown to be distinct from IL-12. Interestingly, IL-18-treated mice were reported to produce significant amount of TNF-alpha and IL-1beta. It was then suggested that IL-18 might have the up regulatory role in the production of the two cytokines (Dai et al., 2004).

The pathogenesis of joint disease in coagulation disorders seems to be multifactorial with changes occurring in the synovium, bone, cartilage and blood vessels associated with the affected joints (Ostergaard et al., 2001; Ovlisen et al., 2009). Iron may be central to these processes, however all these processes may be occurring concurrently rather than sequentially (Ostergaard et al., 2001). Hence, prevention of joint bleeds still seems to be crucial in mitigating the development and progression of joint disease (Ostergaard et al., 2001; Suchitra 2008).

Circulating angiogenic cytokines may provide effective means of detecting early synovial proliferation and identifying candidates for early intervention with therapy (Ostergaard et al., 2001; Ovlisen et al., 2009).Ultimately, anti-angiogenic agents may provide a novel preventative treatment strategy for arthropathy in coagulation disorders (Ostergaard et al., 2001; Suchitra 2008).

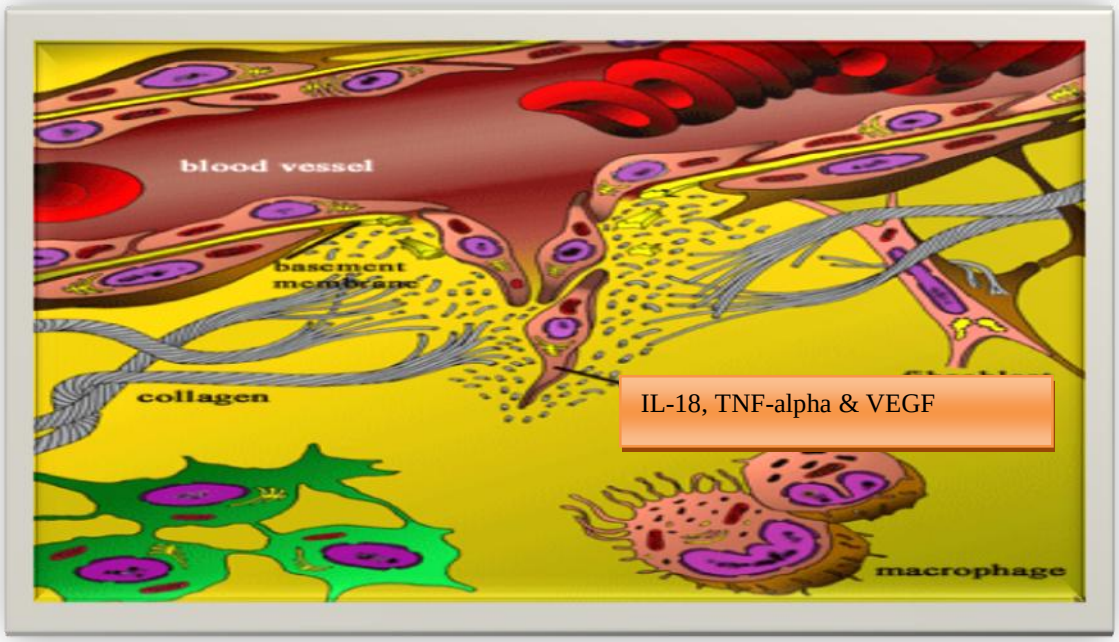

Figure 4. Schematic representation of chronic arthropathy induced by IL-18, TNF-alpha and VEGF. This creates massive haemorrhage and joint degradation. The up regulation of these cytokines is associated with angiogenesis. This results in the formation premature blood vessels that leaks into the joint and worsening the synovitis (http:// www.google.co.za/ image/role of IL-18 in joint inflammation Date of last access 25 June 2009).

1.4.7 Anti-inflammatory cytokines and synovial bleeding

Interleukin-10 (IL-10) and interleukin-4 (IL-4) are modulatory cytokines that have a suppressive effect on TNF-alpha and IL-1beta in *vitro* (De Wall et al., 1991; Yoshiki et al., 2008). They suppress cytokine synthesis by different intracellular mechanisms. IL-4 enhances mRNA degradation of TNF-alpha and IL-1beta, while IL-10 inhibits nuclear factor kappa B (NF-κB) (Schalijo et al., 2009; Yoshiki et al., 2008; Wang et al., 1995). The NF-κB is the intracellular region known to promote the expression of numerous inflammatory genes (Schalijo et al., 2009).

It has been shown that IL-4 and IL-10 stimulate the production of cytokine inhibitors such as interleukin-1receptor antagonist (IL-1Ra) and soluble tumour necrosis factor receptor (TNFR). The up regulation of these anti-inflammatory cytokines was shown to have a positive effect on synovitis (Erik et al., 1998; Jenkins et al., 1995; Shepherd and Nicklin 2005). Sustained treatment with IL-4 was shown to suppress the chronic synovitis and induced the up regulation of IL-1Ra. Elevated levels of IL-10 were found to have the same effect, but the combination therapy with IL-4 has been found to induce a more profound suppression with a major protective effect against arthropathy (Erik et al., 1998; Schalijo et al., 2009).

The IL-1Ra inhibits the actions of IL-1beta by binding to the type I IL-1 receptor (Pan et al., 2000). When delivered systemically as recombinant protein, IL-1Ra was able to prevent the onset of experimental synovitis and reduce the severity of arthropathy (Pan et al., 2000). One study has reported IL-1Ra to have an anti-angiogenesis effect in cancer patients (Dganit et al., 2003). It remains to be seen whether this cytokine might have a significant impact in treating synovitis induced by haemathrosis in FXD patients and in haemophiliacs.

Anticytokine therapy offers new hope to those suffering from synovial bleeding (Bae et al., 2009). The prospect of specifically targeting the effects of key pro inflammatory cytokines in synovitis network may represent a new therapeutic era (Musacchio et al., 2009). From the evidence presented, it could be speculated that all frequent bleeding patients might present with significant high levels of pro inflammatory cytokines (TNF-alpha, IL-1beta and IL-18). These cytokines were shown to be involved in acute inflammatory process leading to cellular infiltrate and joint swelling (De Jager et al., 2007).This enhances synovitis cycle and leads into massive joint bleeding (Ovlisen et al., 2009). On the other hand, infrequent bleeding patients might present with high levels of IL-10 and IL-1Ra. This inhibits the inflammatory synovitis and lessens the frequency and severity of joint bleeding (Erik et al., 1998; Schalijo et al., 2009).

1.4.8 Treatment for factor X deficiency

Repeated bleeds into the joints of factor X deficiency could be prevented by the infusion of factor X concentrates on a daily or weekly schedule depending on the activity levels and bleeding phenotype (Auerswald 2006; Nuss et al., 2000). In those patients who developed antibodies against the infused factor X, isotopic synovectomy can provide relief from repeated joint bleeds (Berntorp and Michiels 2003; Nuss et al., 2000). However, the timing at which this can be applied is still not well-defined. Newer strategies to identify early joint disease through the use of serological markers are needed (Berntorp and Michiels 2003; Nuss et al., 2000; Yoshiki et al., 2008).

The treatment options for bleeding in patients with congenital FX deficiency include the administration of fresh-frozen plasma, or prothrombin complex concentrates (PCCs) that contain FX. The administration of fresh frozen plasma may be associated with complications. This procedure carries a high potential of infections, with hepatitis viruses and human immunodeficiency virus (HIV) being the most reported (Auerswald 2006; Nuss et al., 2000).

Prophylaxis is generally not required in patients with factor X deficiency, except in the most severe cases of bleeding where residual factor X activity is less than 1%, or when bleeding symptoms and severity are similar to those observed in patients with severe haemophilia A (Auerswald 2006; Nuss et al., 2000). However, very low residual factor X activity (1%) may not necessarily be associated with severe bleeding. These observations have been made in patients from the same family and in relatives with the same genetic mutation (Auerswald 2006; Kram et al., 2007; Van Den Berg et al., 2007). Therefore, the heterogeneity in bleeding tendency remains widely open for investigations.

CHAPTER 2

PATIENTS AND METHODS

2.1 Study design

This was a cross sectional, uncontrolled study of two unrelated families with factor X deficiency.

2.2 Study population and Sample size

The study population included a total of 14 members of two families with factor X deficiency currently attending the Haemophilia Comprehensive Care Centre at Charlote Maxeke Johannesburg Academic Hospital.

- Patients were provided with a written patient information leaflet, and were verbally informed about the aim, objectives and procedures to be employed in the study.
- Patients were required to sign a consent form.

2.3 Blood sample collection

Blood for the factor X measurement, cytokine studies and genetic studies was collected in the Haemophilia Comprehensive Care Centre. Each blood sample was processed according to the test to be performed. For factor X assay, the samples were collected in citrate tubes and centrifuged at 3500 g for 20 minutes and the resulting plasma stored at -70 ^{o}C if not analyzed immediately. For cytokine studies, the samples were collected in tubes without anticoagulant and centrifuged at 3500 g for 20 minutes and the resulting serum stored at -70^{o}C until analyzed. For genetic studies, a buffy coat was prepared from an EDTA blood sample and stored at -70 ^{o}C prior to analysis.

2.4 Sample analyses

2.4.1 Cytokine studies

The ELISA kits from BioSource were used for the quantitative determination of IL-1beta (BioSource,IL-1beta Europe,SA),TNF-alpha (BioSource,TNF-alpha Europe, SA), IL-10 (BioSource, IL-10 Europe, SA), IL-18 (BioSource, IL-18 Europe, SA) and IL-1Ra (BioSource, IL-1Ra Europe, SA).These kits uses the same experimental procedure with the only difference being the microtiter plate provided.

Principle

To detect the quantity of cytokine, purified antibody specific for the measured cytokine is linked chemically to an enzyme. Standards and samples react with captured monoclonal antibody (Mab 1) coated on the microtiter well and with a biotinylated monoclonal antibody (Mab 2). After an incubation period allowing the formation of a sandwich, an enzyme Streptavidin -Peroxidase is added and this binds to the biotinylated antibody. The unbound enzyme is removed by washing and a substrate solution is added. This induces an enzyme catalyzed reaction. This reaction is stopped with the addition of stop solution and the microtiter plate is then read at 450nm.The amount of colour change is determined calorimetrically by measuring the absorbance which is proportional to the concentration of the measured cytokine. A standard curve is plotted and the concentration of the measured cytokine is determined by interpolation from the standard curve. This basic assay is shown in Figure 5.

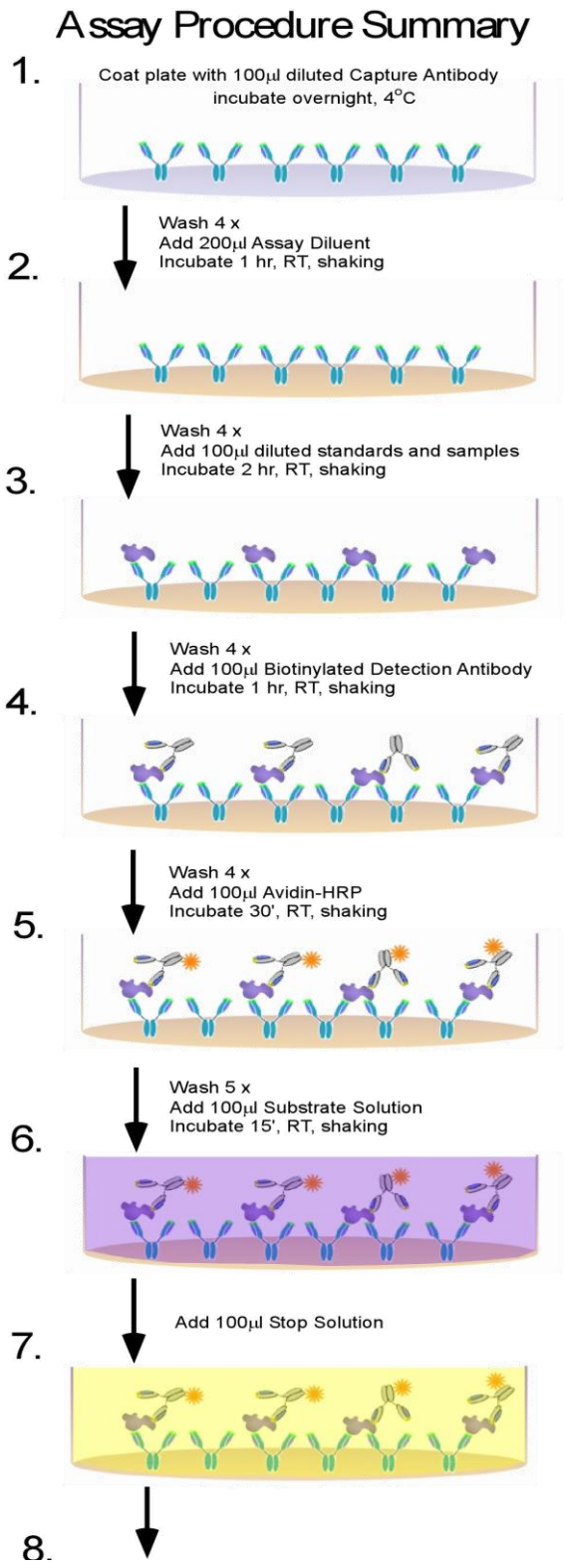

Figure 5.Schematic showing of ELISA. The amount of substrate turnover is determined colorimetrically by measuring the absorbance which is proportional to the concentration of the measured cytokine (BioSource, Europe, SA).

Sample and reagent preparation

Stop solution, substrate reagent, microtiter plate, and assay diluents were ready to use in all the kits. 1X washing solution was prepared by diluting 100ml of 25 X washing solution with 2400ml of distilled water. HRP-conjugate solution was diluted in a 1:101 ratio with conjugate diluent. Standards were prepared after reconstituting the calibrator specific for the measured cytokine with 1ml of assay diluent.The 2500pg/ml of the calibrator was then serially diluted as shown in the table.

Standard concentration	IL-18/IL-1Ra/TNF-alpha/IL-1beta/IL-10	Assay Diluent
1000pg/ml	200µl of 2500pg/ml standard	300µl
400 pg/ml	200µl of 1000pg/ml standard	300µl
160 pg/ml	200µl of 400pg/ml standard	300µl
64 pg/ml	200µl of 160pg/ml standard	300µl
25.6 pg/ml	200µl of 64pg/ml standard	300µl
0.0 pg/ml		300µl

Procedure

Hundred and fifty micro liters (150µl) of prepared standards and samples were pipetted into a new 96 well polyvinyl plate in the same order as the assay to be run. All samples and standards were prearranged in the polyvinyl plate. Hundred micro liters (100µl) of each sample was transferred to the appropriate antibody coated wells simultaneously using a multi channel pipette. The plates were then incubated for 30 minutes. This incubation step ensured that the measured cytokine bind to the coated monoclonal antibody. After incubation, the content of each well was discarded and the plates were washed four times with wash solution.

Hundred micro liters (100µl) of diluted conjugate reagent (biotinylated monoclonal antibody) and Streptavidin-Peroxidase were added followed by 60 minutes incubation at room temperature. This incubation step completed the sandwich and the attachment of Streptavidin-Peroxidase to the biotinylated monoclonal antibody. The supernatant content of each well was discarded and the plates were washed three times with wash solution. Hundred micro liters (100µl) of substrate reagent was added to each well followed by 30 minutes incubation. During this incubation, the enzyme induced a coloured reaction to its substrate. This reaction was stopped by 100µl of stop solution. The plates were then measured at 450nm using a micro plate reader.

Interpretation of results

The absorbencies of the standards were used to plot a standard curve. A straight line graph was obtained and results for each cytokine were extrapolated from the appropriate graph. An example is presented below (Figure 6).

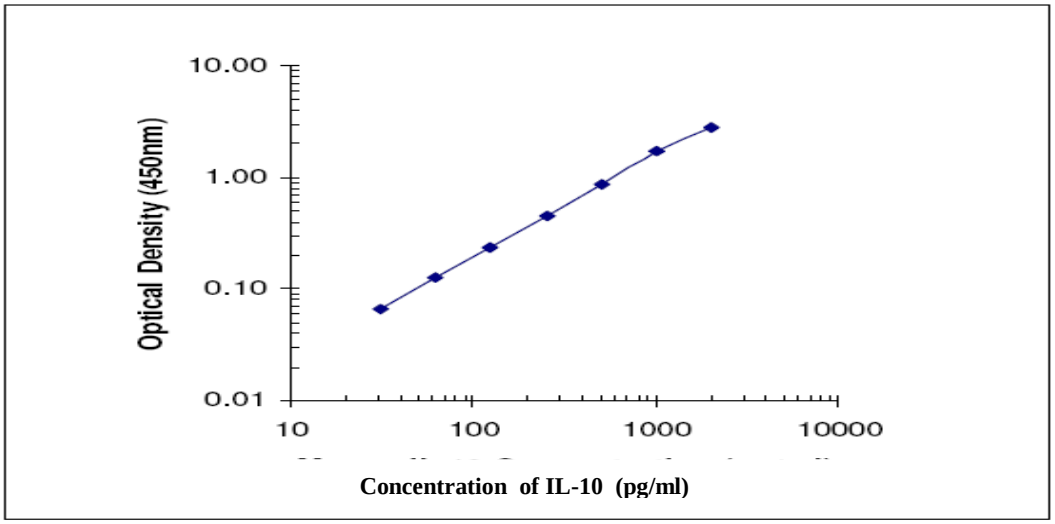

Figure 6. Straight line graph obtained from IL-10 standards. Sample results were extrapolated from this graph.

2.4.2 Determination of coagulation factor X

The factor X assay was used for the quantitative determination of coagulation factor X activity levels (Schafer 2007).

Principle

The factor X assay relies upon measuring the degree of correction of the prothrombin time (PT) when patient plasma is added to normal reference plasma. These results are obtained from an activity curve drawn from dilutions of normal reference plasma and factor X deficient plasma.

Sample and reagent preparation

The factor X deficient plasma was reconstituted with 1ml of distilled water and allowed to equilibrate for 10 minutes. The same procedure was done with assayed reference plasma (Pentapharm, Basel, Switzerland). The assayed reference plasma and factor X deficient plasma were diluted into 1:10, 1:100 and 1:1000 using the Owrens buffer. The procedure is as follows.

1:10	0.9 ml of Owrens buffer + 0.1 ml of reference plasma/test plasma
1:100	9.9 ml of Owrens buffer + 0.1 ml of the reference plasma/test plasma
1:1000	9.99 ml of Owrens buffer + 0.01 ml of the reference plasma/test plasma

Procedure

Thirty microlitres (30µl) of factor X deficient substrate (Pentapharm, Basel, Switzerland) was mixed with 30 µl of the diluted reference plasma. The tubes were incubated at 37°C for 30 seconds and *vipera russelli*-platelet substitute (Pentapharm, Basel, Switzerland) was added to activate factor X. The venom of *vipera russelli* activates factor X directly, and so it will detect deficiencies of factor X, factor V, prothrombin and fibrinogen. However, if factor X deficient plasma is used as a substrate, then the assay will be specific for factor X. After 30 seconds, clotting was initiated by the addition of 0.2 ml of 0.025M Calcium Chloride. The time for clot formation was recorded. This procedure was repeated using 30µl of patient plasma added to normal reference plasma.

Interpretation of results

The clotting times of the test and assayed reference plasma were plotted against the concentration of factor X on a double-log graph paper, taking the 1: 10 dilution as equivalent to 100% of the normal activity. A linear plot was obtained and the patient's lines were parallel to the reference graph .A vertical line was drawn from the dilution that represents 100% of normal reference. This line was drawn until it intercepts the plasma sample being assayed. This vertical line was then dropped until it intercepts the X-axis. The activity level was read at that point. This is well presented in Figure 7.

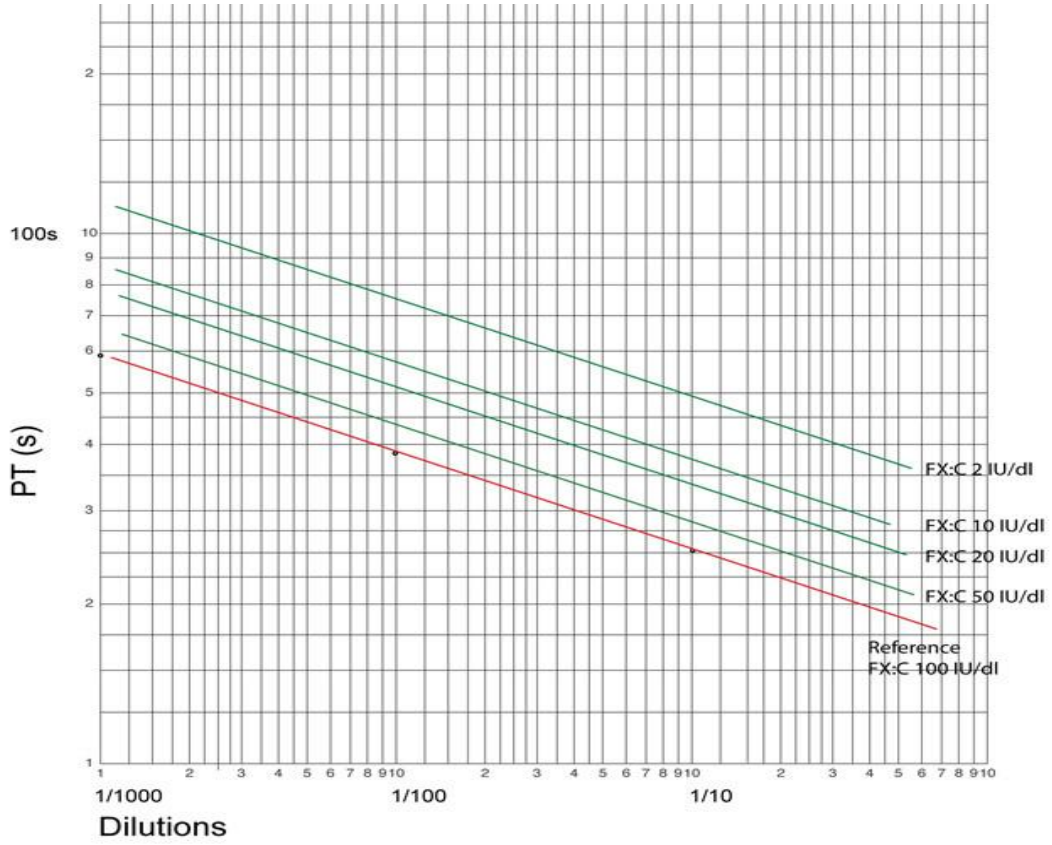

Figure 7.The graph above shows the results of a factor X assay with varying factor X levels. The red line represents the reference plasma and green line represents the test samples. Results were extrapolated from the x-axis and multiplied by the dilution factor 100. 1/2 dilution was assigned a value of 50 IU/dl (50%), 1/4 dilution was assigned a value of 25 IU/dl (25%) and 1/5 was assigned a value of 20 IU/dl (20%) (Schafer 2007).

2.4.3 DNA extraction

DNA was extracted using a Nucleon Resinant kit from Amersham. (Nucleon® BACC2 kit, GE Health Care Europe- Amersham).

Principle

The system from Amersham was designed to give high yields of pure DNA from blood, cultured cells and hard tissue or paraffin sections. The procedure has been optimized to give maximum recoveries of high molecular weight DNA using a low shearing protocol. The principle includes cell lyses, deproteinisation, extraction and precipitation.

Procedure

Nine hundred microlitres (900 µl) of EDTA blood was transferred to the 1.5 ml micro tubes. Three hundred and fifty micro liters (350 µl) of reagent A (sodium perchlorate) (Nucleon® BACC2 kit, GE Health Care Europe-Amersham) was added. The mixture was centrifuged at 600 rpm for 5minutes. This step lysed the cells. The supernatant was removed and 700 µl of sodium perchlorate (Nucleon® BACC2 kit, GE Health Care Europe- Amersham) was added to the pellet and centrifuged again at 600 rpm for 5 minutes.

Six hundred micro liters (600µl) of reagent B (chloroform) (Nucleon® BACC2 kit, GE Health Care Europe- Amersham) was added and the tubes were inverted 7 times. This step removed the proteins (histones) from pellet. Hundred and fifty micro liters (150µl) of Nucleon Resin suspension (Nucleon® BACC2 kit, GE Health Care Europe- Amersham) was added to the mixture, the system was allowed to mix for 5 minutes and centrifuge at 350 rpm for 1 minute. This step extracted the DNA.

Without disturbing the Nucleon Resin layer (brown in colour shown below), the upper solution was pipetted to another 1.5ml micro tube. Nine hundred micro liters (900 µl) of cold absolute ethanol (80% (v/v) Vietnam) was added and the tubes were inverted several times to precipitate the DNA. The DNA was then removed and allowed to air-dry for 10 minutes. Later, the DNA was re-dissolved in sterile water.

Figure 8. 1.5ml micro tube showing the separation of Nucleon Resin layer and DNA during extraction (Nucleon® BACC2 kit, GE Health Care Europe- Amersham). Nine hundred micro liters (900 µl) of cold absolute ethanol (80% (v/v) Vietnam) was added to the upper solution and the tube was inverted several times to precipitate the DNA. The DNA was then removed and allowed to air-dry for 10 minutes. Later, the DNA was re-dissolved in sterile water.

2.4.4 Genetic studies

2.4.4.1 Gene amplification

Due to limited budget for this study, genetic studies could only be done on frequent bleeding patients. Exon VIII (8) of the factor X gene was amplified using PCR and sequenced with Spectrumedix SCE 2410 Genetic analysis system (Spectrumedix ILL, USA). The PCR reaction was performed in a GeneAmp PCR system 9600 thermocycler (PE Biosystems, Foster City, USA). Two sets of primers were used for the amplification of Exon 8. PCR for exonic 8A and 8B was performed in a 25 µl volume containing 300-500 ng of genomic DNA, 7.5 pmoles of each primer, 250 µM of each dNTPs, 0.87 units of Expand High Fidelity PCR system in supplied buffer-2 (Roche Diagnostics, GmbH, Mannheim, Germany). The expand high fidelity PCR system is composed of thermostable Taq DNA polymerase with proofreading function. This results in PCR products with high yield, high fidelity and high specificity. PCR conditions for each fragment are detailed below. All reactions were carried out under the following conditions: 4 minutes at 95°C, 35 cycles at 95°C for 30 seconds, 57°C for 1 minute, 72°C for 5 minutes with a final extension for 5 minutes at 72°C (Jahandharan et al., 2005).

Exon	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)	Forward and reverse primers (pmoles)	Final concentration of $MgCl_2$ (mM)	Annealing temperature (°C)
8A	GATGTGCGAGAGCATGTCC	CTTGAAGCGGGTGACGTG	496	7.5	1.5	57
8B	GCTACGACACCAAGCAGGAG	GTATCTGGGGAAAGGAATGC	495	15	1.5	57

PCR product analysis

PCR products were analysed by agarose gel electrophoresis. Subtyping was achieved through separation of different size PCR bands on a 2 % agarose gel (50 ml of 0. 4 M Tris acetate EDTA, 1g of agarose and 1 µl of 10 mg/ml ethidium bromide). The DNA was visualized by addition of 6 µl of 10 mg/ml ethidium bromide. A 50bp DNA was used as the molecular weight marker (50bp DNA molecular marker Roche Diagnostics, GmbH, Mannheim, Germany). The gels were visualized and photographed on UVP trans-illuminator using Grab IT annotating Grabber 2.51 software (UVP (Inc), USA).

2.4.4.2 DNA sequencing

Purification of PCR products

PCR products were purified using the QIAquick PCR purification kit (Qiagen, Germany) before the samples were sent for sequencing. The QIAquick purification kit is for direct purification of double or single stranded PCR product (100bp-10Kb) from amplification reactions. This kit is suitable for fast cleanup of up to 10 µg of DNA fragments from enzymatic reactions and agarose gels. Using proven microspin technology, the kit delivers highly concentrated purified DNA.

Procedure

Eight hundred micro liters (800µl) of buffer PB (guanidine hydrochloride, isopropanol) (Qiagen, Germany) was added to the PCR products and mixed by pipetting. This buffer dissolved the agarose gel. The solution was added to a QIA spin column placed in a 2ml collection tube. The column was then centrifuged at 13000 rpm for 50 seconds. The filtrate was discarded and the column was placed back to the same collection tube. Seven hundred and fifty micro liters (750µl) of buffer PE (ethanol) (Qiagen, Germany) was added to the spin column to wash the samples and centrifuged at 13000 rpm for 1 minute. The filtrate was discarded and the spin column was placed back to the same tube and centrifuged for an additional 1 minute for complete removal of ethanol from buffer PE. The spin column was then placed in a clean 1.5ml microcentrifuge tube and 50µl of buffer EB (10 mM Tris-Cl, pH 8.5) (Qiagen, Germany) was added to elute the DNA. The purity of the eluted DNA was checked on a NanoDrop spectrophotometer.

DNA sequencing

The PCR products were sequenced on an automated Spectrumedix SCE 2410 (Spectrumedix ILL, USA) from Inqaba Biotech (Pty) Ltd, South Africa. This automated sequencer, incorporates the ABI Big Dye terminator cycle sequencing kit version 3.1 (Applied Biosystem, Foster city, CA) with the primers. The reaction was carried out in a 20 µl reaction. Forward and reverse primers were the same as those used for PCR amplifications.

In brief, approximately 50-90 ng of PCR product was mixed with 5.0 µl of Terminator Ready Reaction mix (A,C,G and T-dye terminators; dNTPs, Tris-HCL (pH9.0), $MgCl_2$, thermal stable pyrophosphatase and Amplitech DNA polymerase FS) (Biosystem, Foster city, CA), 2 µl of 5 X sequencing buffer and 3.2 pmol primer. Sequencing conditions were similar to the one used for gene amplification. Excess DyeDeoxy terminators were removed from the DNA sequencing products with Centri-Sep Spin columns (Biosystem, Foster city, CA). The purified products were dried in a speedivac at -60 °C and stored at -20 °C. Sequencing data was analysed with (MEGA) software version 4.0 (Tamura et al, 2007). Results were compared with FX sequence as reported in the GenBank database (GenBank accession no. M57285).

CHAPTER 3

3. RESULTS

3.1 Cytokine analysis

The sample population consisted of fourteen (14) patients that were divided into two groups according to their families. Age, gender, factor X activity, clinical manifestations and cytokine profiles are shown in Table 3.1.1 and Table 3.1.2 respectively.

Table 3.1.1: Coagulation factor X activity levels, clinical manifestations and cytokine profiles of the first factor X deficient family.

Patient ID	Age	Gender	FX Activity	Clinical Manifestations	IL-1beta Pg/ml	TNF-alpha Pg/ml	IL-18 Pg/ml	IL-1Ra Pg/ml	IL-10 Pg/ml
CFXD 1	42	M	35%	Frequent bleeder	70.72	123.04	62.51	335.00	57.12
CFXD 2	51	M	9%	Infrequent bleeder	0.01	0.07	31.21	398.02	87.41
CFXD3	83	M	44%	Infrequent bleeder	0.01	0.03	31.20	278.01	86.21
CFXD 4	34	M	3%	Infrequent bleeder	0.01	0.10	43.23	550.00	79.31
CFXD 5	40	M	43%	Infrequent bleeder	0.02	0.01	48.22	665.01	91.21

High levels of IL-1beta, TNF-alpha and IL-18 were noted in a frequent bleeding patient CFXD 1 compared to the other family members. These cytokines are reported to induce synovitis and lead into massive joint bleeding. High levels of IL-10 were noted in the entire infrequent bleeding patients. This cytokine was reported to inhibit the inflammatory synovitis and lessen the severity of joint bleeding.

Table 3.1.2: Coagulation factor X activity levels, clinical manifestations and cytokine profiles of the second factor X deficient family.

Patient ID	Age	Gender	FX antigen	Clinical Manifestation	IL-1beta Pg/ml	TNF-alpha Pg/ml	IL-18 Pg/ml	IL-1Ra Pg/ml	IL-10 Pg/ml
CFXD 6	26	M	30%	Infrequent bleeder	0.02	0.07	160.01	43.12	98.3
CFXD 7	18	M	25%	Infrequent bleeder	0.01	0.06	850.03	40.23	89.47
CFXD 8	41	F	1%	Frequent bleeder	146.75	84.21	665.01	82.51	63.91
CFXD 9	67	F	36%	Infrequent bleeder	0.01	0.03	685.02	42.40	93.52
CFXD10	54	M	4%	Infrequent bleeder	0.01	0.09	278.21	42.72	88.34
CFXD 11	28	F	50%	Infrequent bleeder	0.01	0.04	875.01	105.61	89.01
CFXD 12	18	F	80%	Infrequent bleeder	0.01	0.07	555.10	65.23	93.12
CFXD 13	14	M	4%	Infrequent bleeder	0.01	0.01	278.00	31.24	101.24
CFXD 14	42	M	26%	Infrequent bleeder	0.10	3.29	398.00	98.33	87.23
CFXD 15	34	F	22%	Infrequent bleeder	0.01	0.08	678.10	101.35	87.91
CFXD 16	55	F	4%	Frequent bleeder	204.2	97.41	866.01	42.13	66.21

Low levels of IL-1beta and TNF-alpha were observed in infrequent bleeding patients. High levels of IL-1beta, TNF-alpha were noted in CFXD 8 and CFXD 16. High levels of IL-10 were noted in all infrequent bleeding patients. No pattern was noted with IL-18 in both infrequent and frequent bleeding patients.

3.2 Genetic analysis

The PCR results of the first and second amplicon fragment are shown in figure 9 and figure 10 respectively. These amplicons were carefully cut from the gel, purified with QIAquick PCR purification kit (Qiagen, Germany) and sequenced using the Spectrumedix SCE 2410 (Spectrumedix ILL, USA).Sequencing results are shown in figure 11 and figure 12 respectively.

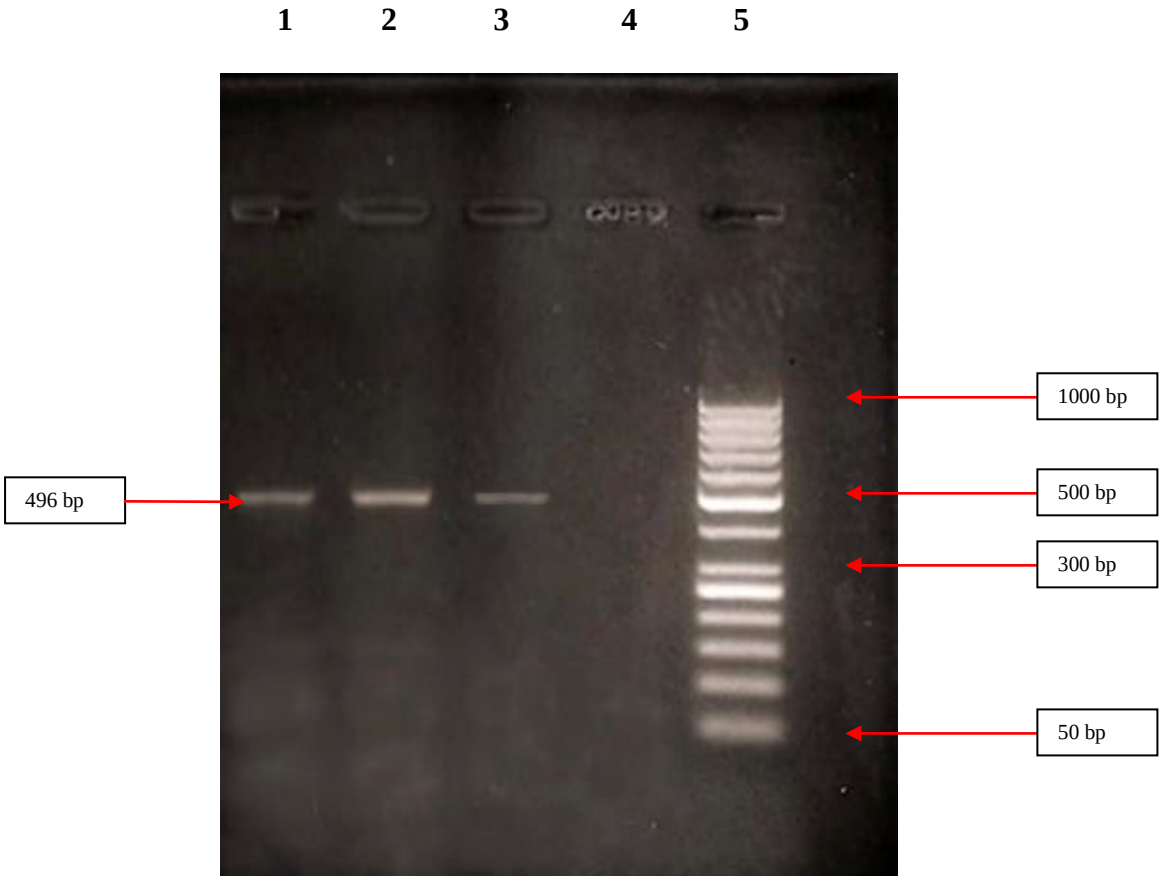

Figure 9. PCR results showing the first portion of exon VIII (8A). The amplicon size was 496 bp. Lane 1 represents CFXD1, lane 2 represents CFXD8, lane 3 represents CFXD16, lane 4 represents the control and lane 5 represents the 50 bp DNA Ladder (50bp DNA molecular marker Roche Diagnostics, GmbH, Mannheim, Germany).

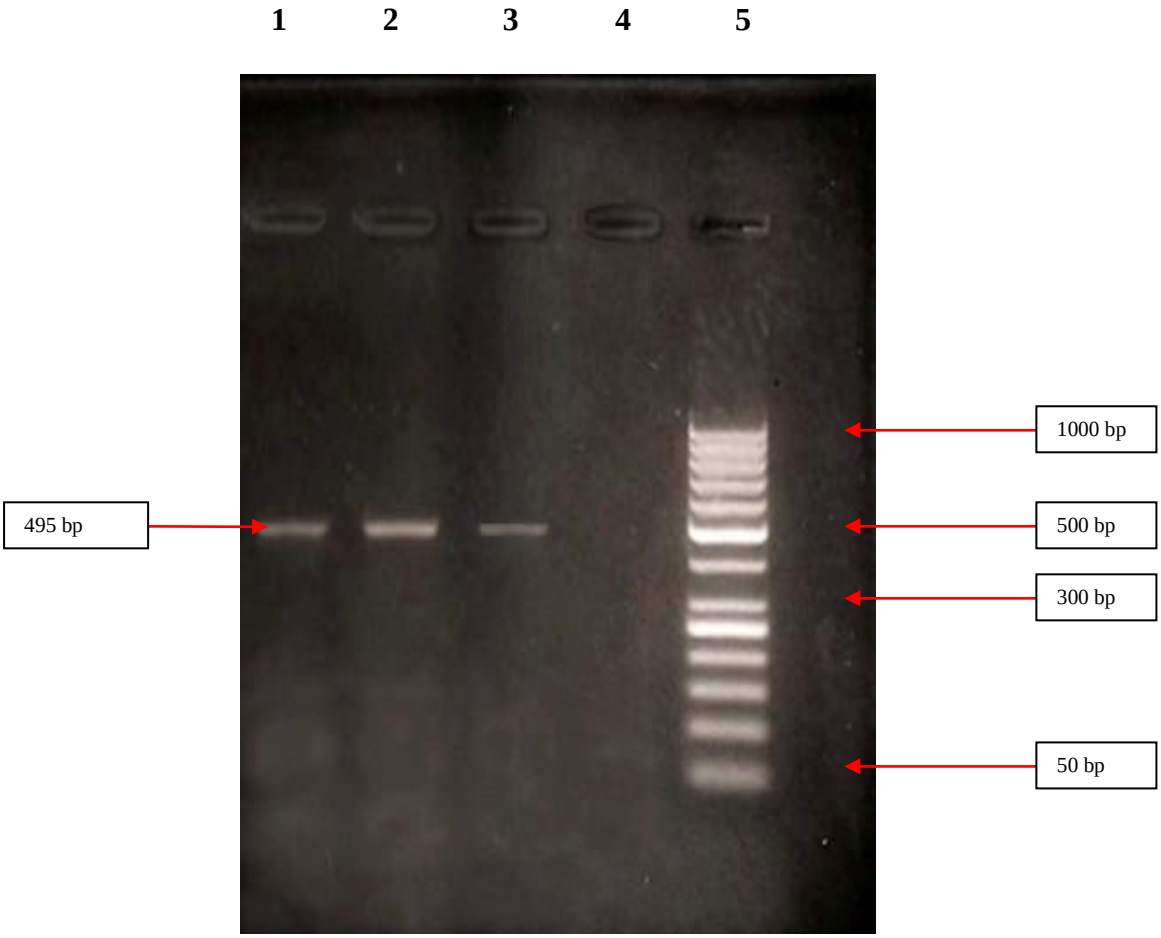

Figure 10. PCR results showing the second portion of exon VIII (8B). The amplicon size was 495 bp. Lane 1 represents CFXD1, lane 2 represents CFXD8, lane 3 represents CFXD16, lane 4 represents the control and lane 5 represents the 50 bp DNA Ladder (50bp DNA molecular marker Roche Diagnostics, GmbH, Mannheim, Germany).

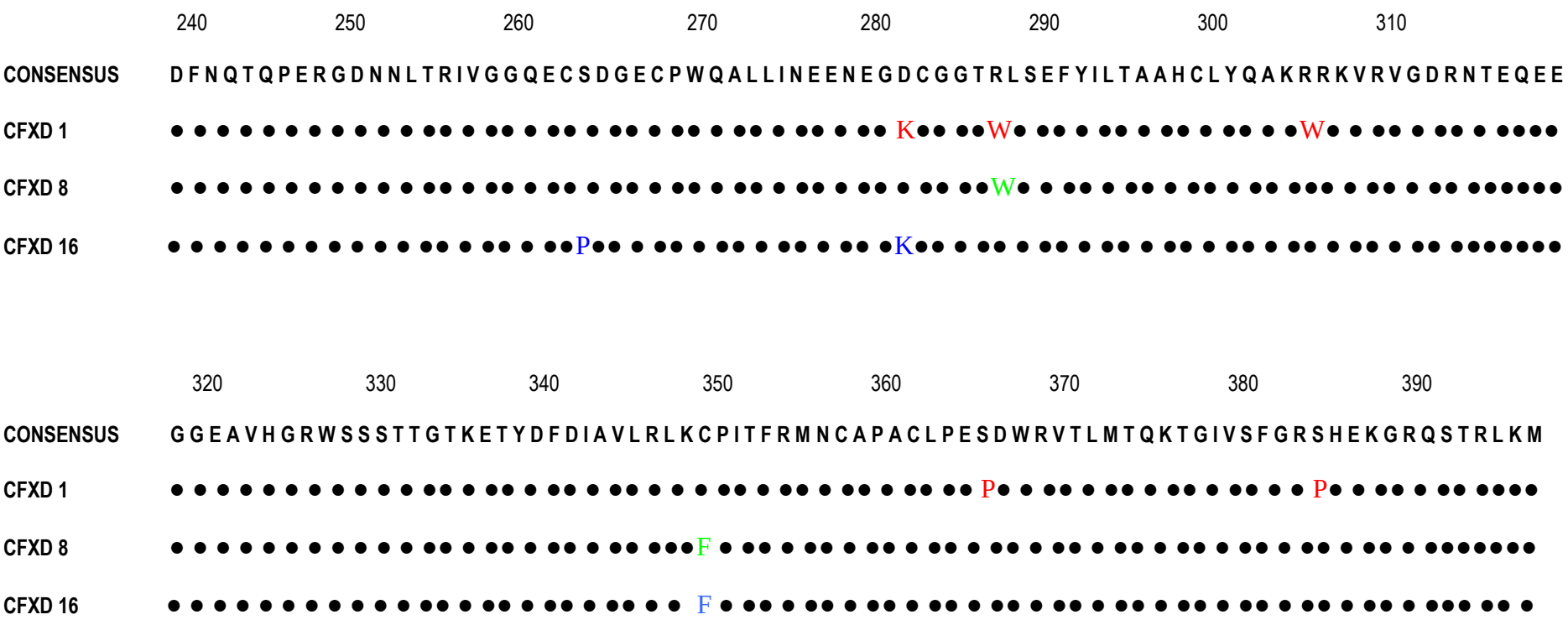

Figure 11. Predicted amino acid sequence alignment of the three patients compared with their respective consensus sequence obtained from *H. sapiens* (GenBank accession no. M57285). The dotted line represents similarity with the consensus and the change in amino acid is presented. Multiple sequence alignment was performed with CLUSTALW file using the Mega 4 software (Tamura et al, 2007).

Differences were observed between the amino acid sequence of the three frequent bleeding patients and the consensus. In addition, a novel mutation Cys350Phe was detected in CFXD 8 and CFXD 16 (figure 12). This mutation is characterized by G>C change in position 350 (Andrea and Fatima 2008; ISTH factor X database, 2009; Vianello et al., 2003). Therefore, the severity of bleeding might be related with these changes. It is possible that after post translation processing the catalytic domains of these patients becomes inactive and destroyed in the plasma. This results in low factor X activity levels and contributes to the massive joint bleeding.

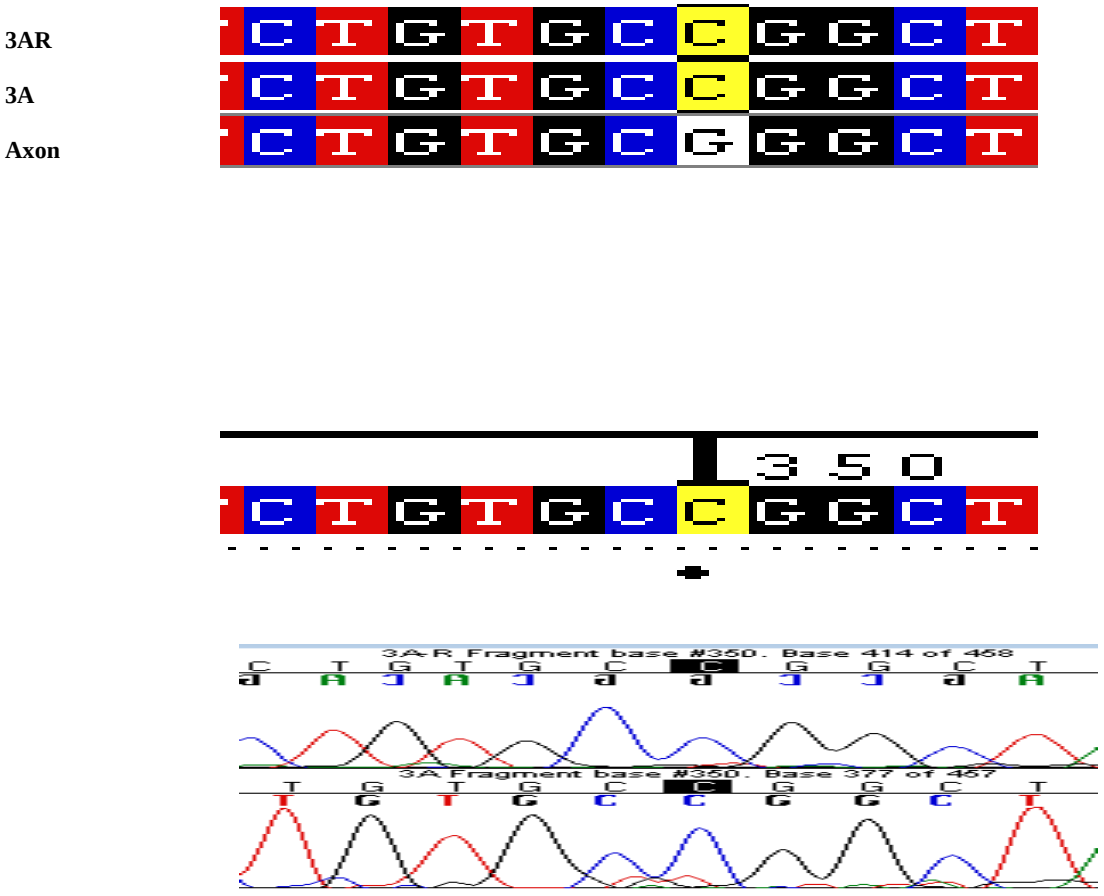

Figure12. Nucleotide sequences showing G>C mutation that results into Cys350Phe mutation (Andrea and Fatima 2008; ISTH factor X database, 2009; Vianello et al., 2003). 3AR represents the reverse sequence, 3A represents the forwards sequence and axon represents the consensus sequence.

CHAPTER 4

4. DISCUSSION AND CONCLUSION

4.1 Cytokine analysis

4.1.1 Cytokines and Clinical manifestations

The present study has explored the association between cytokine profiles and the clinical manifestations of the two factor X deficient families. The high expression of IL-1beta and TNF-alpha in CFXD 1, CFXD 8 and CFXD 16 is consistent with ongoing joint inflammation in haemophilia. These cytokines are known to be involved in the acute inflammatory process leading to cellular infiltrate and joint swelling. This result in chronic synovitis and the creation of a vicious cycle of bleeding → synovitis → bleeding (Ovlisen et al., 2009).The current data has supported our hypothesis that the intensity of bleeding from these patients was related to the expression of the two cytokines. This enhances the synovitis cycle and creates massive joint bleeding.

Given the potential independent etiologic basis for pro-inflammatory cytokines, it would not be surprising if the source of the inflammatory cytokines generated in these patients were different. The source of IL-1beta in the inflamed joint may be generated from invading macrophages, or via local production from the fibroblast-like synoviocytes (FLS) (Hana et al., 2007; Kaneyama et al., 2002). One of the stimulants may also be a recently discovered cytokine, IL-32 which is expressed constitutively in FLS (Fukui et al., 2003; Joosten et al., 2006).

The overwhelming down regulation of IL-1beta and TNF-alpha in infrequent bleeding patients appears to be related to the high expression of IL-10 and IL-1Ra. Therefore, the source for mediators of inflammation in these patients may be more dependent on the local production of anti inflammatory cytokines by cells residing within the joint and the external influx. IL-1beta may be produced locally within the joint by stimulated chondrocytes but, due to high expression of these anti-inflammatory cytokines, this inhibits the inflammatory synovitis and lessens the severity of joint bleeding. Once again, the current data seems to supports such evidence.

There are ranging debates about the role of IL-18 during inflammatory synovitis. Most studies indicates that IL-18 plays an important role in synovitis and joint destruction via T cells and macrophages (Dai et al., 2004; Leo et al., 2004; Smeets et al., 2003). Most authors believe that IL-18 up regulates the production of IL-1beta and TNF-alpha, but it does not have a direct effect on FLS (Dai et al., 2004; Leo et al., 2004; Smeets et al., 2003). It was demonstrated that high levels of IL-18 were associated with the production of IL-1beta and TNF-alpha .This was then found to result in chronic synovitis (Dai et al., 2004; Leo et al., 2004; Smeets et al., 2003).

The current data has not found any association between the expression of IL-18 and the production of TNF-alpha and IL-1beta. This creates questions about the validity of this association. According to Kenji et al. (2001), it was not true to justify IL-18 as a pro inflammatory cytokine. It was shown that IL-18 is a unique cytokine that stimulates both pro and anti- inflammatory responses depending on the surrounding cytokines (Kenji et al., 2001).

It was found that stimulation with IL-12 and IL-18 was associated with the production of IFN-γ and other anti-inflammatory cytokines in a synergistic manner (Dong et al., 2009). Moreover, naive T cells were found to produce both IL-4 and IL-13 in response to stimulation with IL-18 (Tomohiro et al., 1999). IL-4 and IL-13 are cytokines that are known to have suppressive effect on pro-inflammatory cytokines (Kenji et al., 2001; Nathan and denizot 1998). It was then concluded that the actions of IL-18 were influenced by the surrounding cytokines. In conditions with high expression of pro-inflammatory cytokines (IL-1beta and TNF-alpha), IL-18 was more likely to support the actions of pro-inflammatory cytokines, while in conditions with high expression of anti-inflammatory cytokines the opposite effect was expected (Kenji et al., 2001).

The quantitative determination of IL-4, IL-3 and IL-12 are beyond the scope of this investigation, but it would have enabled means to assess the clear picture of IL-18 in these patients. It could be possible that the expression of IL-18 in frequent bleeding patients is associated with pro-inflammatory actions because of the high expression of IL-1beta and TNF-alpha. However, in infrequent bleeding patients IL-18 might support the actions of anti-inflammatory cytokines because of the high expression of IL-10 and IL-1Ra. This leaves an open field for research to predict the exact role of IL-18 in these patients.

4.1.2 Cytokines and Coagulation X factor levels

In FXD, the bleeding diathesis stimulates the coagulation cascade to ensure the culmination of bleeding. The lack of factor X ensures the massive expression of tissue factor which could be stimulated by TNF-alpha and IL-1beta (Charles 2001; Emanuela et al., 1997; Sarah and Machin 2007). It was expected that patients with low factor X activity levels will present with high levels of the two cytokines to ensure the production of the tissue factor. However, the current data has showed no significant pattern between factor X activity levels and the expression of these two cytokines. Therefore, the patho-physiological mechanisms predisposing a particular individual to these inflammatory cytokines requires further elucidation. It could be possible that the discrepancy in the expression of these cytokines lies with the individual genotype in maintaining the balance between pro and anti -inflammatory cytokines (Figure 13).

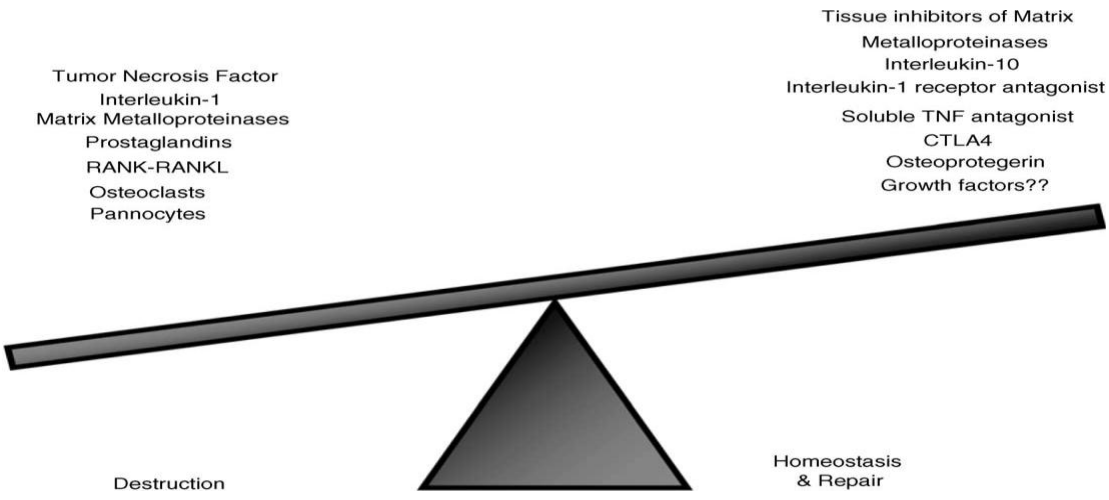

Figure 13. The outcome and severity of chronic synovitis is determined by the balance between destructive or pro-inflammatory and anti-inflammatory or regenerative signaling pathways (Rick et al., 2004).

The coagulation process is down regulated by inhibitors such as anti- thrombin, protein C and protein S (Charles 2001; Sarah and Machin 2007). Defects in these inhibitors often lead to a pro thrombotic status. Some studies have suggested that this status would change the clinical manifestation and make the bleeding tendency less severe (Escuriol et al., 2001; Nichols et al., 1996). Nichols et al. (1996) observed that haemophiliac patients with FV mutation had less severe phenotype. This mutation resulted in a lack of protein C and S thus creating the pro thrombin status. It was argued that the pro-thrombotic risk factors compensated for the low factor levels and result in more efficient thrombin generation and clot formation. This is one area that needs to be investigated in FXD populations (Nichols et al., 1996).

4.2 Genetic analysis

A number of substitutions of proline (P) residues to serine (S) were predicted in CFXD 1 and CFXD 16. According to Bereczky et al. (2008), differences associated with these changes include the lack of cleavage of factor X, altered orientation and a reduction in the catalytic efficiency toward the substrate prothrombin. It was suggested that substitution of proline (P) residue to serine (S) could be responsible for these multiple effects (Bereczky et al., 2008). It was unfortunate that without availability the three-dimensional structure of activated factor X, it was difficult for them to attribute these mutations (Bereczky et al., 2008).

A substitution of Aspartate (D) 282 was observed in CFXD 1 and CFXD 16. Molecular modelling studies have suggested that Aspartate (D) 282 is one of the fully buried residue at the centre of β-strand that is adjacent to the catalytic triad. (Fabrizio et al., 2001; James and uprichard 2002). It was suggested that a change in this residue might pertub the catalytic triad resulting in misfolded protein which will be destroyed in the plasma. (James and uprichard 2002).Therefore, the low levels of factor X activity in these two patients might result from these changes.

A change in Arginine 287(R) was observed in CFXD 1 and CFXD 8. Arginine 287(R) is a highly solvent accessible residue. This residue is implicated in factor V binding during the activation of factor X (James and uprichard 2002; Marchettgi et al., 1995). It was suggested that the introduction of tryptophan (W) was unlikely to affect the conformation of the protein, but it may alter the electrostatic profile of activated factor X. This may in turn compromise the factor V binding (James and uprichard 2002).

The Cys350Phe detected in both CFXD 8 and CFXD 16 is a novel mutation. This mutation is characterized by very low factor X levels which sometimes are not detectable in circulation. According to the molecular modeling study it was shown that Cys350 is positioned in the primary substrate binding pocket (Jayandharan et al., 2005). The side chain of the substituted cystine was shown to cause defect in the substrate binding, leading to the loss of enzyme activity (Vianello et al., 2003). Therefore, despite the influence of cytokines, the severity of bleeding might be exerted by these mutations.

The goal of understanding the relationships between structural and functional aspects of factor X has been limited by the small number of reported factor X abnormalities (Millar et al., 2000). Investigation of the normal and abnormal variations in the factor X gene had been limited by the elucidation of restriction fragment length polymorphisms (Millar et al., 2000).Some preliminary studies have succeeded in characterizing mutations evolving from different regions of the factor X gene. However, much still needs to be done (ISTH factor X database, 2009).

Based on structural homology of the vitamin K-dependent serine protease, naturally occurring mutations in the factor IX and prothrombin genes should complement our understanding of the structure-function relationships of factor X (Millar et al., 2000). The predicted amino acids changes reported in this study produced no significant clinical characterization of these patients. However, these changes provide a model for future structure-function studies of factor X and new insights into the molecular bases of FX deficiency.

4.3 Limitations of the study

The results for the cytokine analysis were extrapolated from the standard curve. It was a challenge to extrapolate precise values that were below the zero standard. Cytokine secretion is influenced by the number of factors such as diet and physical activity. It would have been more appropriate to have fresh samples from these patients at least every month. This would have help in assessing the consistency of the current results. However, due to on going migration of some family members, this was an impossible mission to embark on.

Forward and reverse sequences for each of the segments sequenced were checked for background noise at the beginning and end of the sequence. Those parts of the chromatogram that did not show well defined peaks were edited before the sequences were analyzed. This has resulted into sufficient amount of data being lost.

The sequencing was performed only on frequent bleeding patients. It would have been better to sequence the whole 27 kb in all the participants. This would have enabled us to compare the mutations between and within the family members. In future, more funding should be invested to fully characterize these patients. It is still possible that the discrepancy of the bleeding series might be explained by individual mutations.

4.4 Conclusion

Large heterogeneity in bleeding pattern and arthropathy is observed among patients with coagulation disorders. Because the distinction of phenotypes has implications for both clinical practice and basic science, it is an important subject for study (Pencharz 2006). Understanding the reasons for the heterogeneity in clinical manifestations can give clues that can lead to identifying factors which influence the final haemostasis in FXD. Although there seems to be an overall acceptance that there is a large clinical heterogeneity among patients, no data is available to explain how to identify patients at different bleeding risk.

The results of this current study have shown that levels of IL-1beta and TNF-alpha are high in frequent bleeding patients compared to infrequent bleeding patients with Factor X deficiency. However, much work is still needed. This was a small study intended to elucidate the cytokine profiles of the two families with FXD. More large studies are still needed to justify the statistical difference on these cytokines. From the evidence presented, one might conclude that the origin of the heterogeneity of clinical manifestations in FXD is multifactorial. Cytokines such as TNF-alpha and IL-1beta seem to be involved in the induction and the perpetuation of synovitis. This leads into circle of massive joint bleeding.

High levels of IL-10 in the entire infrequent bleeding patients suggest that these patients might be switching on genes for anti-inflammatory cytokines. This inhibits the inflammatory synovitis and lessens the frequency and severity of joint bleeding. Based on these findings, it could be suggested that the administration of anti-inflammation drugs might be of benefit to the bleeding tendencies of these patients.

The differences observed between the amino acid sequence of the FX protein of the three patients and the consensus may provide a model for future structure-function studies of FX. The severity of bleeding might be related to these structural changes. It might be possible that after post translational processing the catalytic domains of these patients becomes inactive and destroyed in the plasma. This results in low factor X levels which contributes to the increased bleeding frequency.

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APPENDIX

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Thwala

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M071129**
PROJECT	Association between Coagulation Factor Level Cytokine Profiles, Clinical Manifestations and
Genotypic Features	in Factor X Deficiency
INVESTIGATORS	Mr C Thwala
DEPARTMENT	Molecular Med & Haematology
DATE CONSIDERED	07.11.30
DECISION OF THE COMMITTEE*	APPROVED UNCONDITIONALLY

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

DATE 07.12.13 **CHAIRPERSON** .....
(Professors PE Cleaton-Jones, A Dhai, M Vorster, C Feldman, A Woodiwiss)

*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