APPLICATIONS OF DNA DIAGNOSIS TO INHERITED COAGULATION DISORDERS

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STATEMENT OF ORIGINALITY

Since the haemophilia patients were already registered at either the Royal Hospital for Sick Children or the Royal Infirmary in Glasgow, a certain amount of pedigree information was already available and Drs. Anna Pettigrew and Gordon Lowe helped me to obtain further information. Dr. Keith Spowart at the Royal Infirmary in Glasgow collected many of the blood samples from the von Willebrand patients and Dr. Anna Pettigrew collected many blood samples from the haemophilia B patients. All other blood samples were collected by myself. When I started working at the Duncan Guthrie Institute of Medical Genetics, many of the samples from haemophilia B patients had already had DNA extraction done. All DNA extractions were subsequently performed by myself as were Southern blots and autoradiography. The preliminary analysis of haemophilia B pedigrees was done by Professor Connor. All other results were analysed by myself.

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ACKNOWLEDGMENTS

I wish to thank all the people who encouraged me during the time that I was undertaking this project. In particular, I would like to thank Dr. Ian Hann who encouraged my initial interest and who found the funds to support my research; Professor Mike Connor who supervised me with continued enthusiasm and unfailing good humour; Ms. Lindsay Pirritt for help in the laboratory. Coagulation assays were undertaken by several MLSOs in the haematology department of the Royal Hospital for Sick Children and I am grateful to them for carrying out this work. The illustrations could not have been completed without the assistance of the departments of medical illustration in both the Royal Hospital for Sick Children, Glasgow, and St. James's University Hospital, Leeds, and also the artistry of Dr. Robert Chapman and I thank all these individuals for their time and talent. I would like to thank Dr. Richard Jones and Dr. Steve Scott for advice and help with computing. Finally the enormous chore of typing the many versions of this manuscript was kindly undertaken by Mrs. Sue Norfolk. I could not have completed this thesis without her help and I thank her for both her skill and her endurance.

SUMMARY

Haemophilia A, haemophilia B and von Willebrand's disease are the three commonest inherited disorders of coagulation. Genetic counselling at the time this project was undertaken (1985/86) was hampered by the inability of coagulation tests to clearly distinguish carriers from non-carriers for the X-linked haemophilias and by the need for second trimester fetal blood sampling in those families who opted for prenatal diagnosis. The present project aimed to utilise the newly available gene probes for these three conditions to measure their clinical value for improved genetic counselling within affected families.

DNA was extracted from 216 individuals from 16 families affected by haemophilia A of whom 40 were affected males, 38 were obligate carrier females and 71 were females at risk. 4 restriction fragment length polymorphisms (RFLPs), two intragenic, exon 17-18/Bcl I (F8A) and exon 26/Bgl I (F8B), and two extragenic, DX13/Bgl II and St14/Taq I, were applied. Allele frequencies were 0.76/0.24 for F8A, 0.81/0.19 for F8B and 0.625/0.375 for DX13. Allele frequencies were not calculated for St14 which is a highly polymorphic system. There was no evidence of gross gene deletion. Two families studied had other X-linked disorders, one had G6PD deficiency, the other red-green colour blindness. Linkage between these disorders, the factor VIII gene and the two extragenic probes was supported by results found. 29 of 71 females at risk (41%) could be offered definitive determination of carrier status from the result of intragenic RFLPs; a further 22 (31%) had refined genetic information from extragenic RFLPs. However with the probe St14, 3 recombinants were observed amongst 28 phase known meioses (10.7%) highlighting the risks associated in using information obtained from extragenic probes in genetic counselling.

DNA analysis was unhelpful for 20 females at risk (28%) either because of non-informative RFLPs or lack of samples from relatives. A further disadvantage of this technique which became apparent was that results depended upon fathers tested being true biological fathers.

24 females at risk who had carrier status assessed by RFLP analysis had also had conventional coagulation testing to assign carrier status. 13 women were identified as non-carriers using DNA techniques and all 13 had coagulation factor assay results which would also have supported non-carrier status. 11 women were identified as carriers, 8 from intragenic RFLPs, 3 from extragenic. Only 6 of these 11 women would have been identified as carriers from coagulation testing and the other 5 would have been given "odds" from 3:1 to 16:1 that they were not carriers. 5/24 (20.8%) women had

coagulation tests results at variance with RFLP analysis suggesting that coagulation tests are a less accurate method of assigning carrier status.

During the study, one first trimester pre-natal diagnosis was undertaken using an intragenic RFLP. The fetus was shown to be a carrier female.

128 individuals from 12 families affected by haemophilia B were investigated of whom 30 were obligate carrier females, 32 were possible carrier females and 31 were affected males. 3 intragenic RFLPs, factor 9(VIII)/Taq I, factor 9(VIII)/Xmn I and factor 9(XIII)/Dde I were used. The allele frequencies identified were 0.63, 0.68 and 0.77 respectively for each major allele. No gross gene deletions were identified. 22 of 32 women (66%) could be offered definitive assignment of carrier status from the results of RFLP data. 34% of women were unable to have carrier status determined either due to non-informative RFLPs or lack of DNA from relatives. Since the probes used were all intragenic, the information obtained is clearly more reliable than coagulation data.

One pre-natal diagnosis performed during the study showed the fetus to be an unaffected male.

38 individuals from 11 families affected by von Willebrand's disease were tested with the RFLP pvWF1100/Bgl II. In 10 families, the disease was autosomal dominant and in 1 family it was autosomal recessive. The observed allele frequency was 0.64 for the major 9kb allele. No evidence of gross gene deletion was observed. A new RFLP was identified in four individuals from 3 families (10.5% of individuals tested) which showed that a 13kb band, thought to be a constant band, was replaced by a band of 15kb. Co-segregation observed in individuals tested was consistent with chromosome 12 and the von Willebrand gene isolated being the disease locus. The DNA results obtained supported the hypothesis that the individual presenting with severe von Willebrand's disease was homozygous for an abnormality of the von Willebrand factor gene.

The study clearly showed in a large number of families the utility of DNA probes in these three common disorders of coagulation. The advantages and disadvantages are compared with other studies and the utility is compared to conventional approaches to carrier testing and pre-natal diagnosis which were available when the study was undertaken.

CHAPTER I - INTRODUCTION

Inherited coagulation disorders are all rare. However, for the affected individual they are lifelong, may be crippling and can affect all aspects of that individual's life. Treatment is expensive and has associated side effects. Particularly with X-linked conditions even unaffected female members of the family may be concerned that they carry the defective gene and can transmit it to their own children. Thus these disorders may cast a shadow over a whole family.

Clinicians managing affected patients see treatment as their immediate response to the problem but prevention as the ultimate goal. Increasing understanding of molecular genetics has opened the way to improved diagnostic and research methods. The studies described in this thesis use recently developed molecular techniques to investigate family members in families affected by haemophilia A, haemophilia B or von Willebrands disease with the main aim of allowing improved carrier detection and prenatal diagnosis

I.1 NORMAL HAEMOSTASIS

In 1964 two groups independently suggested that blood coagulation was achieved by a series of enzyme reactions which function as a biological amplifier. This they termed the "cascade" or "waterfall" hypothesis [Macfarlane, 1964; Davie and Ratnoff, 1964]. In the ensuing 25 years much more has been learnt about the individual clotting factors involved in this cascade, their interactions and the involvement of calcium and platelets but overall the hypothesis still stands.

The cascade is divided into two pathways, the intrinsic and the extrinsic, which have a common endpoint with the activation of factors X, V and II and ultimately the formation of fibrin. The intrinsic pathway is further divided into the initial contact activation system followed by activation of factor IX and the interaction between factors IXa, VIIIa and X (Fig 1).

The coagulation cascade is only part of a complicated haemostatic network. Primary haemostasis is achieved through the interaction of platelets and the damaged vessel wall. The initiation of coagulation also triggers off fibrinolytic pathways preventing widespread blood coagulation and limiting the coagulant response to a wounded area. There are also links between the haemostatic system and other body defence systems such as the complement system and the kinin generating system [Hutton, 1989].

Intrinsic Pathway of Coagulation

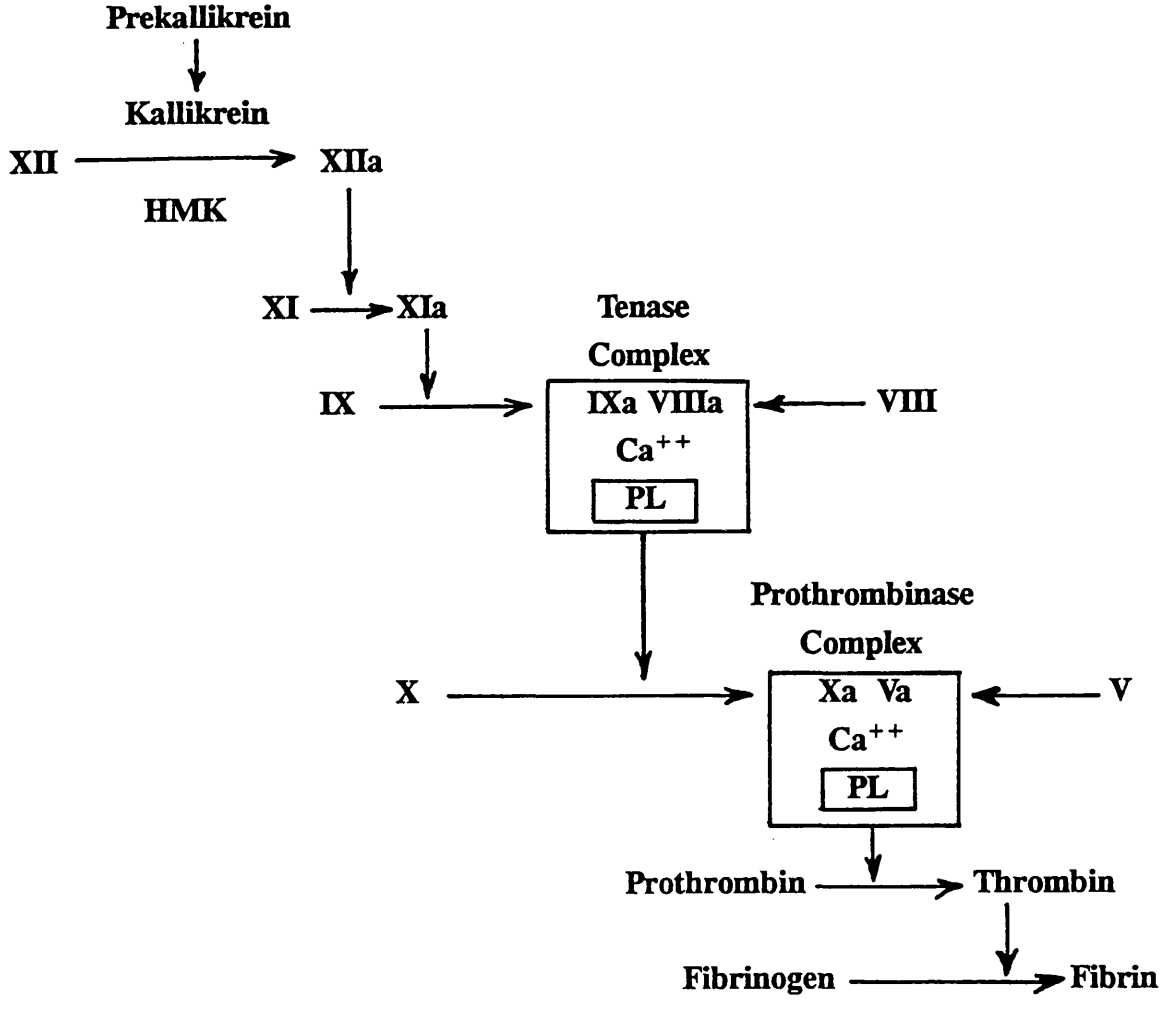


Figure 1.

Schematic representation of the intrinsic pathway of coagulation. Acivated coagulation factors are represented by their Roman numerals. Activated coagulation factors are represented by their Roman numerals followed by a small case a . Other abbreviations : high molecular weight kininogen, HMK; calcium ions Ca^{++}; phospholipid, PL

As this thesis concentrates on abnormalities of factor IX and VIII, this part of the cascade will be described in further detail here.

Factor IX is a protein synthesised in the liver which is dependent upon the presence of Vitamin K to function properly. It is activated in a two step calcium-dependent mechanism through the enzyme activity of factor XIa. First an initial cleavage of the Arg145 -Ala146 bond gives rise to a two chain molecule linked by disulphide bonds. This intermediate product has no detectable clotting activity but cleavage of the Arg180 - Val181 bond results in the release of an active peptide (IXa) with a molecular weight of about 10,000 daltons [McGraw et al, 1985A]. IXa proceeds to activate factor X. This reaction will take place in the presence of IXa alone but the speed of the reaction is greatly increased in the presence of co-factors phospholipid, calcium and activated factor VIII [van Dieijen et al, 1981].

Sequencing of the gene for factor IX has shown it to consist of about 33.5kb of DNA which code for eight exons and seven introns [Yoshitake et al, 1985; Anson et al, 1984]. The eight exons code for a pre-pro-leader sequence and 415 amino acids which make up the mature protein. The exons have been numbered a to h. The first exon consists of a leader sequence and the signal for initiation. Translation begins with the code for a Met residue which is 46 bases before the codon for the amino terminus of the mature protein. The pre-pro sequence code for a hydrophobic portion and two cysteine residues are contributed by the first exon. This is followed by a hydrophilic portion coded for by part of the second exon. The first and second exons are separated by an intron of about 6kb whereas the second and third have only short intervening sequences. The remainder of the second and third exon code for the domain of factor IX which contains its gamma carboxyglutamic acid (GLA) residues which contribute the calcium binding sites. Exons four and five are separated by a large intron and each codes for a region of the protein which is homologous to epidermal growth factor. The sixth exon codes for the two activation cleavage sites with flanking sequences. The last two exons occur at the end of the genomic sequence, separated by a short intervening sequence. Together they code for the balance of the heavy chain of factor IXa which shares considerable homology with other serine proteases [Thompson, 1986].

Factor VIII is a single chain protein. Its main site of synthesis is uncertain but is believed to be the hepatic sinusoids. It circulates in close association with the von Willebrand factor molecule. In the presence of small amounts of thrombin, proteolysis of factor VIII produces an activated form of the molecule, factor VIIIa. Larger amounts of thrombin destroy factor VIII activity [Bloom et al, 1985]. Activated factor VIII is not itself an enzyme but acts as a co-factor. It potentiates the activation of factor X by

aligning factor IXa and factor X on the phospholipid platelet surface to promote an efficient interaction between the trio [van Dieijen et al, 1983].

The factor VIII gene was cloned and sequenced in 1984. The entire gene is 186 kilobases of DNA which is estimated to be 0.1% of the human X chromosome, and it consists of 26 exons and 25 introns. The coding DNA is only 9 kilobases long and codes for 2351 amino acids of which the first 19 comprise the secretory leader peptide. Computer analysis of factor VIII protein sequence has revealed three homologous sequences (the A domain). The second and third domain are separated by a B domain of 983 amino acids which is cleaved off during activation. After the third A domain, there are 2 C domains of 150 amino acids which have about 40% homology with each other [Gitschier et al, 1984; Toole et al, 1984; Wood et al, 1984; Vehar et al, 1984].

Von Willebrand factor (vWF) is a protein active in primary haemostasis, mediating platelet adhesion via the platelet membrane glycoprotein Ib to the subendothelium at sites of vascular injury. vWF also binds to the platelet membrane IIb/IIIa complex and may help to stabilise the initial platelet aggregate. The endothelial cell is the principal site of synthesis of vWF. The vWF molecule is synthesised initially as a 260k precursor called pre-vWF. Before release into the circulation, it undergoes post-translational modification to form dimers in which the sub-unit has a molecular weight of 220k. These dimers are packaged into an array of multimers with molecular weights ranging up to 10^6k. The multimeric form of vWF is the form which is active in primary haemostasis. It is also the form which binds non-covalently with factor VIII in the circulation, protecting it from non-specific proteolysis [Mackie and Bull, 1989].

The cDNA for human vWF has been isolated and mapped to chromosome 12 by in-situ hybridisation [Ginsburg et al, 1985; Lynch et al, 1985; Sadler et al, 1985; Verweij et al, 1985A]. The gene for human vWF is approximately 178kb long. It consists of 58 exons and 51 introns. The fact that this is a long gene may increase the opportunity for polymorphism and explain the relatively high frequency of autosomal dominant vWD [Mancuso et al, 1989]. The full length cDNA construct includes a coding sequence of 8439 nucleotides which encode a single chain precursor of 2813 amino acid residues representing a putative signal peptide, a pro-sequence and mature vWF of 22, 741 and 2050 amino acids respectively. The pro-sequence is released into the circulation cleaved from the vWF molecule and has been referred to as vWAgII [Shelton-Inloes et al, 1987A]. It is hypothesised that dimerization of vWF occurs via disulphide bonding of the C terminal region and that polymerisation occurs when the pro-vWF specific sequences are removed, revealing reactive disulphides which can then participate in interdimer polymerisation.

About 90% of the precursor vWF protein is constructed of repetitive regions, indicating that the precursor vWF gene has evolved from a series of duplicative events of at least four different regions. The precursor consists of two duplicated (B,C), a triplicated (A), a quadruplicated (D) and a partly duplicated domain D' in the following order:

H - D_1 - D_2 - D' - D_3 - A_1 - A_2 - A_3 - D_4 - B_1 - B_2 - C_1 - C_2 - OH [Verweij et al, 1986].

Sequencing and RNA mapping analysis of the 5' non-coding region of the vWF gene have revealed the site of transcription initiation and of two structures, known as "CCAAT boxes", which play a regulatory role in transcription. One of these CCAAT boxes lies in an unusual position, downstream of the transcription initiation site. This has also been described in the prolactin and α–1 antitrypsin gene. All these genes encode secreted proteins that are synthesised by a limited repertoire of cells [Collins et al, 1987]. Whether the unusual position of the CCAAT box is related to this fact and in some way promotes the production of a secretory protein is as yet uncertain. Specific proteolytic fragments of vWF have been found to bind to platelet glycoprotein Ib, glycoprotein IIb, IIIa and collagen [Girma et al, 1986].

I.2 MOLECULAR BIOLOGY

I.2i From DNA to Protein

Clinical geneticists from Mendel onwards defined genes as the biological units of heredity and proved that they are aligned linearly on the chromosomes. Subsequently it was shown that genes control the structures of protein and it is now known that genes consist of DNA or deoxyribonucleic acid. DNA is a double-stranded molecule made up of chains of sugar-phosphate molecules attached to one of four nucleotide bases: adenine (A), thymine (T), guanine (G), and cytosine (C). The two strands are held together in a double helical structure, linked by hydrogen bonds, by complementary base pairing whereby an A molecule is always paired with a T molecule and C with G. This specific base pair complementarity is fundamental to many of the particular properties of DNA and to many of the laboratory techniques used in molecular biology.

DNA is essential for protein synthesis. Genetic information is encoded by the sequence of the bases; it is a triplet non-overlapping code in which three bases determine a particular amino acid. DNA replication involves the copying of a pre-existing DNA strand to produce a complementary sequence; a copy of this sequence will then produce the original (or template) sequence. In the replication of DNA both strands are copied. DNA can also be copied to make the messenger molecule ribonucleic acid or mRNA in a process called transcription. mRNA is similar to DNA but the sugar involved is ribose rather than deoxyribose and thymine is replaced by uracil. Transcription of mRNA from DNA is the first step in protein synthesis and is brought about in the nucleus by an enzyme called RNA polymerase II. It starts on a specific triplet (ATG) or initiator region and stops at a termination triplet (TAA, TAG or TGA). The initial RNA strand produced is long and biologically inactive. The coding sequences or exons are interrupted by non-coding intervening sequences or introns. Processing of mRNA involves excision of introns and splicing of exons. It is chemically modified at its 5' end by the addition of a so-called CAP structure, and at its 3' end by the attachment of a long string of adenylic acid residues (poly A). mRNA then moves into the cytoplasm where translation from RNA to protein takes place on polysomes, groups of ribosomes which themselves are structural units consisting of ribosomal RNA and protein. The triplets of nucleotides coding for an amino acid are recognised by a third type of RNA, transfer RNA or tRNA. There is a specific tRNA present in the cytoplasm for each amino acid. The sequence of the mRNA is 'read' from the 5' end to the 3' end and amino acids are attached sequentially and form peptide bonds between each amino acid until the whole message has been translated into a protein which is then released.

Because the code for each amino acid is specific an alteration in one base of the DNA molecule may alter the protein produced. Similarly an abnormality at the initiator or terminator sequence may abolish RNA transcription. This is complicated by the fact that close to the coding gene on the DNA strand are areas known as promotors and enhancers. Abnormalities within this stretch of DNA may also affect RNA transcription. An omission of one base will affect all the subsequent triplets coding for amino acids and may result in an abnormal or very short protein molecule being produced which may be non-functional. Any alteration that affects the excision of introns and splicing of the RNA molecule may also cause abnormal proteins to be produced. Understanding the process of normal protein production helps to explain the molecular pathology involved in abnormal or dysfunctional protein production (Fig 2).

I - 2ii DNA Electrophoresis and Probing

In order to separate DNA electrophoretically it first has to be cut into smaller fragments. This is achieved using enzymes isolated from bacteria known as restriction enzymes. Each restriction enzyme will cleave DNA only through a given nucleotide sequence which it recognises. The smaller fragments of DNA produced by digestion of DNA with a restriction enzyme can be separated electrophoretically in an agarose gel. The DNA is transferred from the gel to nitrocellulose or nylon membrane by a process known as Southern blotting.

DNA probing relies upon the specific binding of complementary sequences of DNA. A DNA probe is simply a segment of DNA taken from, or close to, a gene of interest. The electrophoresed DNA can be rendered single stranded by exposures to an alkali solution. A DNA probe made single stranded by boiling and radio-labelled with ^{32}P will bind specifically to complementary DNA sequences. Non-specific binding can be washed off. The specifically bound probe is then visualised on an autoradiograph.

I.2iii Restriction Fragment Length Polymorphisms (RFLPs)

Throughout the human genome there are harmless mutations present, often in the form of single base substitutions, which do not affect DNA transcription. They may however alter a restriction enzyme site. These mutations are inherited and so an individual may have different patterns of restriction enzyme cutting in the two alleles of a gene which they have inherited. Different sizes of DNA fragment, known as restriction fragment lengths, will be detected on the autoradiograph. This is the phenomenon of restriction fragment length polymorphism. It is possible to use the different size of fragments to

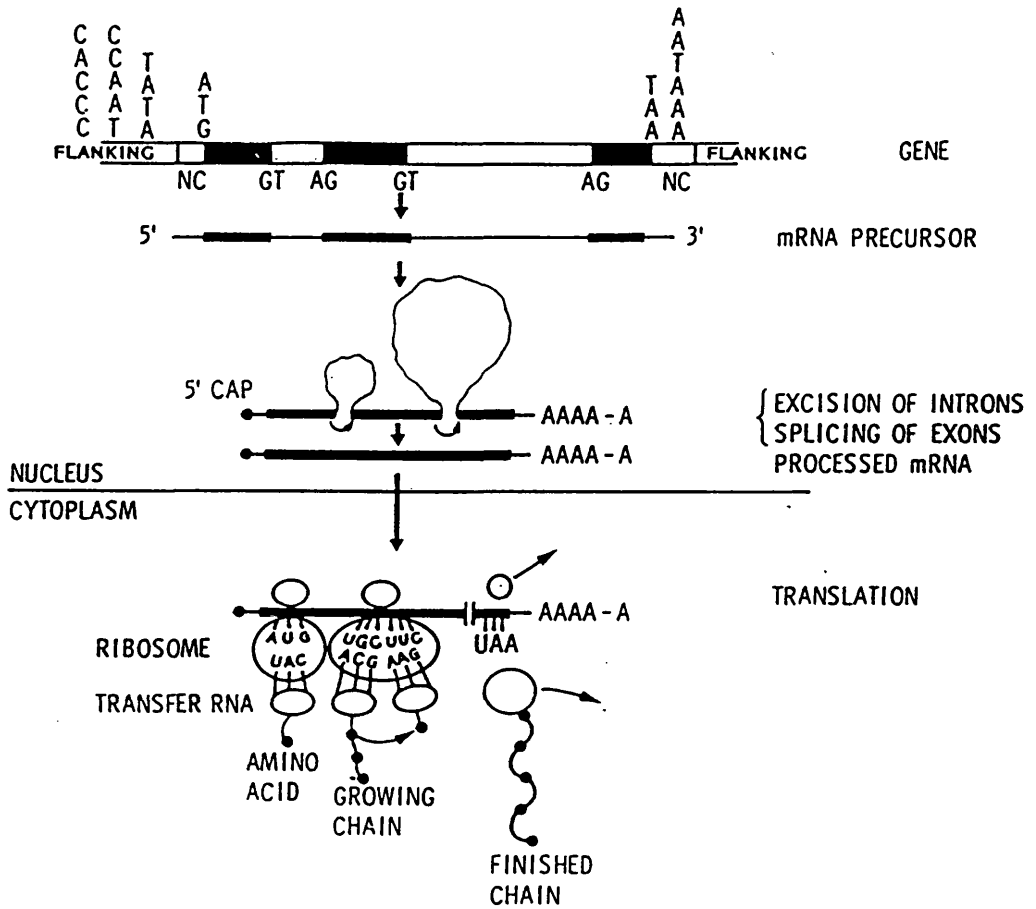


Figure 2

From DNA to Protein

Transcription from DNA to RNA in the cell nucleus followed by translation from RNA to protein in the cytoplasm.

The dark shaded areas of DNA are the exons, the pale areas between are introns.

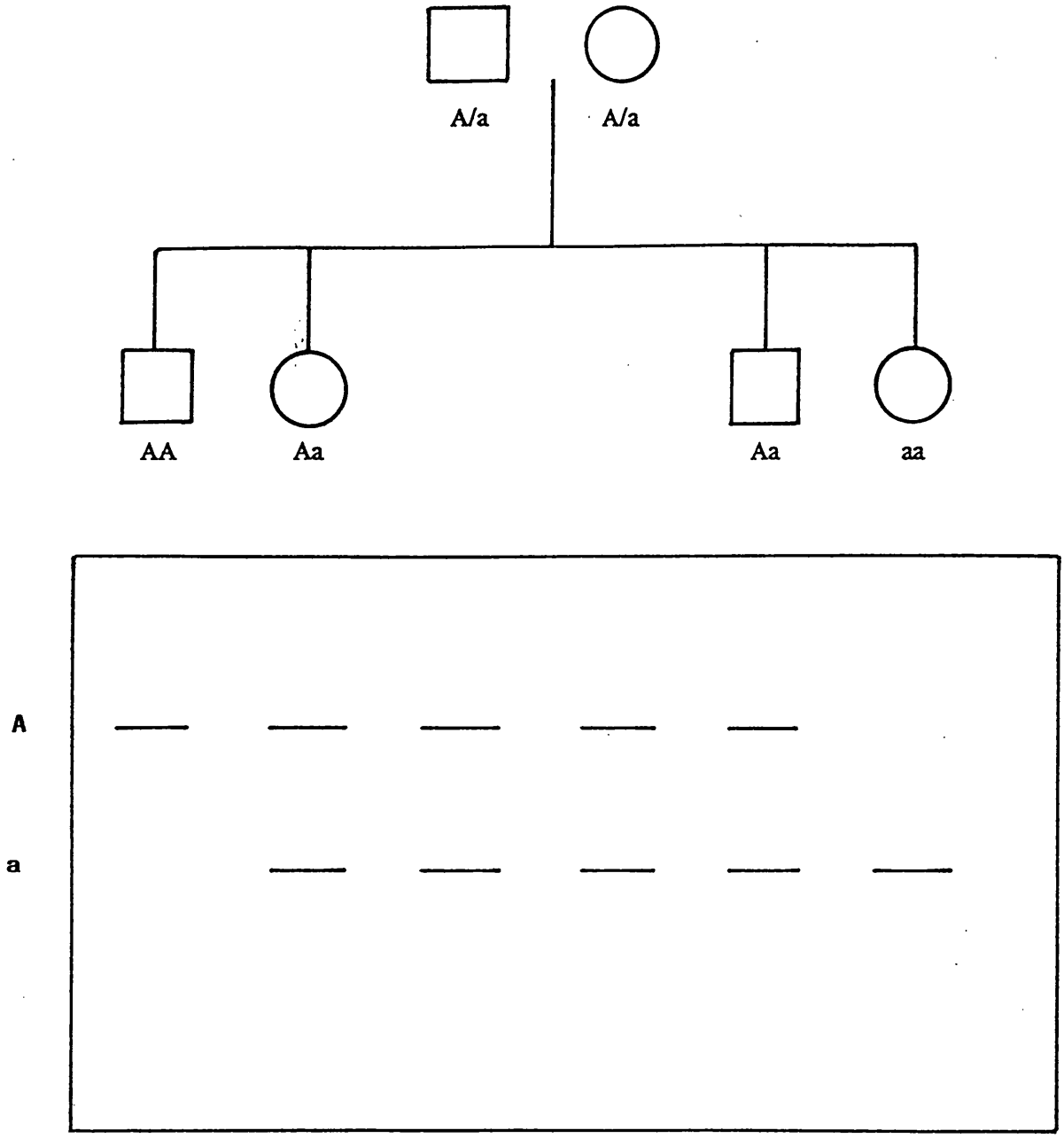


Figure 3.

Diagram of typical autosomal RFLP. Both parents are heterozygous, one child is homozygous for the larger fragment A, one is homozygous for the smaller fragment a, and two children are heterozygous.

track the inheritance of an allele throughout a family without needing to know the precise molecular abnormality of the disease in that family (Fig 3).

If the RFLP lies within a known gene then there will be a close correlation between the inheritance of an RFLP allele and an allele of the gene. This association is described as linkage. Linkage is very strong when the RFLP is intragenic but if the RFLP lies close to but outside the gene of interest there is a possibility of genetic cross-over at meiosis between the RFLP and the gene site resulting in loss of linkage or genetic recombination. Linkage of an RFLP to a gene of interest can be calculated by the number of recombinations observed between the two when a large number of families are studied and expressed as a recombination fraction, called θ .

Much of the information derived from RFLP analysis depends upon studying families and family pedigrees. Interpretation of data relies upon the accuracy of the information. A particular problem arises in pedigree analysis when a blood sample obtained from a male who believes himself to be the father of a child is shown not to come from the true father. This is known as non-paternity and is a not uncommon occurrence. Non-paternity may completely destroy the validity of RFLP analysis.

DNA investigations with a clinical application were first described in prenatal diagnosis of haemoglobinopathies [Kan and Dozy, 1978]. However, methods for identifying carriers in the various haemoglobinopathies have been well established prior to the development of recombinant DNA techniques since carriers for these disorders are usually phenotypically abnormal. This was not the case for carriers of haemophilia A and an early application of the new molecular techniques was the use of DNA probes and restriction fragment length polymorphisms (RFLPs) to identify carriers of haemophilia A and to allow early pre-natal diagnosis of disease. In 1984, Harper et al described a DNA probe, DX13, linked to haemophilia A which was clinically useful and a second linked probe, St14, was soon described [Oberle et al, 1985A]. Within a short period of time, two intragenic probes were also described [Gitschier et al, 1985A; Antonarakis et al, 1985A]. It was these four probes which were used in the present study.

The first RFLP to be described in the factor IX gene was the Taq I polymorphism. A preliminary description of this polymorphism relied upon a cDNA probe [Camerino et al, 1984]. This was rapidly followed by a description of the same polymorphism identified with a genomic probe, known as probe VIII, which identified a constant band of 5.3kb and a polymorphic site giving a band of either 1.8kb or 1.3kb [Giannelli et al, 1984]. This is the first probe and enzyme combination used in this study.

Two further intragenic RFLPs were quickly described [Winship et al, 1984]. The first was the same F9 probe (VIII) but the enzyme is Xmn I. A polymorphic site close to exon c, within intron 3, showed an 11.5kb band in the majority of cases but in some instances this band was absent being replaced by two bands at 6.5 and 5.0kb. The Xmn I polymorphism is the consequence of a single C and G base transversion resulting in the formation of a Xmn I site in this region of the gene. In contrast to this, the Dde I polymorphism described (which is also apparent with the enzyme Hinf I) represents an insertion of 50 bp forming the rarer allele. This in turn probably represents a duplication of the flanking simple repeat sequence of the form [(CA/TA)mGT]n where m and n are independent integers. The original method used the probe XII although in this study now reported we used probe XIII.

The sequencing of the cDNA for the vWF gene has enabled partial length cDNA probes from the entire length of the vWF gene to be developed. The RFLP used in this study is a Bgl II polymorphism described by Verweij et al [1985B] using an 1100bp vWF probe.

I.3 INHERITED DISORDERS OF COAGULATION

Historical Aspects

That a bleeding disorder exists which has X-linked inheritance has been recognised for over 150 years [Hay, 1813] and the term haemophilia was introduced in the nineteenth century. There are earlier references to a bleeding disorder affecting males from the second century AD when the Babylonian Talmud describes the decision of Rabbi Judah that the son of a woman whose three previous sons had bled to death following circumcision be excused from this rite [Wintrobe, 1985]. Addis in 1911 described how the abnormality in haemophiliac plasma could be corrected by adding normal plasma. He attributed this to a deficiency in prothrombin and when this was shown not to be the case his work was largely forgotten. The so-called "classic" theory of coagulation at that time proposed by Morawitz in 1904 only envisaged four coagulation factors: thromboplastin derived from damaged tissue or blood cells converting prothrombin to thrombin in the presence of calcium; thrombin then reacting with fibrinogen to form insoluble fibrin. The development of the one stage prothrombin time test by Quick in 1935 allowed the preliminary classification of inherited disorders of coagulation. In haemophilia the prothrombin time is normal. At about the same time it was shown that a fraction of normal plasma corrected the coagulation defect in haemophilia [Patek and Taylor, 1937]. This factor was referred to as anti-haemophiliac factor (AHF) or anti-haemophiliac globulin (AHG). The thromboplastin generation test described by Biggs and Douglas in 1953 showed that there was plasma thromboplastin in an intrinsic plasma system. Applying this test to haemophiliac plasma detected an abnormality which was presumed to be due to the lack of AHF. A group of patients were identified who apparently had haemophilia but whose plasma would correct the defect of other haemophiliacs. The thromboplastin generation test showed that this subgroup had a defect of a serum factor. This defect was called Christmas disease after the name of the first patient described [Biggs et al, 1952]. At the same time Aggeler et al [1952] describing the same defect called the missing factor plasma thromboplastin component (PTC).

An International Committee devised standard nomenclature for coagulation factors in 1962 based on Roman numerals [Wright, 1962]. AHF became factor VIII and Christmas factor or PTC became factor IX. Haemophilia was divided into Haemophilia A or classic haemophilia due to a defect in factor VIII and Haemophilia B or Christmas disease due to a defect of factor IX.

Von Willebrand disease was first described in 1926 in inhabitants of the Aland islands [von Willebrand, 1926]. Other reports followed and autosomal inheritance was

recognised. The disease was initially referred to as autosomal pseudohaemophilia. Unlike haemophilia, von Willebrands disease is associated with a prolonged bleeding time. For this reason, it was postulated that it was due to a defect in platelets but it is now known to be due to a variety of defects in the von Willebrand factor which is present in plasma.

Other inherited coagulation disorders such as factor VII deficiency are of historical importance since the recognition of these clinically and genetically distinct disorders sheds further light on the physiology of the coagulation cascade. However, these conditions will not be considered further here.

I.4 HAEMOPHILIA A

I.4i Clinical Aspects

Haemophilia A is due to an inherited functional deficiency of coagulation factor VIII. This deficiency affects secondary haemostasis so that bleeding is likely to be a particular problem when larger blood vessels are involved or after initial vasoconstriction resolves [Bloom, 1987]. Although neonates may present with a cephalhaematoma, with excessive bruising at injection sites or with post-operative haemorrhage, more commonly the diagnosis is not suggested until late infancy. At this stage multiple areas of excessive bruising may raise the suspicion of non-accidental injury. In older children and adults haemarthroses predominate. Any joint may be involved although the large weight bearing joints cause most problems. Repeated haemarthroses often lead to secondary arthropathy which itself predisposes that joint to further episodes of haemorrhage [Rizza and Matthews, 1984].

Deep intramuscular locations are the other common sites of haemorrhage and may be spontaneous or follow relatively minor trauma. These haematomas may cause local pressure effects, for example the femoral nerve may be compressed by an iliopsoas haematoma. Inadequate treatment of these deep haematomas may result in cystic collections which are prone to secondary infection unless surgically removed [Valderrama and Matthews, 1965].

Although the incidence of intracranial bleeding appears to have fallen it is still the commonest cause of death following bleeding in haemophiliacs [Biggs, 1977]. The bleeding may be extradural, intradural or intracerebral and may follow relatively trivial head injury. In a recent series 47% of survivors had neurological sequelae [Eyster et al, 1978].

Less common sites of haemorrhage are the renal tract, the upper airway and the gastrointestinal tract and for these sites local alternative pathologies need to be excluded.

I.4ii Treatment

Addis in 1911 showed that blood or normal plasma could correct the abnormality in haemophilia and treatment became a reality with the development of a technique to cryopreserve concentrated plasma factors [Pool et al, 1964]. Cryoprecipitate avoids the earlier problems of volume overloads and allows the preparation of highly concentrated lyophilised factor VIII which is stable for several months.

Mild haemophiliacs require intravenous treatment only if they have a haemarthrosis or if they are to undergo an operation or dental extraction. Those with more severe haemophilia may be candidates for home therapy as they are aware of the onset of haemorrhage before there is clinical evidence [Jones, 1977] and thus treatment can be initiated promptly. In some cases regular prophylaxis of spontaneous haemorrhages is appropriate.

Prior to the availability of factor VIII treatment, the average lifespan of a severe haemophiliac was estimated to between 20 and 30 years. Over that time a haemophiliac would suffer increasing disability and pain. Figures produced in the early 1980's when factor VIII was widely available estimated that individuals suffering from haemophilia A had, on average, a 50 year lifespan. That is that the disease shortened their life by 20 years [Weatherall, 1985]. The current problems with the HIV virus and AIDS may change this situation in the 1990's.

Unfortunately therapy is not without its drawbacks. First there is the need for intravenous injections and second there is the risk of transmission of infectious agents from pooled plasma preparations. Currently over 50% of regularly treated haemophiliacs have abnormal liver function tests and in the majority of cases this is believed to reflect viral hepatitis [Stevens et al, 1983]. The current method of preparation of factor VIII concentrate inactivates Epstein-Barr virus and cytomegalovirus and it is also now routine to screen donors for hepatitis B. However, almost all develop evidence of non-A non-B hepatitis (now known as hepatitis C) after treatment [Fletcher et al, 1983]. A recent study described evidence of progressive liver disease in 21% of haemophiliacs who had liver biopsy for persistently elevated liver function tests [Hay et al, 1985].

Currently the major concern relates to the transmission of human immunodeficiency virus (HIV) and the subsequent development of acquired immunodeficiency syndrome (AIDS). A recent survey found 1,201 UK haemophiliacs to be HIV positive with 85 cases of AIDS and 47 deaths [Darby et al, 1989]. Seroconversion has been reported despite heat-treatment of factor VIII [White et al, 1986] and in the UK all blood donors are now screened for this virus. However, this does not apply to foreign sources of factor VIII concentrates and depends upon the reliability of the current donor screening programme.

I.4iii Laboratory Aspects

Any description of current investigations of coagulation disorders contains a confusing collection of abbreviated terms referring to different types of assays. The nomenclature is somewhat haphazard, reflecting the understanding of the time of development of an assay rather than a rational description of events. These terms are even now in a state of flux and so anyone reading the literature of present day investigations has to be conversant with various terms for the same investigations.

The defect in factor VIII can be measured functionally in plasma using a coagulation test. Factor VIII assayed in this way is referred to as VIII:c. Alternatively factor VIII may be measured antigenically using the autoantibodies which develop in some severe haemophiliacs after multiple treatment. This is referred to as factor VIII:Ag. An individual haemophiliac with a given level of VIII:c is likely to have a slightly lower level of VIII:Ag, but in most individuals the levels are proportionate. However in a few individuals the measured antigen is much higher than the functional assay. These individuals are said to have cross reacting material: they are described as CRM+. Those individuals with antigen levels equivalent to functional VIIIc are CRM- [Peake and Bloom, 1978]. The CRM status will be the same for all affected individuals in a kindred. Confusion arises because early immunological studies, using antibodies raised in animals detected an antigen which was referred to as factor VIII related antigen or factor VIIIR:Ag. It is now realised that this is an antigenic function of the much larger molecule von Willebrand's factor which is bonded non-covalently with factor VIII:c in plasma but does not play a role in haemophilia A.

The clinical severity of haemophilia shows a correlation with the degree of reduction in the functional level of factor VIII with mild cases having VIII:C over 10iu/dl, moderate 2-10iu/dl and severe under 2iu/dl.

Langdell et al in 1953 described the one stage modified activated partial thromboplastin time using factor VIII deficient plasma to assay factor VIII coagulant activity. This test is still used in laboratories today.

I.4iv Genetic Aspects

Haemophilia has been known to run in families since antiquity. In the Jewish Talmud from the 5th century BC boys in these families were excused circumcision [Wintrobe,

1985]. Early descriptions showed the characteristic features of X-linked recessive inheritance [Macklin, 1928]. In 1952 haemophilia B was distinguished from haemophilia A and both were accepted as X-linked recessive traits. In families with red-green colourblindness the locus for haemophilia A but not for haemophilia B showed linkage [Haldane and Smith, 1947].

Hence males with only a single copy of the X chromosome are hemizygous for X-linked genes and are either affected or unaffected with haemophilia. The female with two X chromosomes can carry the mutant factor VIII and will usually be clinically normal. In every female at around the 2000 cell stage one X chromosome is largely inactivated (X-inactivation, Lyonisation), [Lyon, 1962]. This pattern of inactivation remains constant for all descendants of each cell. In some carrier females by chance mainly the normal factor VIII gene will be inactivated and these females may show mild haemophilia. Conversely some carrier females will mainly inactivate the abnormal factor VIII gene and have normal laboratory investigations whilst the majority will occupy an intermediate position.

There are two areas of difficulty in genetic counselling of affected families: the determination of female carrier status and selective prenatal diagnosis. A female has to be a carrier (ie is an obligate carrier) if she is the daughter of a haemophiliac, has two haemophiliac sons or has a haemophiliac offspring and other affected maternally related relatives.

Since haemophilia has been recognised to be a genetic disorder, there has been interest in detection of possible carriers. Identification of heterozygotes for haemophilia A by laboratory means was first attempted by Merskey and Macfarlane in 1951 but the results were not sufficiently discriminating to be useful. Bennett and Huehns [1970] described the production of a rabbit antibody to crude human factor VIII and used this to measure what they believed to be immunologically competent but biologically inactive factor VIII. They noted that carriers of haemophilia A appeared to have lower levels of factor VIII activity measured by a functional assay compared to the protein acting against factor VIII antibody. It became apparent from work published by Zimmerman et al in 1971 that this antibody acted against a protein which was missing in patients who had severe von Willebrand's disease. This protein came to be known as factor VIII related antigen (FVIIIR:Ag). Results published in the early 1970's by various groups [Bennett and Ratnoff, 1973; Rizza et al, 1975; Prentice et al, 1975; Meyer et al, 1975] confirmed the finding that carriers tended to have a low FVIIIC and a normal FVIIIR:Ag and that it was possible in many cases to identify carriers. However, none of these groups could identify 100% of carriers by this technique. Bennett and Ratnoff [1973] looked at 18

daughters of known carriers and identified 9 of these as carriers but at the time of publication none of the 18 women had had any sons and so confirmation of the technique was uncertain. Rizza et al [1975] conducted a blind study whereby those performing the assay did not know the carrier status of the individual tested. Looking at 34 normal individuals and 34 known carriers they found that 71% of carriers could be identified on the basis of the ratio of factor VIII activity to factor VIII related antigen. Prentice et al [1975] used discriminant analysis to calculate betting odds of carrier status. Investigating 32 possible carriers of haemophilia A they could only give relative betting odds greater than 5:1 of carrier status or non-carrier status in 69% of women tested. One woman investigated and shown to have odds of 10.6:1 of not being a carrier subsequently gave birth to a severely affected haemophiliac son. Meyer et al [1975] measured factor VIII activity and factor VIII related antigen in 49 carriers and 31 normal women. The data was analysed by four statistical approaches. Discriminant analysis appeared to be the most useful statistical method but even with this method 16% of normals and 18% of carriers were misclassified, overlapping with the other group. The World Health Organisation published a memorandum on the subject of carrier detection of haemophilia A and B with recommendations for the standardisation of laboratory techniques and statistical methods [Akmeteli et al, 1977]. It was clear that the combination of accurately performed coagulation assays and analysis of pedigree data could alter the calculation of a prediction of likelihood that any particular woman at risk was in fact a carrier. Nonetheless, the uncertainties for many women remained since many could only be offered "betting odds" of carrier status rather than absolute answers. Published studies attempted to determine the specificity and sensitivity of laboratory techniques and to determine the cause of interlaboratory variation but this failed to eradicate the probabilistic value of the calculated results and the associated limitations when applied to carrier detection [Klein et al, 1977; Kirkwood et al, 1977].

Details other than pedigree information and coagulation results may help to distinguish carrier females. As early as 1960, Rapaport, Patch and Moore had shown that the "between day" variation in VIII:C assays was greater than the variation between duplicates on the same day suggesting that averaging of replicates obtained over time would provide a more representative VIII:C level for a woman than a single determination or the average of replicates obtained on a single day. In some parts of the world, the known linkage between haemophilia A and G6PD deficiency can be helpful in a family where both diseases are present [Filippi et al, 1984]. However in the UK, G6PD deficiency is rare. Improved discrimination between carriers and non-carriers was reported in a study where DDAVP was infused into women prior to sampling [Kobrinsky et al, 1984]. Post DDAVP haemophilia carrier detection was increased from 85% with 10% false positive and 20% false negative assignments to 95% with 5% false

positive and 5% false negative assignments. An immunoradioactive assay of the VIII:C antigen (VIIICAg, now called VIIIAg) was shown to give comparable results in carrier detection to the functional assay of VIIIC but the results were more reproducable between samples where plasma had been frozen [Peake et al, 1981]. It has been suggested that the improved stability of this technique would allow samples to be more easily transported to a central laboratory to allow assays where results are difficult to interpret such as during pregnancy [Hoyer et al, 1982]. A recent study has highlighted the importance of the effect of age on both FVIII:C and VWF:Ag. Values of both these factors are higher in the very young and very old and reach their lowest level at age 25 to 30 years. This study also reported significantly higher levels of both factors in women whose blood group was not group O [Graham et al, 1986].

Many women who are obligate carriers or are advised after testing that they are likely to be carriers of haemophilia often choose not to have a family or to limit their family in some way. The earliest method of prenatal diagnosis was fetal sexing following amniocentesis. Any male child terminated following this procedure had only a 50% chance of being affected which made this diagnostic method unacceptable to many female carriers. In 1979, both Firshein et al and Mibashan et al described prenatal diagnosis of coagulation disorders following fetal blood sampling and coagulation factor assays. If amniocentesis showed a male fetus, the fetoscopy was performed at about 19 weeks gestation with fetal blood sampling. Factor assay allowed accurate diagnosis prior to termination. Termination of pregnancy inevitably meant a late termination of pregnancy. The development of chorionic villus sampling in the first trimester of pregnancy allowed DNA analysis and early prenatal diagnosis. If haemophilia is diagnosed in the fetus, termination of pregnancy is offered at a much earlier stage of gestation. Initial reports have shown this to be a more acceptable method of prenatal diagnosis than those which preceded it.

Thus at the time that this study was undertaken women seeking advice about carrier status could only be offered coagulation testing which does not detect all carriers accurately and should they proceed to pre-natal diagnosis they could only be offered second trimester testing.

I.5 HAEMOPHILIA B

I.5i and I.5ii Clinical Aspects and Treatment

Clinically haemophilias B and A are indistinguishable and the clinical features and natural history are as outlined in section I.4i.

Treatment with cryoprecipitate is ineffective and most patients are treated with highly purified factor IX concentrate. As in all coagulation disorders antifibrinolytic agents may have some place in controlling bleeding especially after dental extraction. DDAVP does not increase the level of factor IX and so has no role in the treatment of haemophilia B.

The problems associated with therapy are the same as those encountered in haemophilia A: in particular the development of antibodies to factor IX, liver disease and viral transmission.

I.5iii Laboratory Aspects

Factor IX may also be assayed functionally, factor IX:c, or antigenically, factor IX:Ag. Combining information from functional and immunological assays, haemophilia B families can be divided into B+ where there is a low IX:c level but a normal IX:Ag, B- where a low IX:c is accompanied by a similarly low IX:Ag and Br where a low IX:c is associated with a reduction of IX:Ag to a lesser degree [Parekh et al, 1978].

Factor IX is a vitamin K dependant coagulant factor synthesised in the liver. It has close homology with factors II, VII and X as well as protein C which are also vitamin K dependant [Kurachi and Chen, 1987].

Patients with haemophilia B have a prolonged partial thromboplastin time and factor IX can be assayed using a coagulation assay and factor IX deficient plasma. Unlike haemophilia A the type of assay used may affect the resulting level found [Bloom, 1981]. This may be because some assay methods underestimate factor IX. Haemostasis requires a lower proportion of factor IX than factor VIII.

It has been apparent for some time that there are many variants of haemophilia B as a result of different structural abnormalities of the factor IX protein [McGraw et al, 1985A and 1985B]. These variants began to be recognised when factor IX was assayed by immunological methods as well as by coagulant techniques.

I.5iv Genetic Aspects

Haemophilia B is inherited as an X-linked recessive trait in exactly the same manner as haemophilia A.

Carrier detection in haemophilia B does not have the advantage of the known discrepancy in ratio between VIII:c and vWF (previously referred to as VIIIR:Ag). Measurement of IX:c only in obligate carriers identifies carrier status in less than 50% [Simpson and Biggs, 1962]. Plasma factor IX antigen is present in excess of the amount of factor IX antibody in haemophilia B+ (or CRM+) families but in haemophilia B- (or CRM-), which is the more common form of haemophilia B, factor IX antigen and factor IX activity are present in equivalent amounts. Orstavik et al [1979] measured factor IX antigen and factor IX activity in 40 controls, 18 B- carriers and 10 B+ carriers and constructed statistical tolerance regions graphically for carriers and for controls. Only 17 out of the 40 controls were outside the 90% tolerance region for carriers. In the B- group 10 of the 18 carriers were outside the 90% tolerance region for carriers whereas in the B+ group only 1 individual fell within the 90% tolerance region for controls. Although measuring factor IX antigen as well as activity improved carrier detection, particularly for B- families, there were still many women not detected as carriers by this method and there was considerable overlap with the control group.

Second trimester prenatal diagnosis is possible for haemophilia B although, like other vitamin K dependant factors, factor IX remains at a relatively low level until the third trimester. This makes the cut off point between disease levels and normal less distinct than in the case of haemophilia A. Also factor IX antigen is present in amniotic fluid so that any sample contamination may affect the result. This is not the case in haemophilia A [Thomson, 1984]. As is the case for haemophilia A carrier detection by coagulation assay is unable to give definitive answers. Pre-natal diagnosis must be done late, is technically difficult and has the added risk of sample contamination.

I.6 VON WILLEBRAND DISEASE

I.6i Clinical Aspects

Von Willebrand disease is a heterogeneous group of conditions which in common involve abnormalities of the von Willebrand factor. These disorders affect primary haemostasis so that affected individuals suffer from bruising and mucosal bleeding. Children may present with bleeding from the gastrointestinal or genitourinary tracts and in older women menorrhagia is a common complaint. After surgery or dental extraction bleeding is excessive and occurs immediately after injury [Rizza and Matthews, 1984]. The severity varies both within and between affected families and the most severely affected may have deep intramuscular bleeding and haemarthroses in addition to the mucosal bleeding and rival the haemophilias in severity.

The clinical situation in vWD varies from that in haemophilia. The autosomal dominant form is relatively common. However it is usually mild and it is extremely unlikely that an individual would ever seek pre-natal diagnosis for this condition with a view to termination of pregnancy although it is possible that knowledge of the presence of von Willebrand disease would be useful for planning delivery. Since it is an autosomal dominant condition, affected individuals can be identified by conventional clotting tests and so the issue of carrier detection rarely arises. Occasionally, even after testing on several occasions, clotting studies may produce equivocal results in which case DNA studies may help to elucidate the problem. Similarly, a second disorder interfering with blood clotting, such as thrombocytopenia or drug induced problems may make coagulation studies difficult to interpret and in this situation DNA studies may be helpful. The situation in the rare type III vWD is different. This is a homozygous or double heterozygote disorder which causes a severe bleeding disease. Parents of an affected individual might well seek genetic counselling and pursue the possibility of pre-natal diagnosis. Not surprisingly, published series of studies of vWD in families investigated by RFLP or other DNA based investigations have tended to concentrate on this rare but serious form of vWD.

When looking at the present study it is interesting to note the relatively small number of affected families registered at the Regional Haemophilia Centre. This does not bear a close relationship to the estimated prevalence of vWD but does reflect the experience of other regions. Many individuals with von Willebrand disease are relatively asymptomatic and are therefore less likely to attend clinics for regular follow up. The greater difficulty in obtaining DNA from relatives of probands may also reflect the lower

motivation of a family affected by a disorder which is perceived to be less serious than haemophilia.

I.6ii Treatment

DDAVP stimulates the release of von Willebrand factor from endothelial cells and is the treatment of choice in type I and some type II von Willebrands disease. It is contra-indicated in type IIb vWD where the released abnormal vWF causes aggregation of unstimulated platelets. Until recently cryoprecipitate was recommended for these patients who did not respond to DDAVP as it is rich in vWF [Bennett and Dormandy, 1966], however, with the increasing anxiety about the risk of transmission of the HIV virus, there is now a move to usage of moderately pure FVIII concentrates which may contain lower levels of vWF but which are heat treated and therefore thought to be safer than cryoprecipitate [Berntorp and Nilsson, 1989]. Treatment with cryoprecipitate restores the bleeding time for up to 12 hours. The level of factor VIII is restored for a much longer period and so cannot be used as a guide to therapy [Holmberg and Nilsson, 1985].

Inhibitors of fibrinolysis may be used in the treatment of von Willebrand disease and are of particular value after dental extraction. Since DDAVP also stimulates release of plasminogen activator a fibrinolytic inhibitor such as tranexamic acid is usually administered at the same time [Ludlam et al, 1980].

I6.iii Laboratory Aspects

The vWF protein has two functions. It cross-links platelet membrane to the collagen exposed in damaged endothelium under conditions of high shear rates. However, it also acts as a protective carrier for factor VIII in the circulation. The tests used to identify vWD reflect these two different functions. FVIII is low in vWD not because of abnormalities in the FVIII protein but because is decays more rapidly in the absence of its carrier protein. A prolonged bleeding time reflects the abnormal platelet binding to collagen. vWF:Ag is also measured directly. Finally platelet aggregation in the presence of the antibiotic ristocetin is measured (Ricof). Ristocetin promotes spontaneous aggregation of platelets but this response is reduced in vWD. The exception to this is type IIB vWD where there is increased platelet aggregation even at low concentrations of ristocetin. The basic tests for vWD are completed by checking the platelet count and platelet aggregation to exclude a bleeding disorder secondary to a platelet abnormality. Many laboratories now extend the investigation of vWD to include electrophoresis of the protein. This will identify the vWF multimers and the subtype of vWD will be assayed

according to the abnormality seen. Type I makes up 80% of patients with vWD and is subdivided further in A, B or C. Type II makes up the majority of the remainder and is subdivided A to F according to the multimer analysis and laboratory results. Type III is the rare autosomal recessive severe form of the disease [Tuddenham, 1989].

I.6iv Genetic Aspects

Most families show autosomal dominant inheritance for von Willebrand disease including the original description from the Aland islands [von Willebrand, 1926]. The recent isolation of the gene for von Willebrand factor and its localisation to chromosome 12 is consistent with these observations. In addition several families have been described with apparent autosomal recessive inheritance of particularly severe disease [Holmberg and Nilsson, 1985].

Since, for the vast majority of families, it is a dominant disorder, the problem of carrier detection seen in the X-linked disorders does not arise. There may be difficulties in identifying affected individuals since the clotting studies will show both inter and intra individual variations and it may be necessary to repeat tests several times. Pre-natal diagnosis is unlikely to be requested in the common dominant inherited disorder as the bleeding problems are rarely severe. However the recessively inherited form of vWD may resemble severe haemophilia but with mucosal bleeding as an extra problem. Families with one affected child may then seek PND for a future pregnancy. At the time that this study was undertaken, this could only be achieved by fetal blood sampling and second trimester diagnosis.

CHAPTERII - AIM OF THE PRESENT INVESTIGATION

This project was initiated in 1985/86. As indicated at that time the coagulation tests available for carrier detection of haemophilia were inadequate. Pre-natal diagnosis of any coagulation disorder could only be achieved using second trimester blood sampling. The overall aim of this project was to utilise the newly available DNA RFLPs in families with common inherited coagulation disorders to see if they improved detection of gene carriers and could be used for first trimester pre-natal diagnosis.

Specific aims were as follows:-

1. To obtain DNA samples from a large number of haemophilia A families and analyse them with intragenic factor VIII and linked extragenic probes and to compare the results with the two sets of probes and coagulation test results.

2. To obtain DNA samples from a large number of haemophilia B families and analyse them with intragenic factor IX probes and to compare the results with the probes with historical coagulation data.

3. To obtain DNA samples from a large number of von Willebrand disease families and to analyse them with an intragenic probe and to compare the results with coagulation test results.

III - PATIENTS, MATERIALS AND METHODS

III.1 PATIENTS

Records of patients were obtained from the Regional Haemophilia Reference Centre at the Royal Infirmary, Glasgow, and from the Duncan Guthrie Institute of Medical Genetics, Glasgow. Attempted total ascertainment in the West of Scotland revealed twelve families with haemophilia B with more than one person affected, ten of whom were willing to participate in the study. A further two families were referred for investigation from other centres. All families were of Caucasian origin. Ten families with haemophilia A were selected from the register who had more than one affected member, several possible carrier females and who lived reasonably locally. One family was of Malaysian origin, the others were all Caucasian. A further six families presented themselves during the course of the study requesting investigation. Eleven families were ascertained who had more than one individual affected with von Willebrand disease. Ten families were of Caucasian origin. There was one family with severe recessive vWD who were Asian and the marriage was consanguinous. An extended pedigree was obtained from all families and blood samples obtained for DNA analysis.

DNA from unaffected individuals, used to calculate allele frequencies in the normal local population, was obtained from DNA already stored in the Duncan Guthrie Institute of Genetics or from spouses of family members in the present study.

III.2 MATERIALS

DNA PROBES - Probes factor 9 (VIII) and factor 9 (XIII) were kindly donated by Professor G.G. Brownlee. Probe factor 9 (VIII) is a 2.5kb segment of the factor IX gene containing 2.5kb of introns and the "d" exon. It identifies two bands in Taq I digested DNA from males and two or three bands in Taq I digested DNA from female subjects. The 5.3kb band occurs in all subjects and the polymorphic fragments were 1.8kb or 1.3kb. When DNA is digested with Xmn I and probed with factor 9 (VIII) only a single band is apparent in males, 11.5kb or 6.5kb, whereas females may be homozygous for either fragment or heterozygous, 11.5kb/6.5kb. Probe factor 9 (XIII) similarly only identifies a single band in Dde I digested male DNA, 1.75kb or 1.7kb, whereas females may be homozygous for either fragment or heterozygous.

Four probes were used to investigate haemophilia A, St14, DX13, F8A and F8B. St14 is a fragment of 9kb which reveals a polymorphic system with at least ten allelic fragments in addition to fragments of constant size in Taq I digested DNA (it also recognises a polymorphism on Msp I digested DNA). It lies in the q26-qter region of the X chromosome although not within the factor VIII gene. In this study alleles were numbered 1-10 according to size rather than using the actual kilobase measurement of each allele [method used in Youssoufian et al 1986]. St14 was supplied by Dr. J-L. Mandel. The international marker symbol for it is DXS52. DX13 (international marker symbol DXS15), supplied by Dr. K. Davies, is a cloned sequence of DNA localised to band Xq 28 of the X chromosome which recognises a polymorphism in Bgl II digested DNA. As well as four invariant bands males have either a 2.8kb band or a 5.8kb band, women may be heterozygous 5.8kb/2.8kb or homozygous for either band. DX13 does not lie within the factor VIII gene. F8A (exon 17-18) supplied by Dr. Lawn, is a 647bp intragenic factor VIII probe which recognises a polymorphism in Bc1 I digested DNA. Males have either a 1.2kb band or a 0.9kb band, women may be heterozygous or homozygous. F8B (exon 26) is supplied by Dr J. Toole. It is a 1.8kb fragment of exon 26 of the factor VIII gene which recognises a polymorphism in Bgl I digested DNA. Males have either a 5kb fragment or a 20kb fragment, women may be heterozygous or homozygous.

The probe used to study von Willebrand disease was donated by Dr CL Verweij. It is a 100bp cDNA fragment of the von Willebrand factor gene,named pvWF1100,which recognises a two allele polymorphism in Bgl II digested DNA with a band at either 9kb or 7.4kb as well as two invariant bands at 13kb and 4.9kb. Since the gene is localised to an autosome, chromosome 12, both males and females can be heterozygous or homozygous for the polymorphic alleles.

III.3 METHODS

III.3i DNA Analysis

DNA was extracted from whole blood in EDTA using the method of Kunkel et al [1977]. Eight micrograms of DNA were digested overnight using thirty to fifty units of restriction enzyme. The resulting fragments were electrophoresed in 0.8% agarose gels and then stained with ethidium bromide. The only exception was samples being investigated for Haemophilia B with the Dde I polymorphism and the probe XIII where a long gel of 1.2% agarose was used. Each lane was examined and photographed in ultraviolet light to check for complete digestion before transfer to nitrocellulose or Hybond N nylon based transfer membrane (Amersham) by Southern blotting [Southern, 1975]. DNA was fixed to the transfer membrane by a five minute UV crosslinking treatment.

DNA probes were labelled with either nick-translation (using the Amersham kit) or hexanucleotide labelling [Feinberg and Vogelstein, 1983]. The filters were hybridised with denatured probe at 42°C in 50% formamide, 1x Denhardts solution, 5x SSC, 20mmol NaH_2PO_4 (pH 6.8), 0.1mg/ml heat denatured salmon sperm DNA, 20g/ml poly A and 10% dextran sulphate. Hybridisation was allowed to take place over 48 hours. Excess probe was then washed off in 2x SSC, 0.1% SDS at room temperature for 30 minutes followed by washes at 63°C in 0.5x SSC, 0.1% SDS or 0.1x SSC, 0.1% SDS for 30 minutes, depending upon the final stringency required. Filters were then exposed to X-ray film using intensifying screens at -70°C for various periods of time.

The size of bands produced in each lane of DNA could be identified by running concurrently a lane containing fragments of DNA of known size: a DNA ladder. The data obtained for each RFLP for the individuals tested is contained in the appendix. Interpretation of the data required a knowledge of the family pedigree. RFLPs in this context are used simply as markers of the relevant gene (or area close to the gene in the context of extragenic probes) and allow the inheritance of a particular gene to be traced. Thus a mother with alleles A/B may have a son with either. If the son with allele A has haemophilia, then any daughter inheriting a maternal allele A is a carrier and any daughter with allele B is not a carrier.

III.3ii Haematological Investigations

These methods are all described in detail in a standard haematology laboratory textbook [Dacie and Lewis, 1984].

Bleeding time is measured by the standardised template method using a commercially manufactured blade (Simplate, General Diagnostic). VIIIc in this study is measured functionally using a one stage assay. The immunoelectrophoretic method of Laurell [1966] is used to measure von Willebrand factor antigen (VWF:Ag) and ristocetin co-factor (VWF:RCo) is measured using a platelet aggregometer and applying the method described by McFarlane [1975].

III - 3iii Interpretation and Relaying of Results

Results of DNA studies were interpreted by myself but checked by Dr. Connor. The individuals concerned were informed of the results. Where possible, women were seen by myself, informed of the results and the limitations of the tests explained. Subsequently, they received a letter stating the information presented at interview. Women who were unable to attend a clinic were informed of the results by letter. All women were offered the opportunity of further counselling as required.

CHAPTERIV - RESULTS

IV.1 HAEMOPHILIA A

IV.1i Individuals Tested

In the West of Scotland, 215 males with haemophilia A are registered with the Regional Haemophilia Centre. This represents an estimated prevalence $^{1}/_{6500}$ males. In view of the large numbers involved, it was decided to select local multiplex families who were interested in having the studies performed upon themselves. During the study, individuals presented themselves requesting genetic counselling and these families were also included. In all, sixteen families were studied. The pedigrees of these families are shown in figure 4. Altogether, a total of 216 individuals were tested of whom 40 were affected males, 38 were obligate carrier females and 71 were females at risk. The DNA results and haematological results for tested members of each family are presented in appendix 1.

IV.1ii Allele Frequency

The RFLPs used in this study are illustrated in Fig 5. The allele frequency for each RFLP in unrelated X chromosomes in individuals not affected by haemophilia A was measured. In view of the multiple allele system of the St14 RFLPs and the relatively small numbers in this study, no attempt was made to record allele or heterozygote frequency within the general population although the results obtained from the study will be presented in a later section. The results for the three other RFLPs are contained in table 1.

The allele associated with haemophilia A in the same 3 RFLPs is shown in table 2.

IV.1iii Female Relatives Investigated

Details of all obligate and potential carriers who were investigated in this study are shown in table 3.

Thirty eight obligate carriers were tested although only thirty were tested with all four probes. The information obtained was useful not only as part of pedigree studies but also to show how many obligate carriers in the population studied are informative, ie heterozygous for any one probe and so could be offered prenatal diagnosis should they seek it. See table 4.

50% of obligate carriers were informative with F8A probe whilst only 17% were informative with F8B. There were two women not informative for F8A who were heterozygous with F8B so that in all 55% of obligate carriers were informative with an intragenic probe. 47% of carriers were informative using DX13. Only 35 obligate carriers were tested with St14 and 29 (83%) of these were informative. These 35 obligate carriers were tested with both extragenic probes and in all 32 (91%) of them were informative. Twenty seven (90%) of the thirty women tested with all four probes were informative.

Seventy one females who had at least a $^1/_8$ chance of being a carrier from pedigree were tested with DNA probes. Twenty nine (41%) had definitive determination of carrier status on the basis of the two intragenic RFLPs. 19 of these relied upon information from F8A, 8 from F8B and two from both. A further 22 (31%) had refined genetic information on the basis of extragenic probe information. One of these relied upon information from DX13, two from St14 and 19 from both. In 20 females at risk (28%) DNA analysis was unhelpful. In 9 of these the problem was maternal homozygosity for all four probes tested and in the remaining 11, absence of DNA samples from one or both parents or in one case grandparents, prevented interpretation of the data from the female at risk (Table 5). Use of the St14 probe with MspI digestion of DNA rather than Taq I which was used throughout the study failed to reveal any polymorphic sites amongst the 9 mothers homozygous for all four probes.

19 females were identified as carriers (27%) and 32 were identified as non carriers (45%).

IV.1iv Non Paternity

In two families, the results of DNA studies suggested that siblings had different fathers although the family history had not suggested this. In pedigree 7402, the alleles for the four RFLP probes which are linked with haemophilia A are 1.2kb, 5kb, 2.8kb and 7kb (In the order F8A, F8B, DX13 and St14) See figure 6. These have been inherited by individual II8 from her mother and, deriving information from II8 would give her father the genotype 1.2kb, 20kb, 2.8kb and 5kb. Neither II4 nor II6 have inherited the 20kb allele of F8B and II4 does not have the St14 allele of 5kb.

In pedigree 7405, looking at the results of F8A it is assumed that II2 and II4 are both 0.9kb, 0.9kb. Since the 0.9kb fragment is linked with haemophilia A and has been inherited by III11 and III13 from their mother, it is clear that their father had a 1.2kb

fragment and consequently could not have been the same person as the father of III8 who is homozygous for the 0.9kb fragment. It is, however, still possible to advise individual III8 that she is not a carrier for haemophilia A on the basis of the results obtained from the St14 probe.

IV.1v Recombinants

No recombination between the disease trait and the two intragenic factor VIII RFLPs was observed in 22 phase known informative meioses. Similarly, no recombinants were observed between the factor VIII gene and DX13 in 14 phase known informative meioses. However, for St14, three recombinants were observed amongst 28 phase known meioses. In pedigree 7402, (figure 7), there were two recombinants in a sibship of 13. Individual II8 is a carrier for haemophilia A. With the St14 RFLP, the haemophilia A is linked with allele 7. However, two haemophiliac sons have inherited the allele 5 suggesting recombination between the haemophilia gene and the St14 allele site in the X chromosome. Similarly, in pedigree 2218, individuals II3, II5 and II7 are all obligate carriers of haemophilia A. Individuals II3 and II5 have the St14 alleles 2 and 7 and the haemophilia A is linked with the allele 7. Individual II7 has the alleles 2 and 3 with haemophilia A now linked to allele 3. This would suggest a recombination in the maternal alleles between the gene for haemophilia A and the site of the St14 alleles.

IV.1vi Coagulation Studies

21 obligate carriers had FVIIIc and vWFAg measured and these carriers are listed in table 3 with coagulation results in appendix I. There were 24 possible carriers who had coagulation studies and who could also be assigned as carriers or non carriers as a result of DNA studies(table 6). Most of these women only had coagulation tests performed on one occasion. Where results were already available from the Haemophilia Centre, these were combined with the tests performed during this study and the results presented are a mean of these results. There was one obligate carrier 7319 II3 and one potential carrier 7405 III6 who were pregnant when these studies were made. The relative odds of each possible carrier being a carrier were calculated using the graph contained in the paper publised by Prentice et al 1974. This was chosen as, at the present time, this is the data used locally to advise possible carriers of their risk from information obtained from coagulation studies. Of the 11 assigned carrier status as a result of DNA studies, six would have been given odds greater than even that they were carriers from coagulation studies. However, five would have been given odds greater than even that they were not carriers. Of these five, three were assigned as carriers from information obtained using intragenic probes whereas two relied upon extragenic probes (table 6). All of the 13

women assigned as non carriers would have been given odds against their being carriers from the results of coagulation studies.

These coagulation results are displayed diagramatically (Fig 8) and show clearly how gene probes improve the odds either for or against carrier status which will allow improved carrier detection.

IV.1vii Linkage with G6PD Deficiency

Individuals II1 and II3 in Pedigree 7409 who are both haemophiliacs had undetectable levels of G6PD. Their sister II2 is identified as a carrier of haemophilia from the information obtained from the St14 probe. Her level of G6PD is 3.3iu/gHb (normal range 8.5-16.9iu/gHb) and this would suggest that she is heterozygous for the abnormal G6PD gene. Thus G6PD deficiency, haemophilia A and St14 are linked in all three individuals (Fig 9).

In this study this is the only family where G6PD deficiency was identified. This family originated from Malaysia although they were living in Glasgow at the time of the study.

IV.1viii Linkage with Colour Blindness

There was a history of red/green colour blindness within pedigree 7407 (see Fig 10) which had been confirmed by the local ophthalmologist. Five out of nine males in the third generation were colour blind. Their maternal grandmother (I2 in the pedigree) was an obligate carrier for haemophilia A. She was non informative with intragenic probes but was informative with both DX13 and St14. The daughters of this woman who were the mothers of the colour blind men (II2 and II12 in the pedigree) were shown not to be carriers by extragenic probes. They were both heterozygous for the DX13 RFLP, the 2.8kb allele being the maternal allele from her non affected X chromosome and the 5.8kb allele being paternal. Similarly, they were both heterozygous for St14, the allele numbered 8 coming from the maternal non-affected X chromosome and the allele 3 coming from the father. One colour blind son (III3) and one non-affected son (III5) of II2 were studied and showed that the alleles inherited from I2 were not associated with a diagnosis of colour blindness and the colour blindness must have originated in I1 (their maternal grandfather. The colour blind sons of II12 (III17 and III18) and one non-affected (III19) showed the same results. There was no genetic crossover noted between the gene for colour blindness and the extragenic probes St14 and DX13 in these five individuals.

Lod scores calculated in this family between colour blindness and DX13 and St14 were maximum at a recombination fraction of 0 and gave a result of 0.96. Computer programme LINKAGE version 5.03 was used to calculate the lod score [Lathrop and Lalouel, 1984].

IV.1ix Prenatal Diagnosis

During the course of this study, early prenatal diagnosis was requested by one obligate carrier. The family pedigree is 7319. (See fig 4.) II3 is an obligate carrier as she has an affected brother and has had second trimester prenatal diagnosis and termination of pregnancy of an affected male foetus. As shown in fig 11, she is heterozygous for the intragenic F8A RFLP and her normal sons have the 0.9kb fragment. The foetus was tested at 9 weeks of pregnancy from DNA obtained by transcervical chorionic villus sampling. Since this time, the baby has been born and is a female child. A total of 10μg of DNA was obtained allowing the test to be done in duplicate. It has received the 1.2kb fragment and hence the mutant factor VIII gene. Fig 11 also shows results with a Y specific probe GMGY7 and absence of hybridisation to the DNA from the foetus shows that it is female. This was confirmed cytogenetically.

PROBE	No OF CHROMOSOMES TESTED	RFLP (kb)	No OF EACH ALLELE	% OF EACH ALLELE
		0.9	34	75.6
F8A	45	1.2	11	24.4
		5	21	80.8
F8B	26	20	5	19.2
		2.8	30	62.5
DX13	48	5.8	18	37.5

TABLE 1 - RFLP FREQUENCIES IN NORMAL INDIVIDUALS IN THREE RFLPS USED IN INVESTIGATING HAEMOPHILIA A

PROBE	RFLP (kb)	No TESTED	No OF EACH ALLELE	% OF EACH ALLELE
	0.9		8	50
F8A	1.2	16	8	50
	5		11	85
F8B	20	13	2	15
	2.8		12	75
DX13	5.8	16	4	25

TABLE 2 - PERCENTAGE OF EACH ALLELE LINKED WITH HAEMOPHILIA A IN FAMILIES STUDIED

PEDIGREE No.	LIVING TESTED OBLIGATE CARRIERS	FEMALES AT RISK		
		CARRIERS	NOT CARRIERS	NON-INFORMATIVE
2218	II3 II5 II7	III4 III6 III11	III5 III8 III11 III13	
7406	I2 III1 III3			II4
7409	I2	II1		
7408	I2 II4 III5	II2	III1	
7411	II2 II4	III3 III7	II6 III4 III9	
7401	II2 III2 III4 III10	IV2 V1	III7 IV9 IV10 III14 IV11	IV6 IV13 II7 III12
1065	II2 III4	III2	IV1	
7403	I2 II6		II4 III1	
7413	III2 III3		III7	III6
7412	II2			
7319	II3	III4		
7402	II8 III14	III18	III9 III12	II4 II6
7407	I2 III8 II8	II4 II14 II10	II2 II12 III20 III21	III7 III12 III13 III22 III14 III15 III16
7410	III2 II4 II12 III4		IV3 III8 III9 III10	II6 II8 II10 III7
7399	II2 III8	III3	IV1	III9
7405	II2 III11 III13	IV6 IV8 III9	III4 III6 III8 IV1	
TOTAL	38	19	32	20

TABLE 3 - OBLIGATE CARRIERS AND FEMALES AT RISK OF CARRYING HAEMOPHILIA A INVESTIGATED

THE FEMALES AT RISK ARE DIVIDED ACCORDING TO CARRIER STATUS ASSIGNED BY THIS STUDY

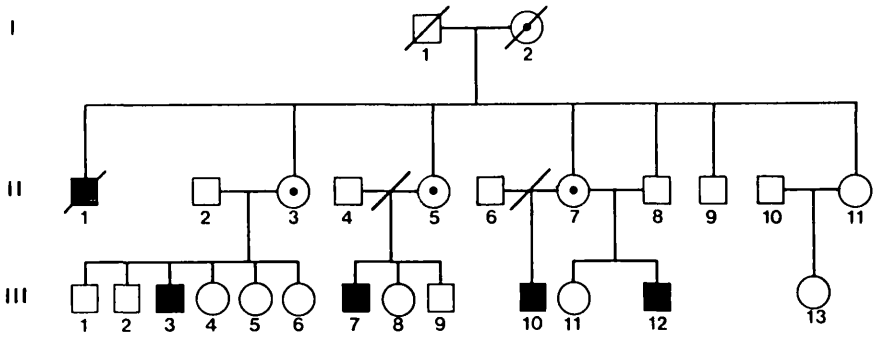
LACK OF PARENTAL DNA		MATERNAL HOMOZYGOSITY		LACK OF GRANDPARENTS DNA	
PEDIGREE	INDIVIDUAL	PEDIGREE	INDIVIDUAL	PEDIGREE	INDIVIDUAL
7406	II4	7401	IV6	7410	III7
7401	II7	7401	IV13		
7401	III12	7407	III7		
7413	III6	7407	III12		
7402	II4	7407	III13		
7402	II6	7407	III22		
7410	II6	7407	III14		
7410	II8	7407	III15		
7410	II10	7407	III16		
7399	III9				

TABLE 5 - 20 FEMALES AT RISK OF CARRYING HAEMOPHILIA A TO WHOM DNA ANALYSIS WAS UNHELPFUL

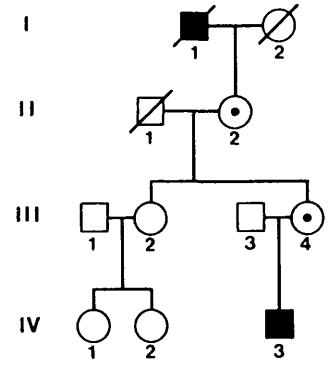
PEDIGREE	INDIVIDUAL	CARRIER STATUS	PROBE	FVIII:Ci (iu/dl)	vWF:Ag (iu/dl)	ODDS
2218	III4	C	IG	130	100	1:16
	III6	C	IG	80	125	8:1
7399	III3	C	IG	110	70	1:16
7401	IV2	C	IG	35	72	2:1
	V1	C	IG	42	84	4:1
7405	III9	C	EG	180	82	1:16
	IV6	C	IG	130	100	1:16
	IV8	C	IG	74	175	128:1
7407	II4	C	EG	19	66	8:1
	II14	C	EG	41	50	1:3
7411	III7	C	IG	77	137	32:1
2218	III5	NC	IG	140	52	1:16
7399	IV1	NC	EG	110	55	1:16
7405	III4	NC	EG	130	115	1:12
	III6	NC	EG	250	185	1:16
	III8	NC	EG	180	165	1:12
	IV1	NC	EG	180	180	1:6
7407	II2	NC	EG	62	41	1:16
7408	III1	NC	EG	90	65	1:16
7410	III8	NC	EG	100	100	1:6
	III9	NC	EG	110	100	1:10
	III10	NC	EG	100	60	1:16
	IV3	NC	IG	140	72	1:16
2218	III8	NC	IG	82	50	1:16

TABLE 6 - RESULTS OF COAGULATION TESTING UPON INDIVIDUAL IDENTIFIED AS CARRIER (C) OR NON CARRIER (NC) USING INTRAGENIC (IG) AND EXTRAGENIC (EG) PROBES.

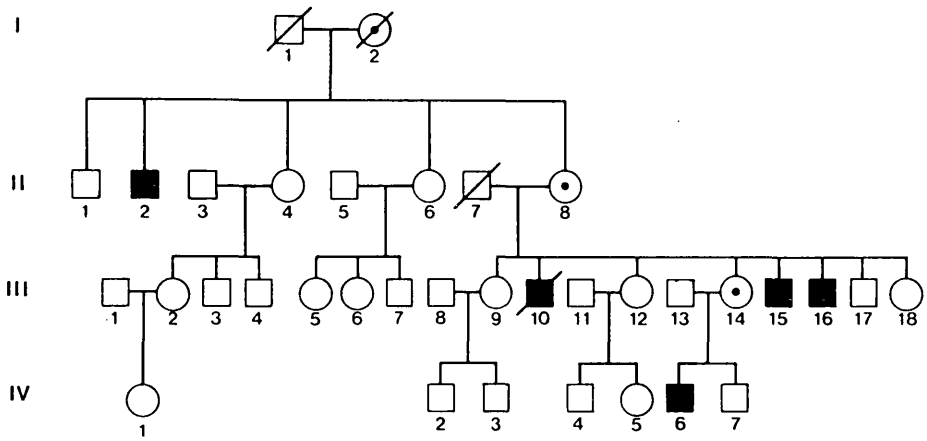
THE FINAL COLUMN SHOWS THE ODDS OF THE LIKELIHOOD THAT THEY ARE CARRIERS FROM THE COAGULATION DATA.[Prentice et al,1975]



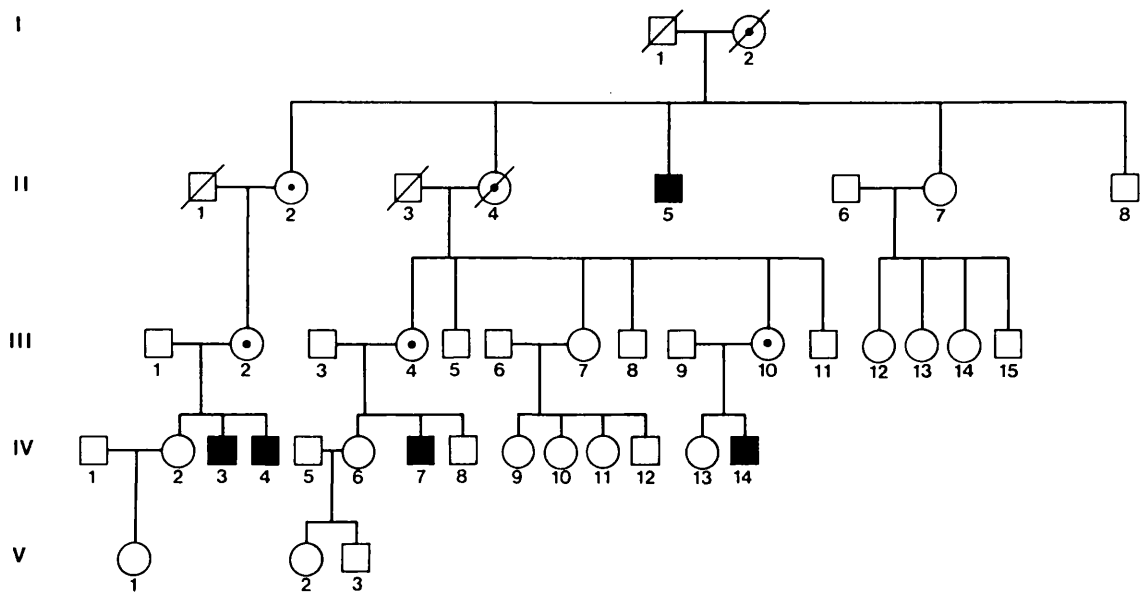
Pedigree 2218



Pedigree 1065

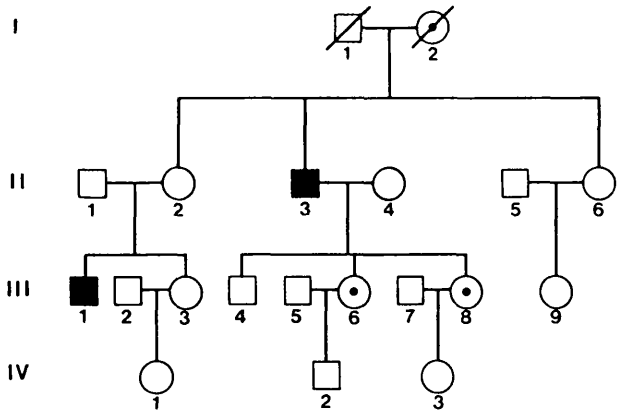


Pedigree 7402

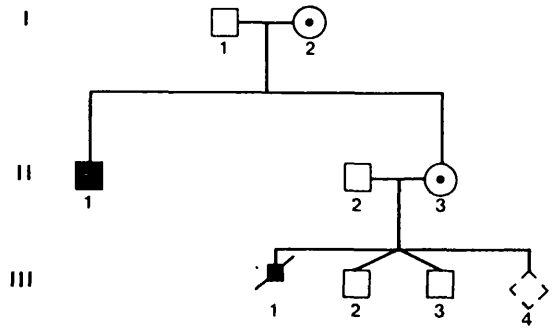


Pedigree 7401

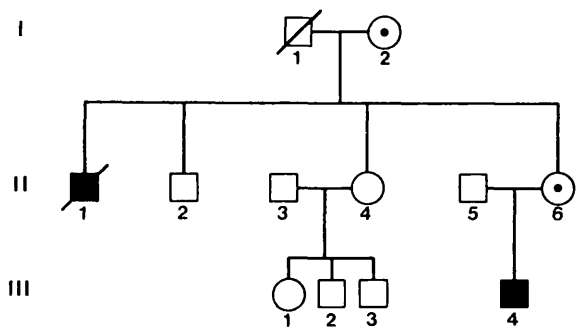
Figure 4 - Pedigrees of Haemophilia A Families



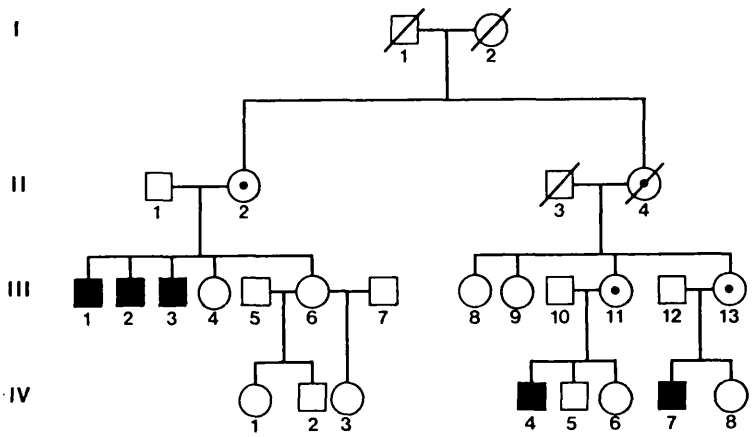
Pedigree 7399



Pedigree 7319

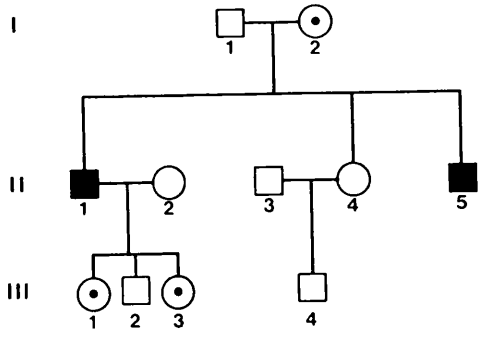


Pedigree 7403

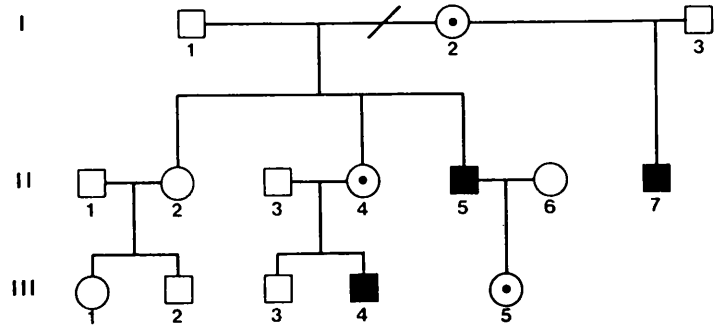


Pedigree 7405

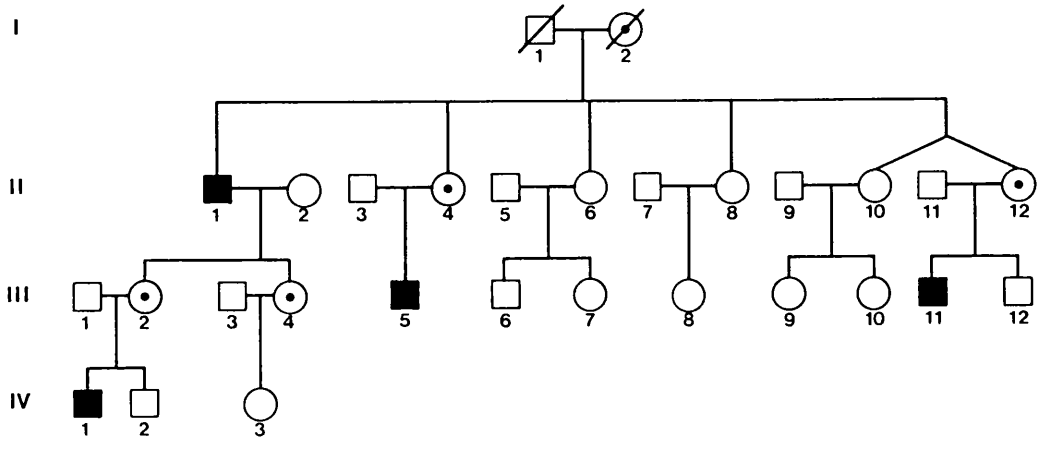
Figure 4 - Pedigrees of Haemophilia A Families



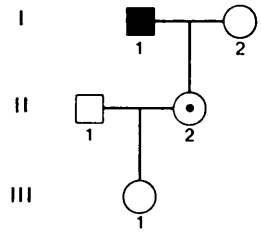
Pedigree 7406



Pedigree 7408

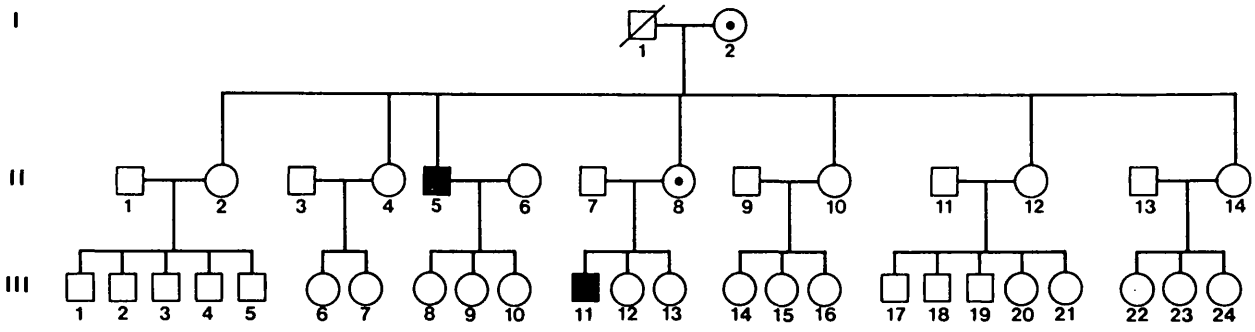


Pedigree 7410

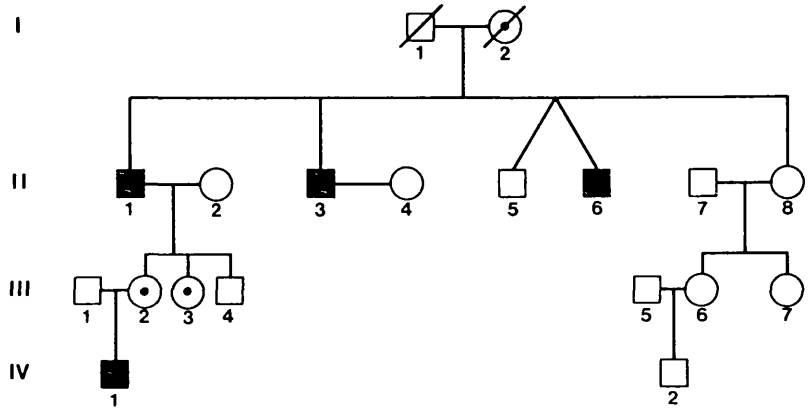


Pedigree 7412

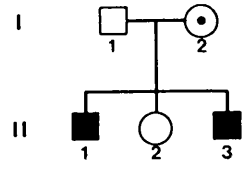
Figure 4 - Pedigrees of Haemophilia A Families



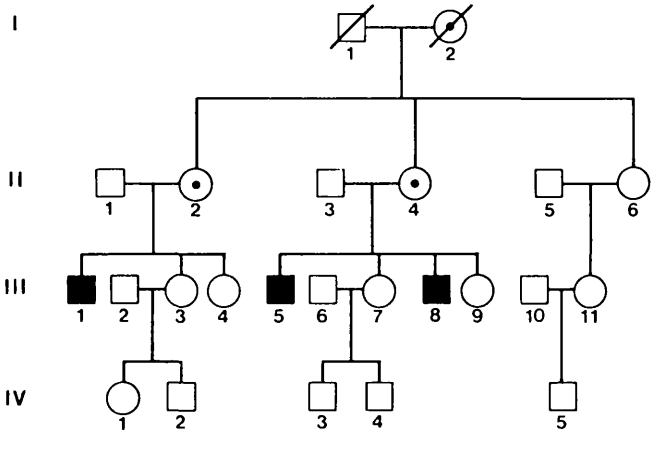
Pedigree 7407



Pedigree 7413



Pedigree 7409

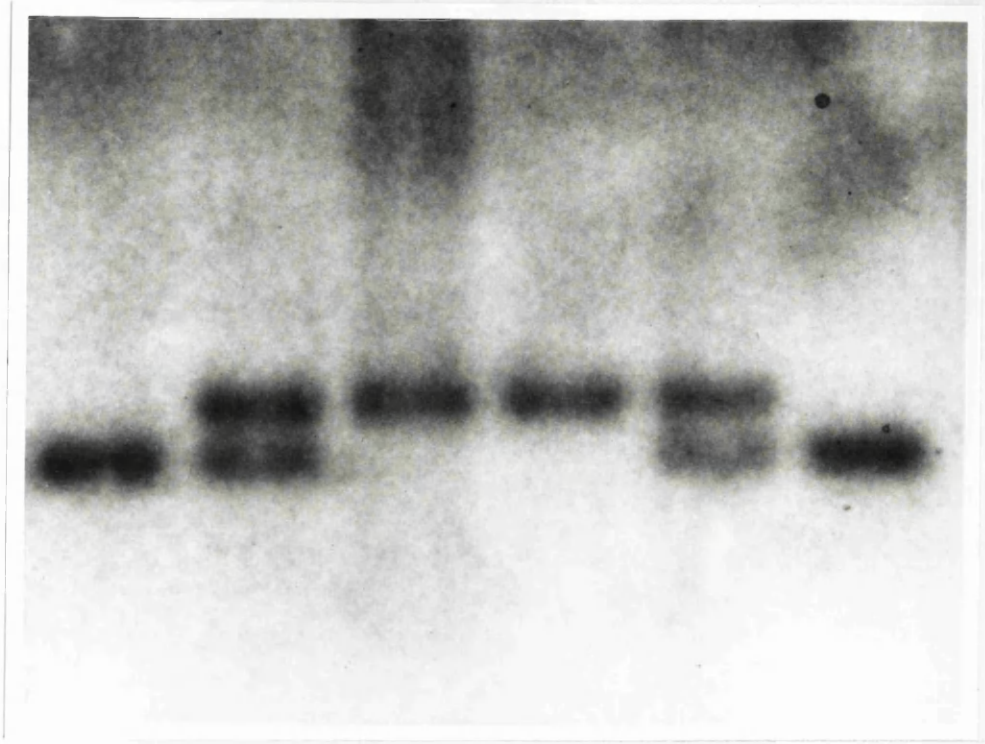


Pedigree 7411

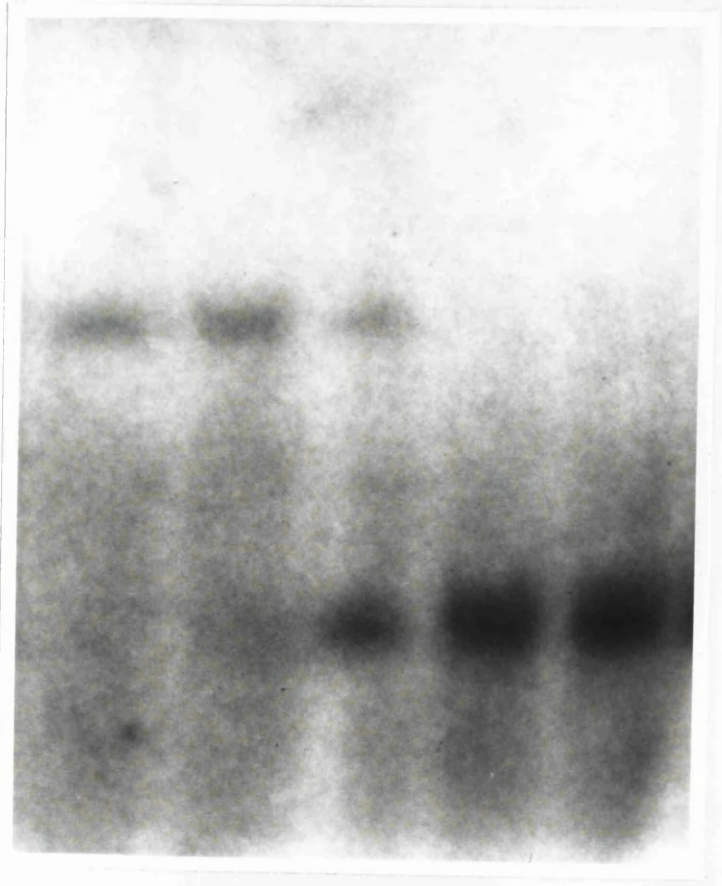
Figure 4 - Pedigrees of Haemophilia A Families

Figure 5(a)

Intragenic RFLPs, F8A/Bcl I and F8B/Bgl I used to investigate Haemophilia A



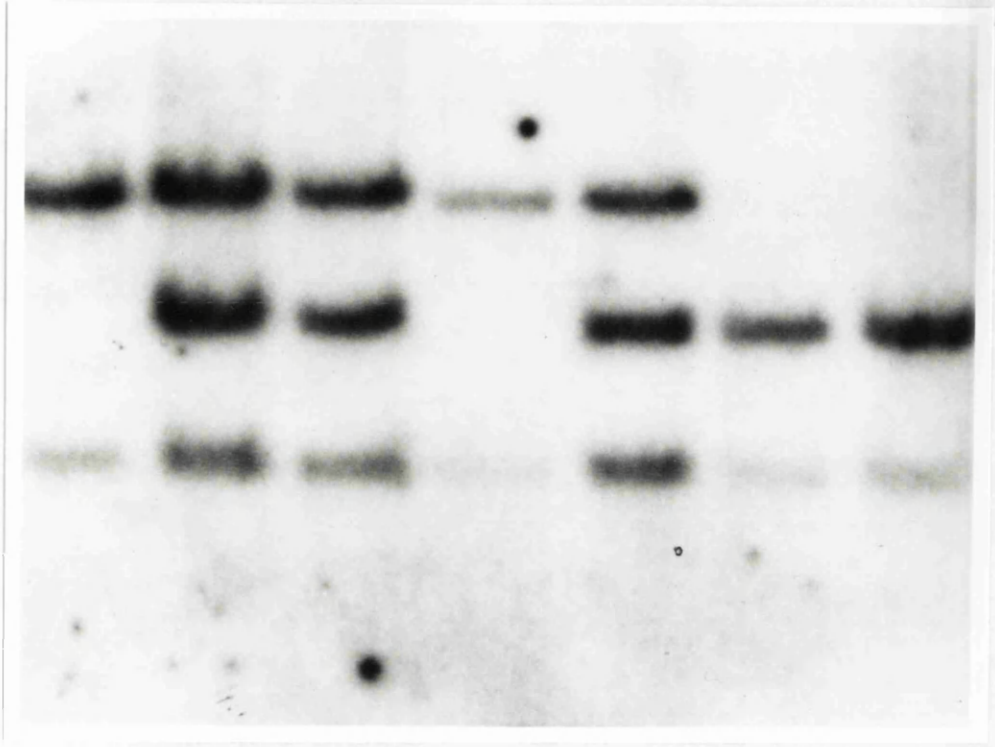
Factor 8A (Exon 17-18)



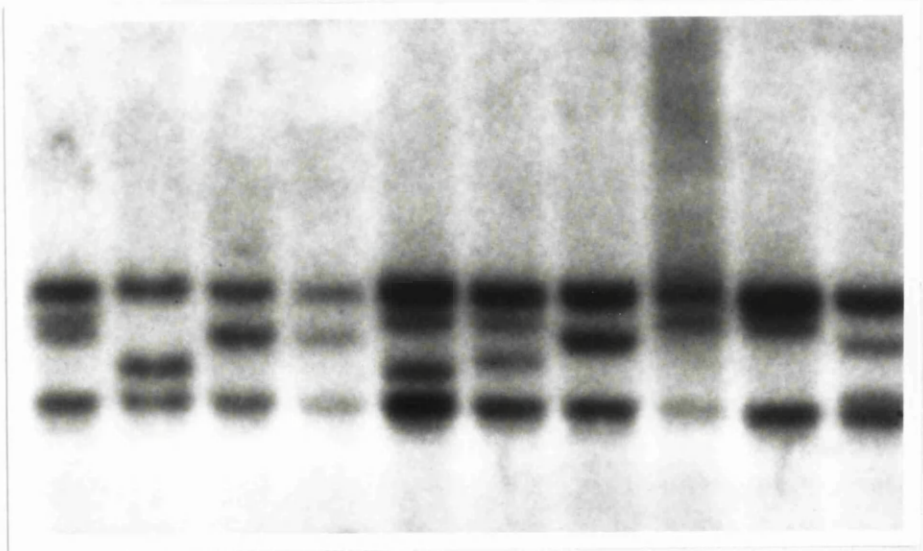
Factor 8B (Exon 26)

Figure 5(b)

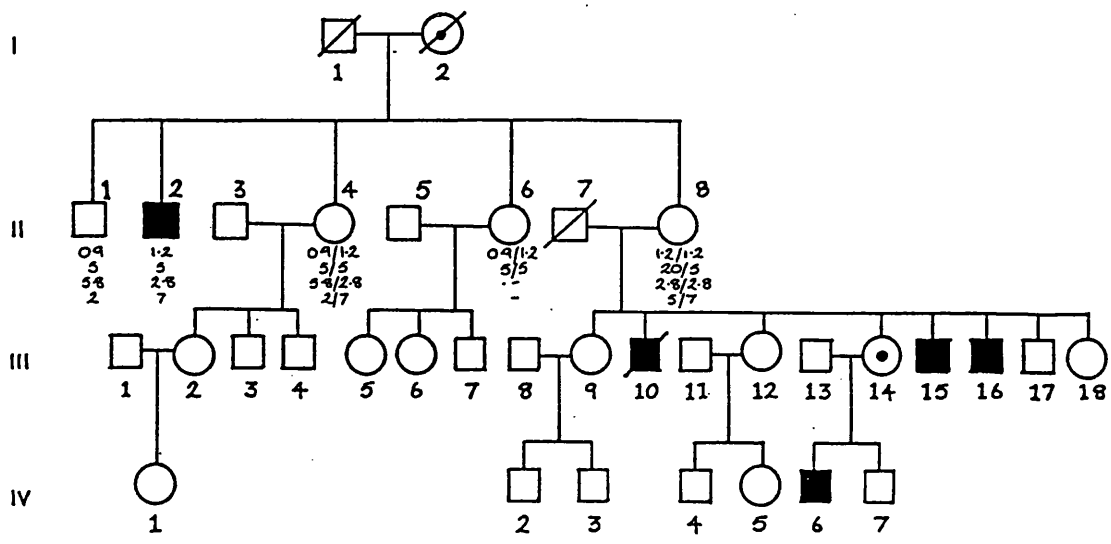
Extragenic RFLPs, DX13/Bgl II and St14/Taq I, used to investigate Haemophilia A



DX13(DXS15)



St14(DXS52)



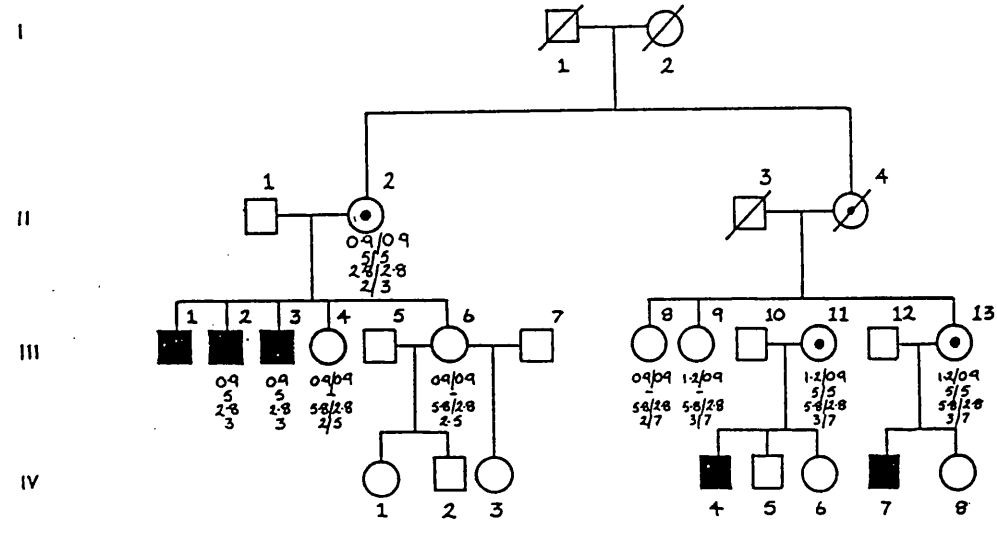
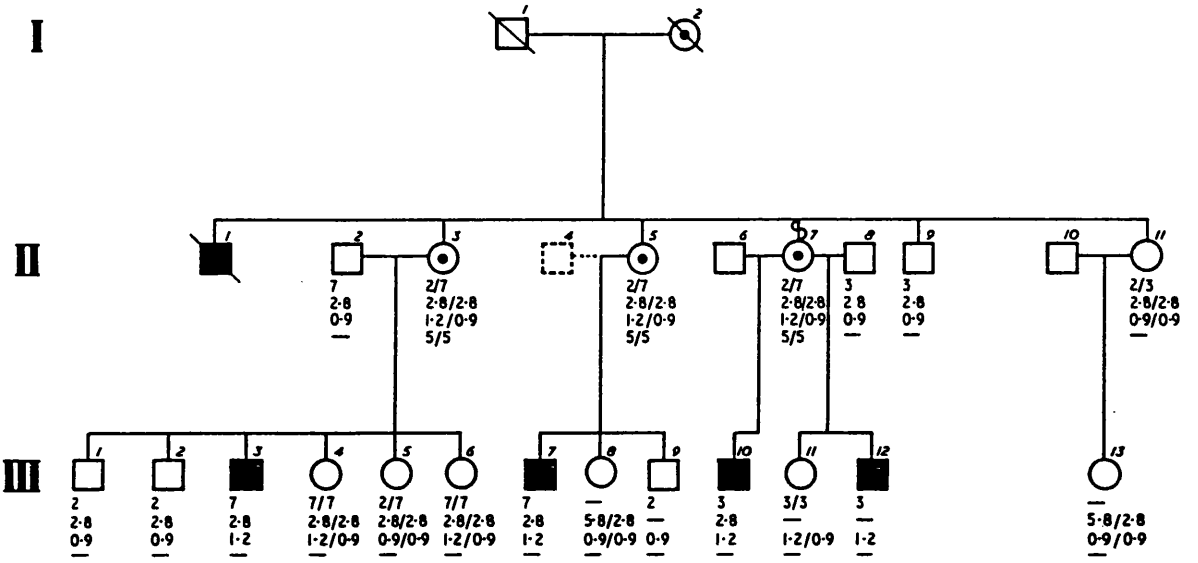


Figure 6

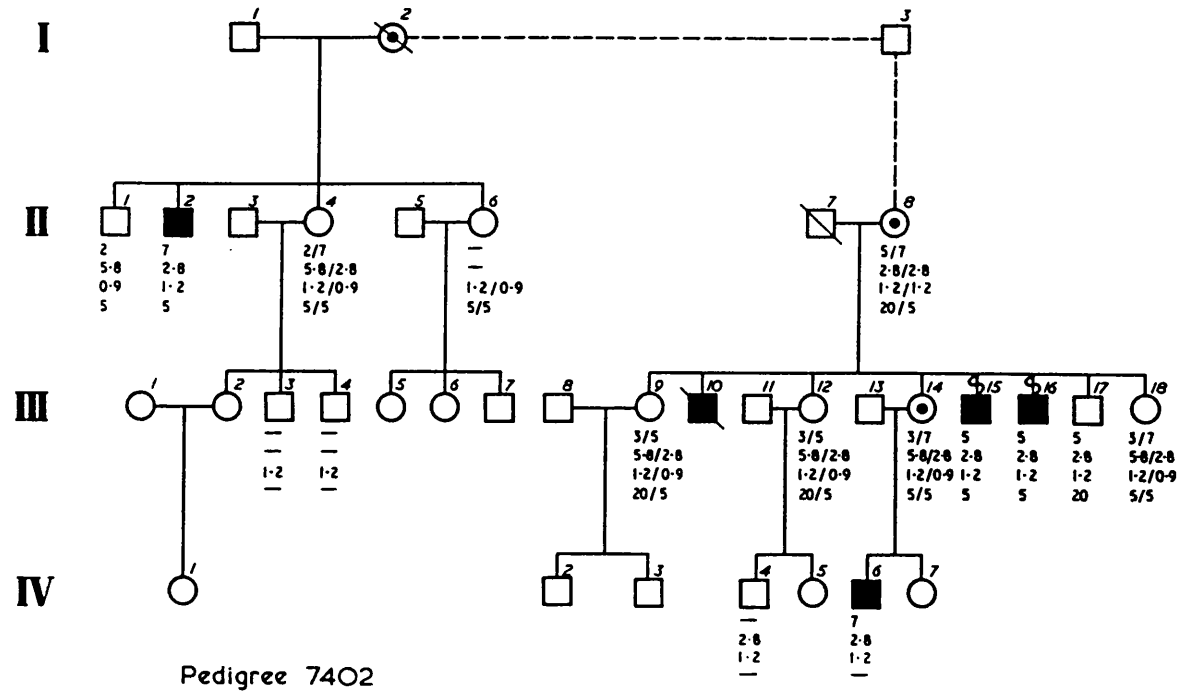
Non paternity demonstrated in two pedigrees: 7402 (individual II_8) and 7405 (individual III_8). Results listed in order: F8A, F8B, Dx13, St14.

Figure 7

Recombination between Haemophilia A and St 14 probe in three individuals in two families, (pedigree 2218-II7; pedigree 7402 III 15 and III 16).



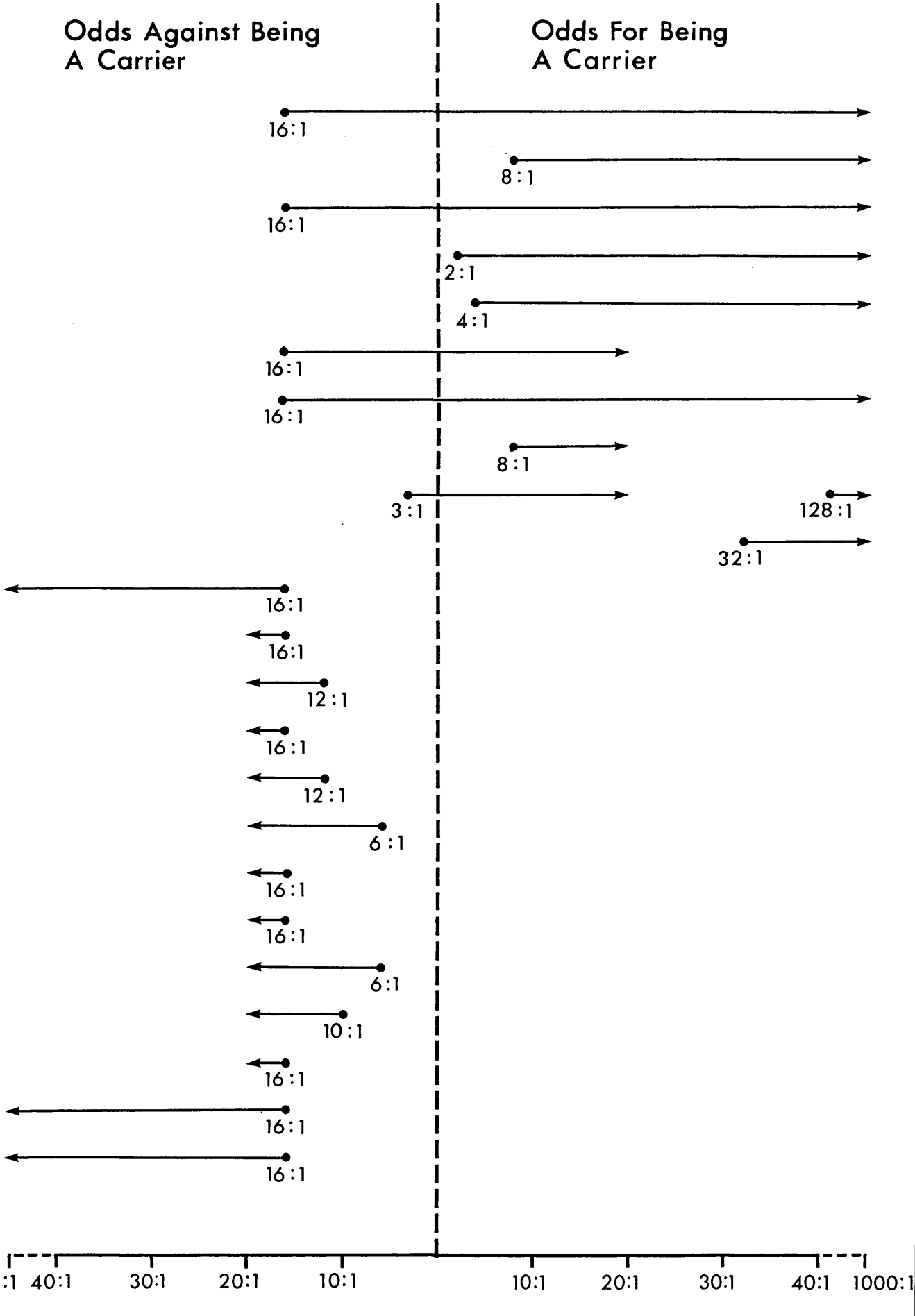
Pedigree 2218



Pedigree 7402

Fig. 8

24 women tested by both coagulation methods and RFLP's for Haemophilia A. Diagram shows odds of carrier status derived from coagulation results and the change in odds after results from RFLP's are obtained. RFLP results from extragenic probes are plotted as 20:1 whereas results from intragenic probes give results of at least 1000:1



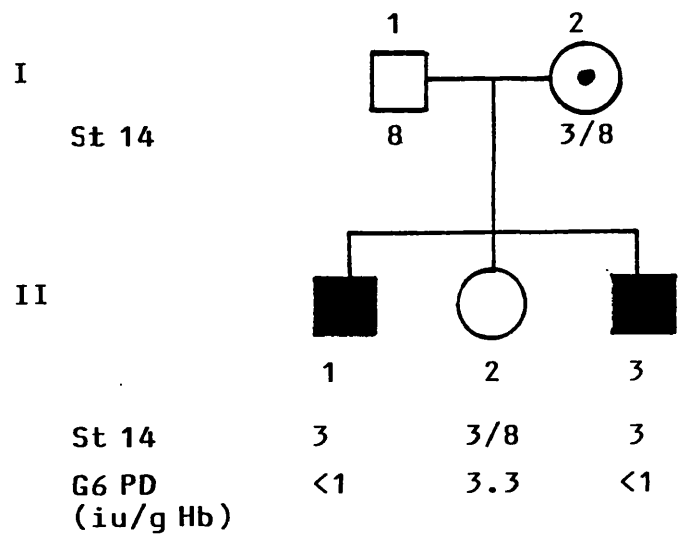


Figure 9

Pedigree 7409. Linkage between St14, Haemophilia A and G6PD deficiency.

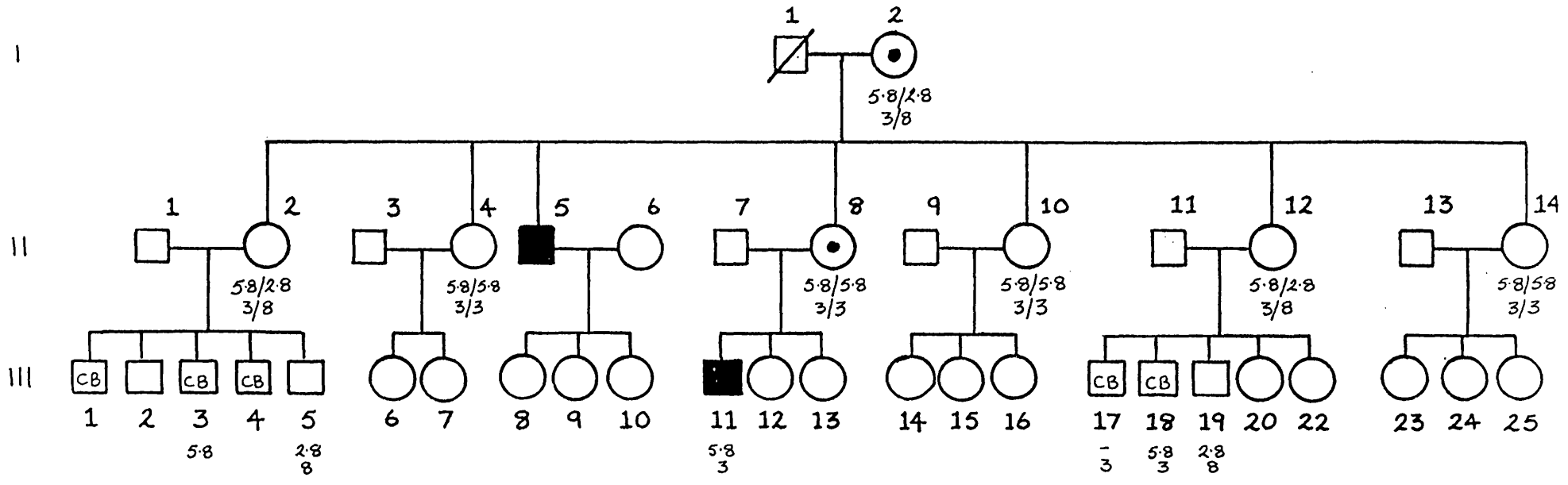
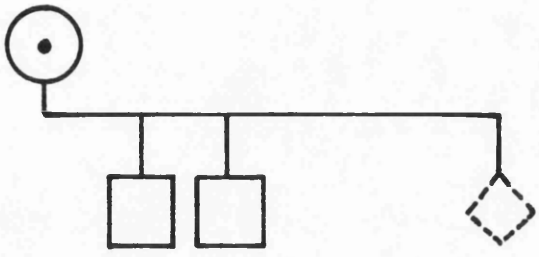


Figure 10

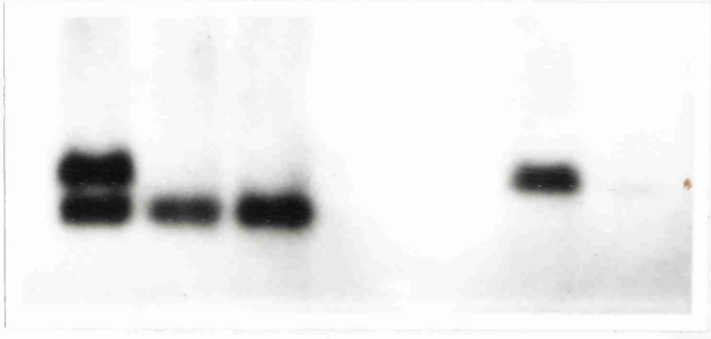
Pedigree 7407. Linkage between Dx13, St14, Haemophilia A and colour blindness.

Figure 11

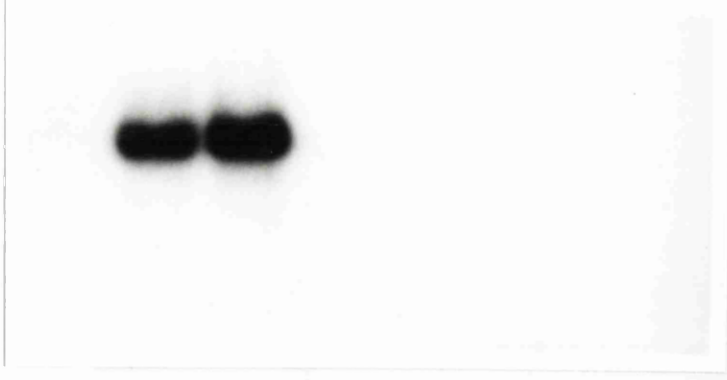
Prenatal diagnosis in Pedigree 7319. Fetus has the allele linked with Haemophilia A but a Y chromosome probe shows that the fetus is female.



F8A



GMGY7
(Y Chromasome
Probe)



IV.2 HAEMOPHILIA B

IV.2i Individuals Tested

Within the West of Scotland 52 males with haemophilia B were identified with an age range of 2 to 76 years. This represents a prevalence of 1 in 26,900 males. Initially, these were considered to belong to 35 separate families but extended pedigrees reveal that several were related. Of the remaining 28 families, 16 contained only a single affected male and 12 had two or more affected subjects. Two of these 12 families were unwilling to participate in this study. However a further two families were referred to the Duncan Guthrie Institute from other centres for investigation during the course of the study and their results are included. A total of 128 individuals were tested of whom 30 were obligate carrier females, 32 were females who were at risk of being carriers of haemophilia B and 31 were affected males. The pedigrees of these families are shown in figure 12. The DNA results and haematological results are presented in appendix II.

IV.2ii Allele and Heterozygote Frequency

Figure 13 illustrate the RFLPs used in this study. The frequency of each allele in unrelated Scottish subjects is indicated in table 7.

The allele linked with haemophilia B in the families investigated is shown in table 8. In the three RFLPs investigated the more common allele found in normal individuals in the West of Scotland was also the more common allele in disease linkage although the preponderance of the 11.5kb fragment in the F9(VIII)/Xmn I RFLP was higher than in normal subjects.

IV.2iii Female Relatives Investigated

The thirty living obligate carriers who were tested are identified in table 9. This table also contains details of thirty two females at risk who were tested. Eight (25%) of these women were shown to be carriers of haemophilia B using these recombinant DNA techniques whilst thirteen (41%) were shown not to be.

The data from obligate carriers and possible carriers is broken down to quantify homozygote and heterozygote frequency in both these groups. There is an increase in the heterozygote frequency for the F9(VIII)/Taq Iand F9(VIII)/Xmn I RFLPs in the possible carrier group compared to the heterozygote frequency in the healthy Scottish population (Tables 10 and 11).

In these families eleven females (34%) could not be counselled as a result of DNA analysis. However three of these females had factor IXc levels at or below the lower limit of normal (normal range 50% - 200%) and had haemorrhagic symptoms (pedigree 4549 III5: factor IXc level 52%; pedigree 4565 IV2: factor IXc level 42% and pedigree 4555 IV4: factor IXc level 50%). Although normal coagulant studies do not exclude carrier status of haemophilia B, the combination of clinical history and low factor IXc levels indicate a high probability that these three females are carriers. Eight females could not be counselled despite DNA analysis and coagulation testing. For three of these females the problem is maternal non-information for the RFLPs tested (pedigree 4542 IV13, pedigree 4551 II5 and pedigree 4551 III3) and for the other five unavailability of one or both parents for testing prevented interpretation of the consultand's DNA result (pedigree 1756 III7, pedigree 3569 II4, pedigree 3569 III5, pedigree 4542 III2 and pedigree 4542 III3).

Thus DNA analysis alone permitted definitive genetic counselling of 21 of 31 females at risk (68%). The addition of coagulation results allowed 24 of 31 females (77%) to be offered genetic counselling.

There were 38 carriers either obligate or identified by DNA analysis. Of these 28 (74%) were heterozygous with at least one probe and so theoretically were in a position to be offered prenatal diagnosis for any future pregnancy. However, only 29 of these 38 carriers were tested with all three probes. 24 of the 29 women were informative for at least one probe (83%), eight out of the 29 were informative with two probes (28%). There was no carrier female who was heterozygous with all three RFLPs.

IV.2iv Prenatal Diagnosis

In family 1756 (Fig 14) III9 underwent prenatal diagnosis after amniocentesis and a male foetus without haemophilia B was predicted by DNA analysis. Unfortunately this pregnancy was also at a 1 in 4 risk for cystic fibrosis as IV10 is so affected. Amniotic fluid biochemistry and ultrasonography suggested a recurrence of cystic fibrosis which was confirmed at necropsy after termination of pregnancy.

RFLP	POLYMORPHIC FRAGMENTS (kb)	FREQUENCY	NO.OF UNRELATED X CHROMOSOMES ANALYSED
F9(VIII)/Taq I	1.8	0.63	46
	1.3	0.37	
F9(VIII)/Xmn I	11.5	0.68	60
	6.5	0.32	
F9(XIII)/Dde I	1.75	0.23	30
	1.7	0.77	

TABLE 7 - ALLELE FREQUENCIES FOR FACTOR IX INTRAGENIC RFLPs IN NORMAL SCOTTISH SUBJECTS

RFLP	POLYMORPHIC FRAGMENTS (kb)	NUMBER LINKED WITH HAEMOPHILIA B	NUMBER OF FAMILIES INVESTIGATED
F9(VIII)/Taq I	1.8	7	12
	1.3	5	
F9(VIII)/Xmn I	11.5	10	11
	6.5	1	
F9(XIII)/Dde I	1.75	1	6
	1.7	5	

TABLE 8 - ALLELE LINKED WITH HAEMOPHILIA B IN FAMILIES INVESTIGATED

PEDIGREE No.	LIVING TESTED OBLIGATE CARRIERS	FEMALES AT RISK		
		IDENTIFIED CARRIERS	IDENTIFIED NON-CARRIER	NON INFORMATIVE
1756	III8 III9	IV10	II4 III3 II6 III4 II1 III5	III7
1832	I2	II1		
2377	II2 III2 III13 II9 III8 IV5	IV2		
3569	II2 III2	IV2	IV3	II4 III5
4542	III1 III7 IV10 V3		V1	III2 III3 IV13
4549	I2 II4 II6 III1 III2		III6	III5*
4551	I2 II2 II4			II5 III5
4555	II4 IV5	III6		IV4*
4559	II2 II4	III2	III3 III4	
4569	IV7		V1	IV2*
5215	I2		II4	
5292	IV3	V2 V4		
TOTAL	30	8 (+ 3 COAG)	13	11 (- 3 COAG)

* = BY COAG CARRIER

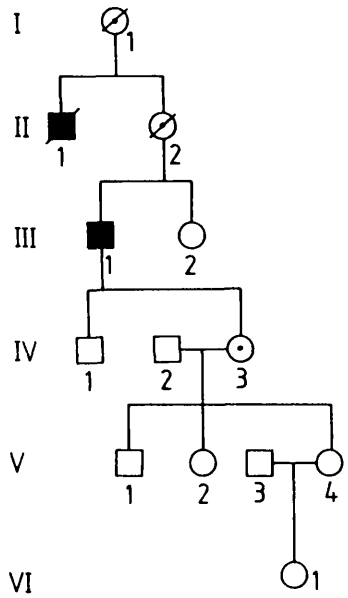
TABLE 9 - OBLIGATE CARRIERS AND FEMALE RELATIVES AT RISK OF CARRYING A HAEMOPHILIA B GENE

PROBE	No.TESTED	HOMOZYGOUS ALLELES		NUMBER OF HETEROZYGOTES	HETEROZYGOTE FREQUENCY
		1.8kb	1.3kb		
F9(VIII)/Taq I	30	7	10	13	43%
		ALLELES			
		11.5kb	6.5kb		
F9(VIII)/Xmn I	29	21	1	7	24%
		ALLELES			
		1.75kb	1.7kb		
F9(XIII)/Dde I	27	0	15	12	44%

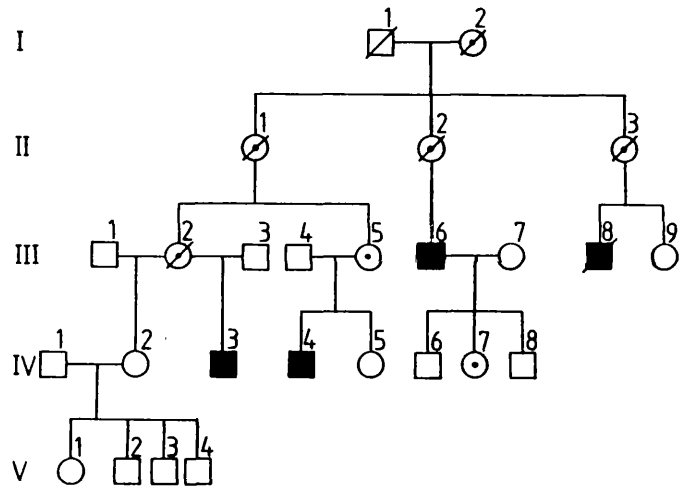
TABLE 10 - HOMOZYGOTE AND HETEROZYGOTE FREQUENCY OF THREE INTRAGENIC RFLPs IN 30 OBLIGATE CARRIERS OF HAEMOPHILIA B

PROBE	No TESTED	HOMOZYGOUS ALLELES		NUMBER OF HETEROZYGOTES	HETEROZYGOTE FREQUENCY
		1.8kb	1.3kb		
F9(VIII)/Taq I	28	3	5	20	71%
		ALLELES			
		11.5kb	6.5kb		
F9(VIII)/Xmn I	17	7	0	10	58%
		ALLELES			
		1.75kb	1.7kb		
F9(XIII)/Dde I	10	0	6	4	40%

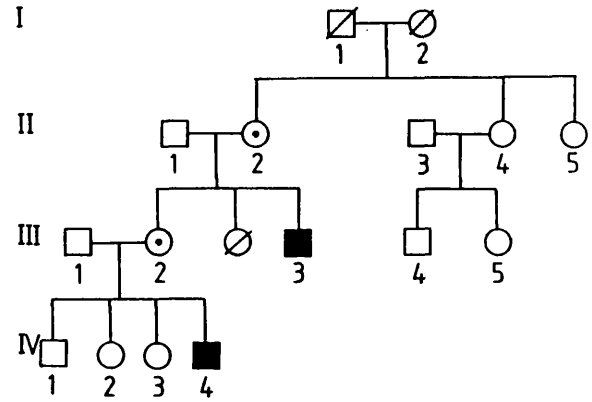
TABLE 11 - HOMOZYGOTE AND HETEROZYGOTE FREQUENCY OF THREE INTRAGENIC RFLPs IN 32 FEMALES AT RISK OF CARRYING THE HAEMOPHILIA B GENE



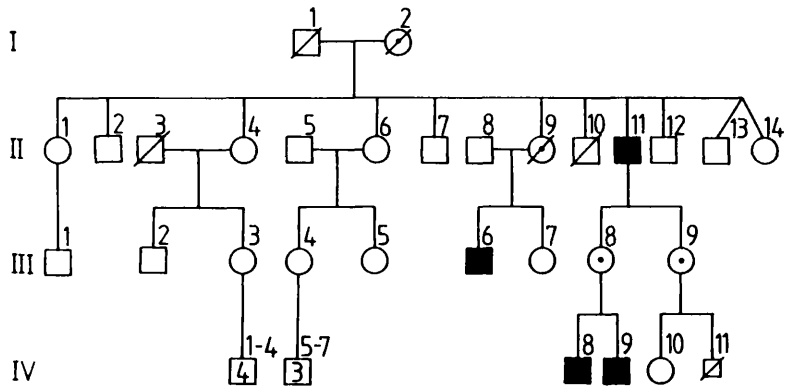
Pedigree 5292



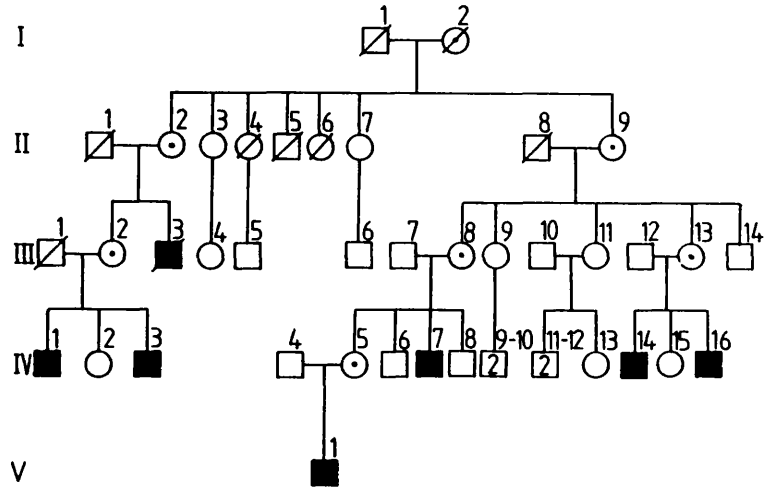
Pedigree 4569



Pedigree 3569

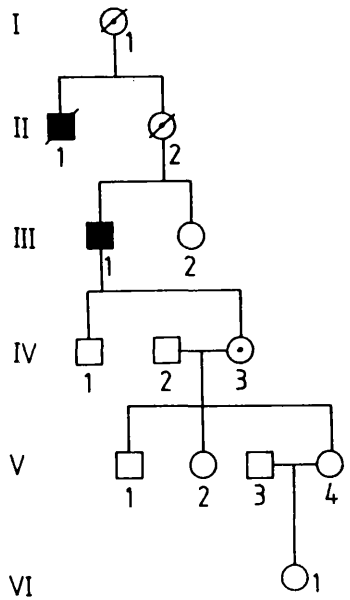


Pedigree 1756

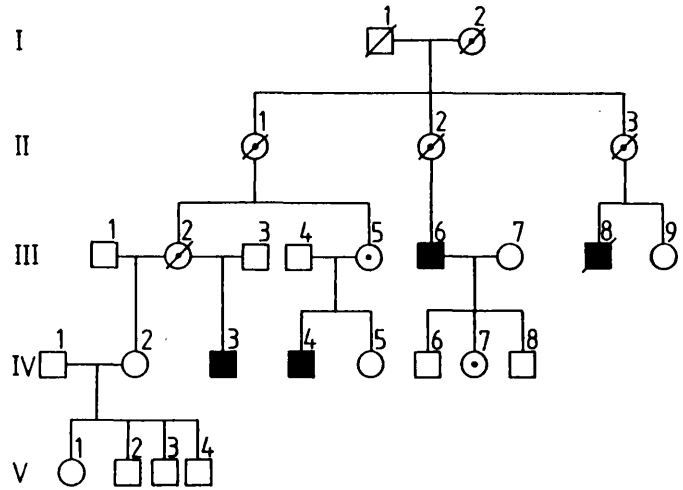


Pedigree 2377

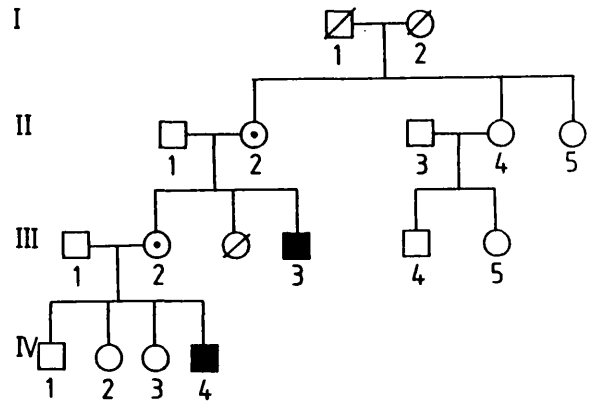
Figure 12 - Pedigrees of Haemophilia B Families



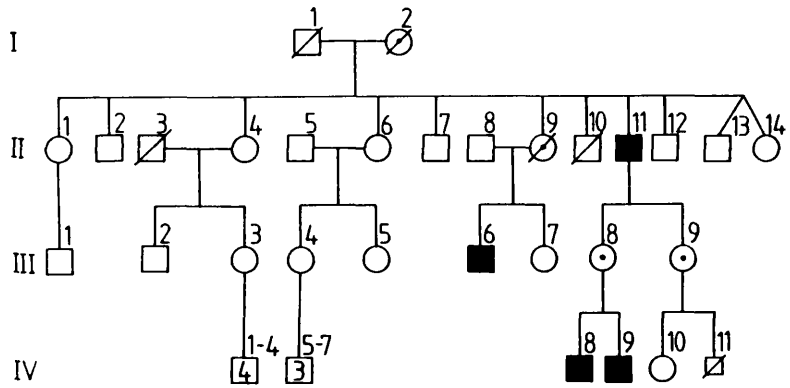
Pedigree 5292



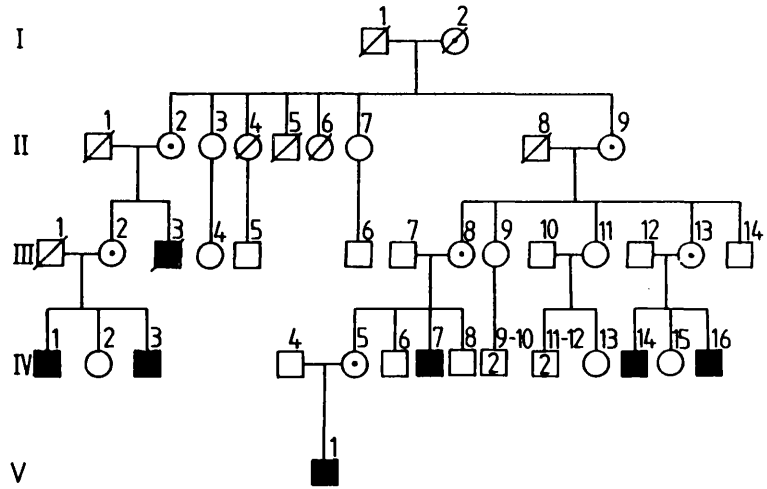
Pedigree 4569



Pedigree 3569



Pedigree 1756

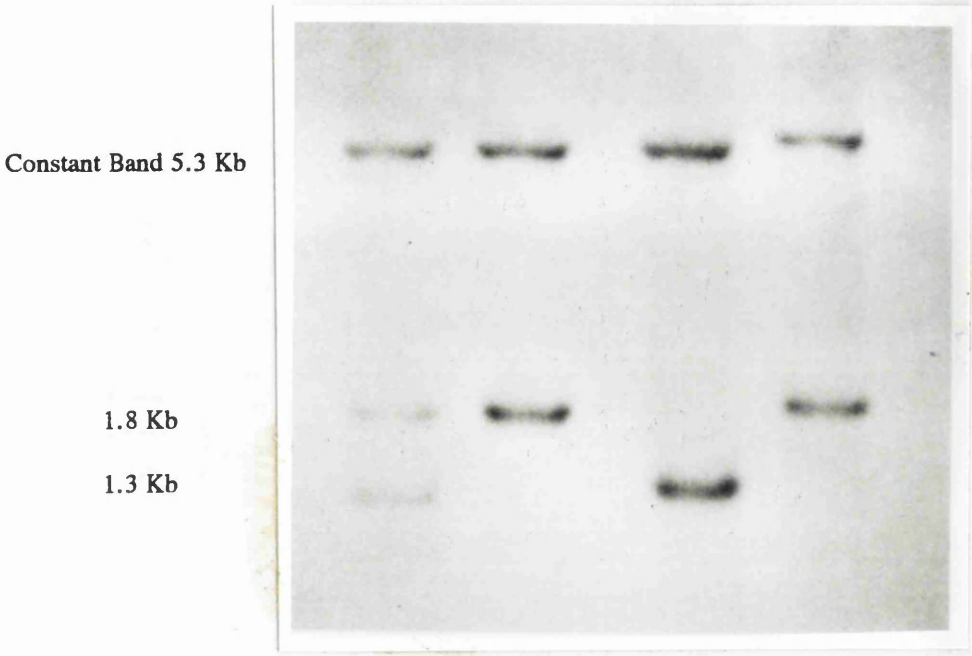


Pedigree 2377

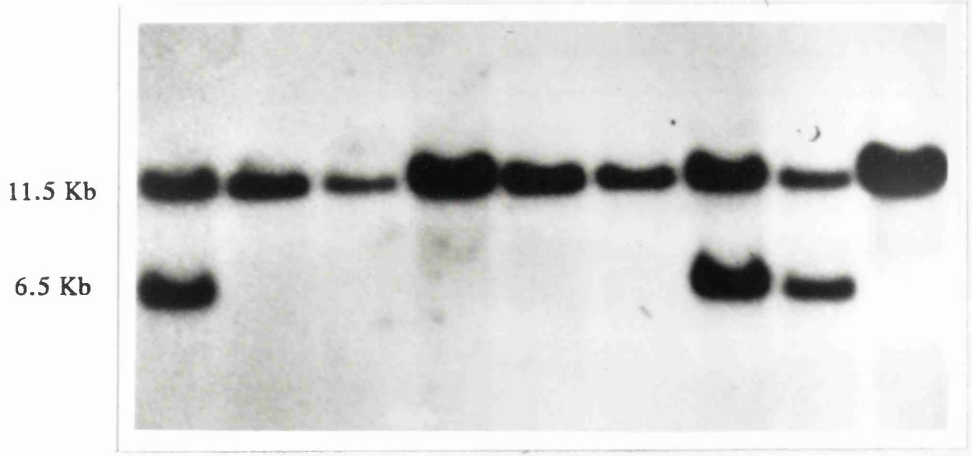
Figure 12 - Pedigrees of Haemophilia B Families

Figure 13

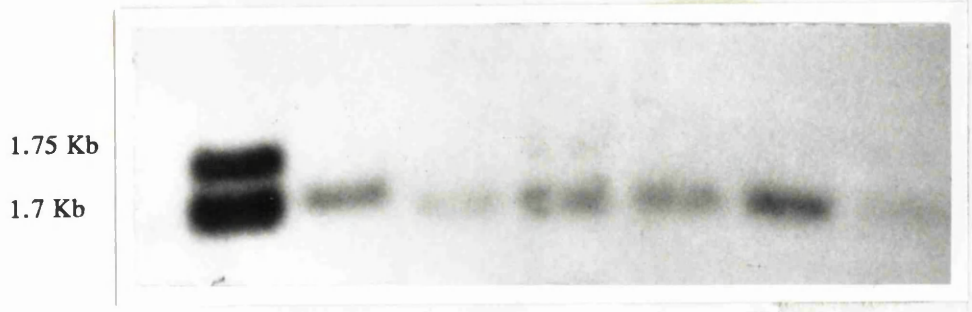
Three RFLPs used to investigate Haemophilia B



F9(VIII)/Taq I



F9(VIII)/Xmn I



F9(XIII)/Dde I

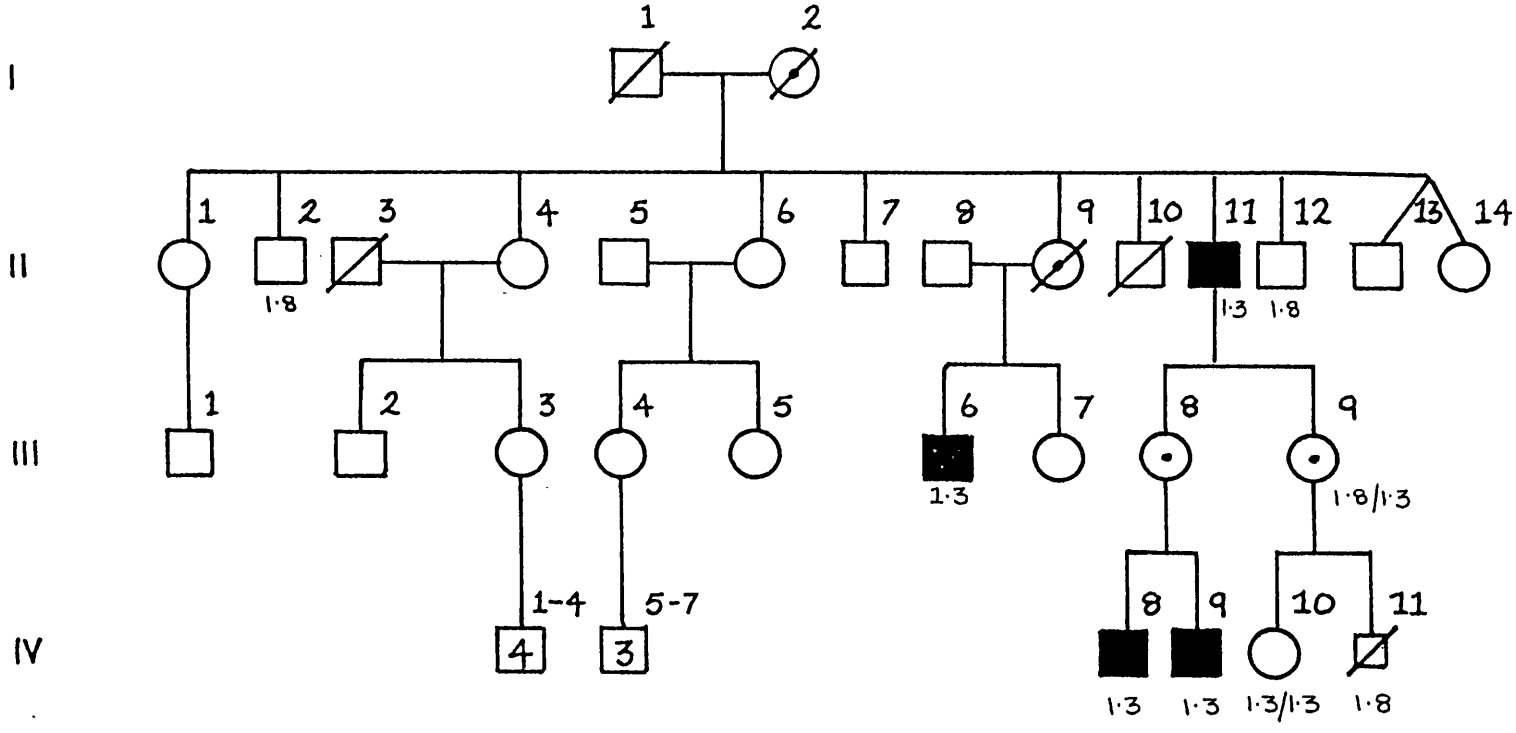


Figure 14

Prenatal Diagnosis - Pedigree 1756 Haemophilia B

Results of probe F9 (VIII)/ TaqI RFLP show that IV_{11} does not carry allele linked with Haemophilia B.

IV.3 VON WILLEBRAND DISEASE

IV.3i Individuals Tested

Thirty eight individuals from eleven different families were studied. These represented all the individuals registered at the Regional Haemophilia Centre who were affected by von Willebrand disease, had more than one individual member in their family affected and were willing to participate in the study. Of these eleven families, six families provided DNA samples from only two members and a further two families from three members. The pedigrees of these families are illustrated in figure 15. The complete DNA results obtained and all haematological results are presented in appendix III.

IV.3ii Allele Frequency

The RFLP used in this study is shown in Fig 16. Examining twenty two unrelated individuals the allele frequency was 0.64 for the 9kb allele and 0.36 for the 7.4kb allele. In this study twenty of thirty eight individuals were heterozygous. In eight families studied the disease was associated with the 9kb allele in six and the 7.4kb allele in two.

IV.3iii Inheritance

In 10 families autosomal dominant inheritance was apparent and in the informative meioses in families 7512 and 7517 there was co-segregation of the disease trait and the vWF polymorphism. However, in all other families the results were not informative and could not have been used in a predictive fashion for any future pregnancy.

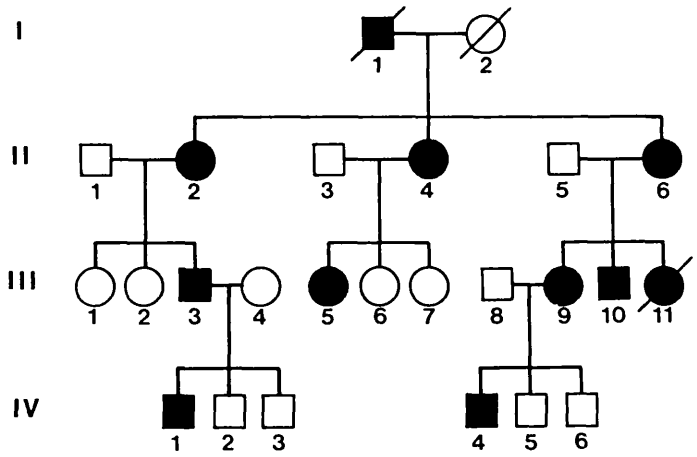
IV.3iv A New RFLP

There was no evidence of gross gene rearrangements or deletions in affected individuals from these families but four individuals from three families showed replacement of one 13kb invariant band by a fragment of 15kb (I2 pedigree 7514, II5 pedigree 7514, II4 pedigree 7516, III6 pedigree 7522, Fig 17). This is a further RFLP using the same probe and enzyme combination which will give additional information in informative families.

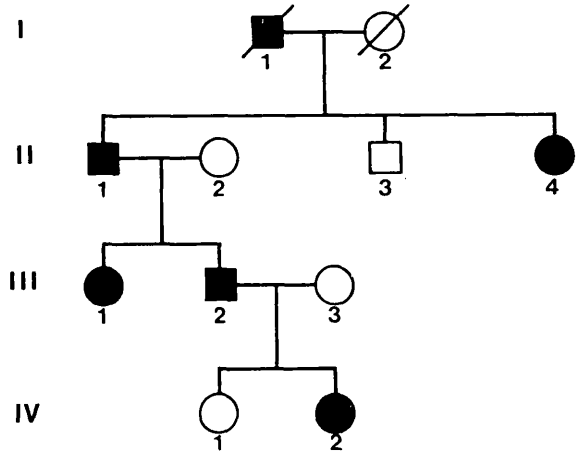
IV.3v Severe vWD

In one family autosomal recessive inheritance was suspected. The parents are Asian first cousins. The mother has no symptoms nor any relatives with coagulation problems, the

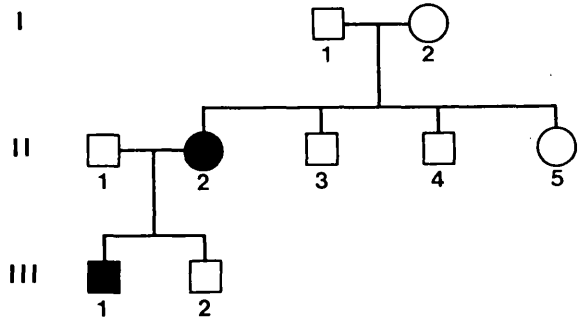
father has symptoms of mild von Willebrand disease (pedigree 7517, Fig 15). Their eldest daughter II1, has severe vWD with repeated haemorrhagic problems. A younger daughter and a son are asymptomatic. However as seen from the results in the appendix both parents and the asymptomatic daughter had haematological evidence of mild vWD. Fig 18 shows the results of DNA analysis in this family. Both parents are heterozygous for this RFLP (9kb/7.4kb). The severely affected child is homozygous for the 9kb fragment, the mildly affected child is heterozygous and the normal child is homozygous for the 7.4kb fragment.



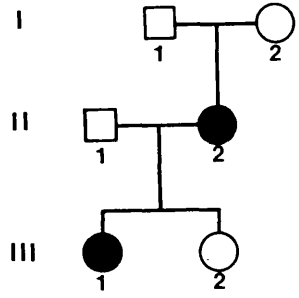
Pedigree 7512



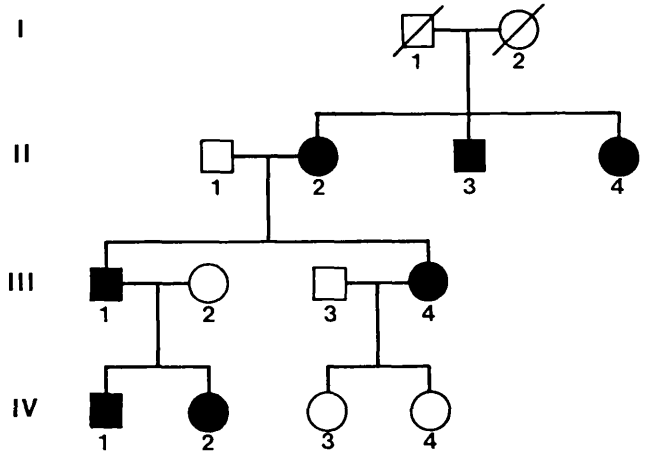
Pedigree 7513



Pedigree 7514

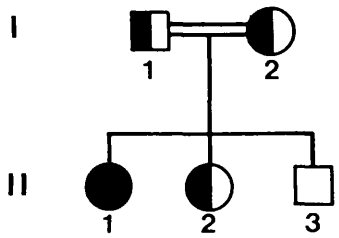


Pedigree 7515

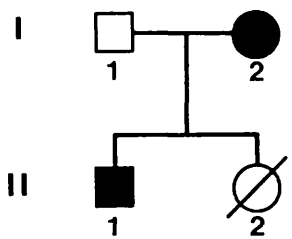


Pedigree 7516

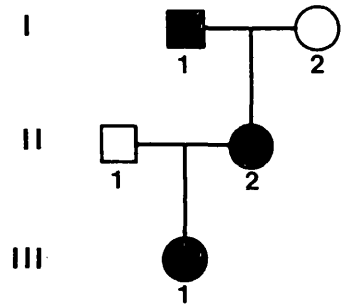
Figure 15 -Pedigrees of Families with Von Willebrand Disease



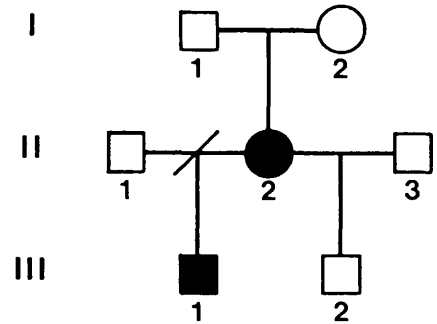
Pedigree 7517



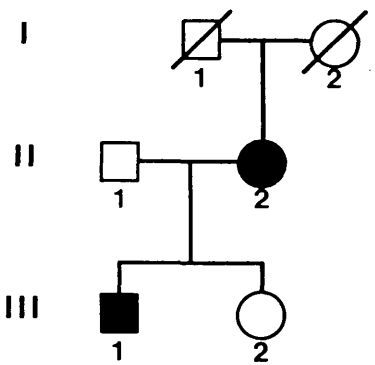
Pedigree 7518



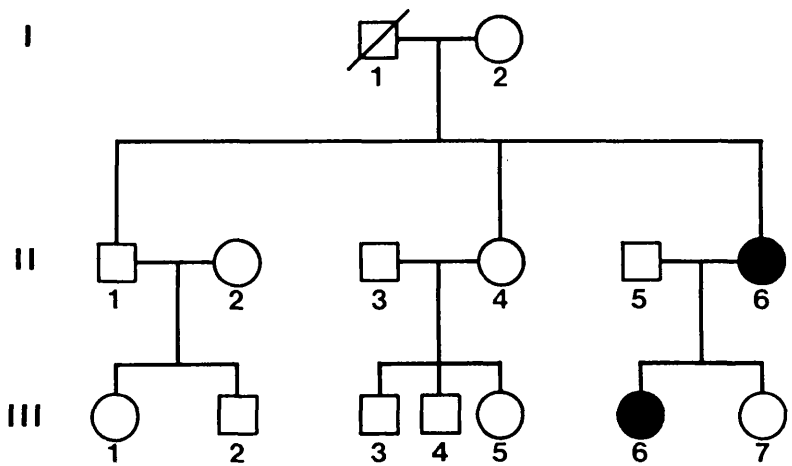
Pedigree 7519



Pedigree 7520



Pedigree 7521

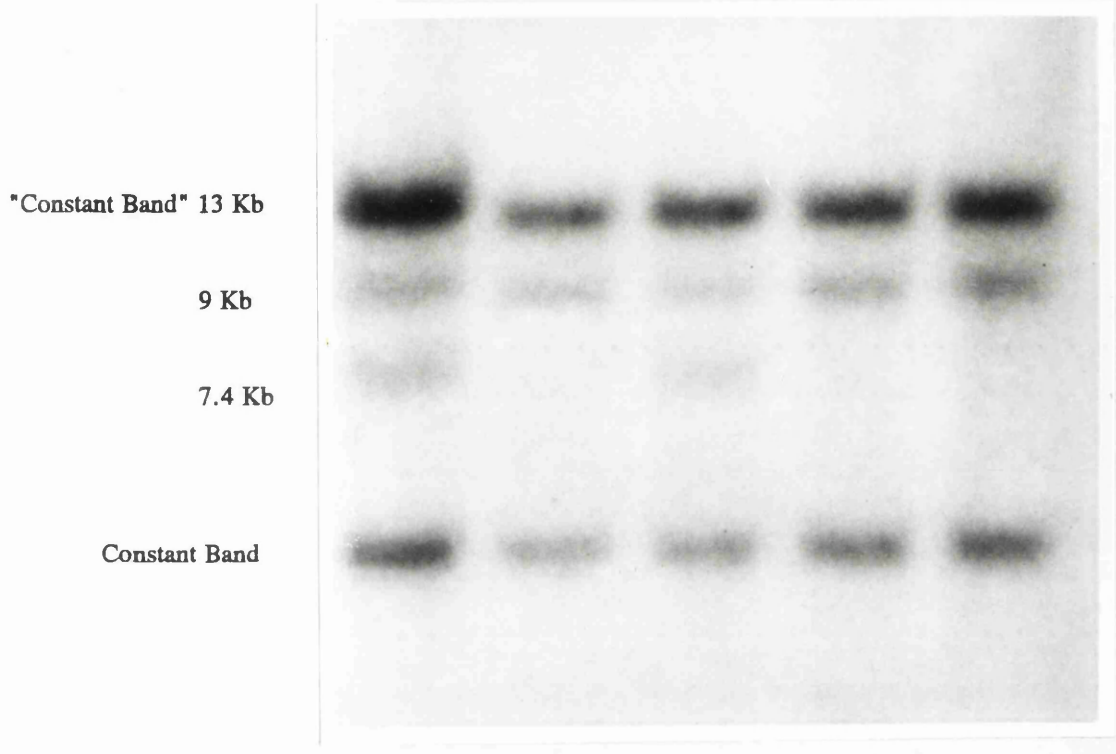


Pedigree 7522

Figure 15 -Pedigrees of Families with Von Willebrand Disease

Figure 16

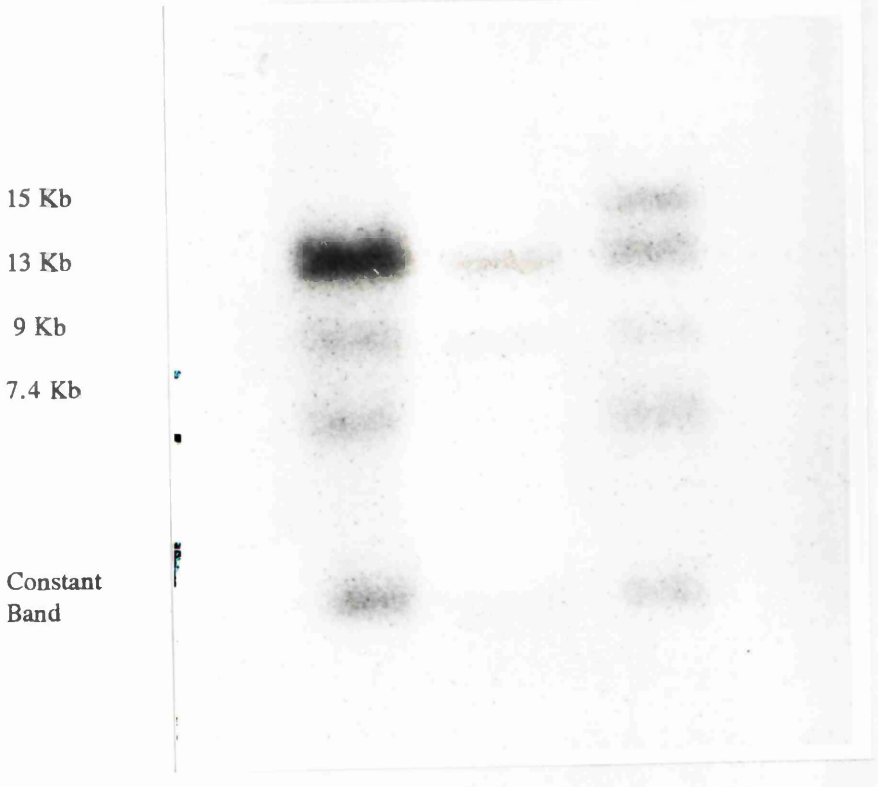
RFLP used to investigate von Willebrands disease.



vWF/Bgl II

Figure 17

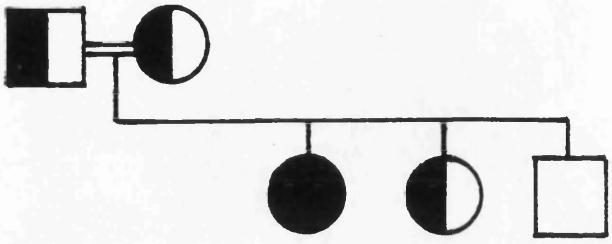
A new RFLP with vWF/Bgl II. The 13 Kb Band thought to be constant is half the usual density in individuals where there is a 15 Kb Band.

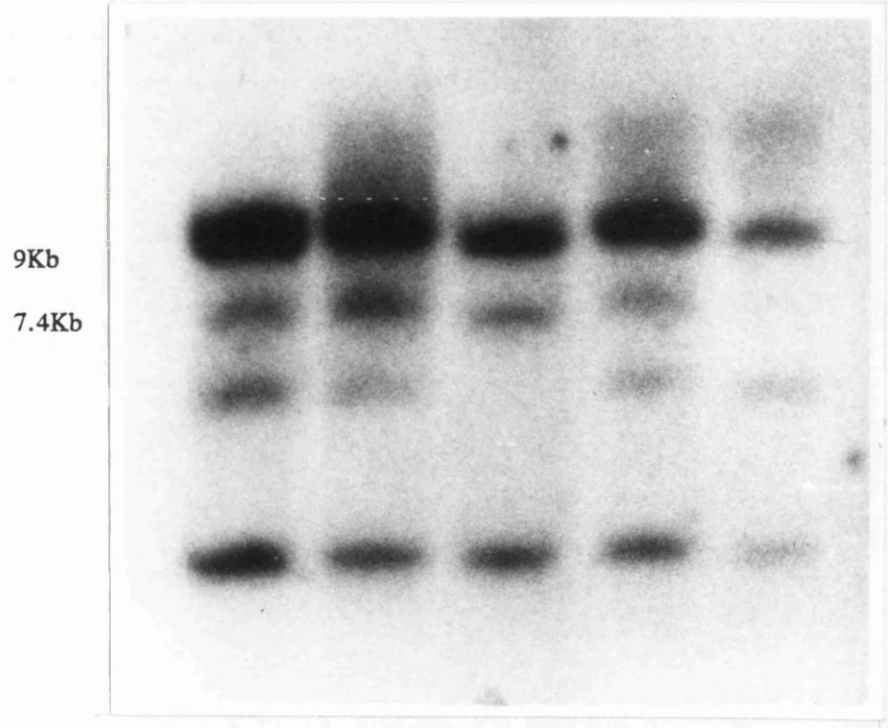


vWF/Bgl II

Figure 18

Pedigree 7517. Severe vWD in II is linked with the 9Kb allele inherited from both parents in a consanguinous marriage. II, who has mild vWD has one 9Kb allele and one 7.4 Kb allele.





vWF/Bgl II

CHAPTER V - DISCUSSION

V.1 HAEMOPHILIA A

V.1i Allele Frequency for RFLPs

The allele frequency for the three bi-allelic RFLPs, F8A, F8B and DX13, were measured in individuals from the West of Scotland unaffected by haemophilia A and in obligate carriers. The allele linked with haemophilia A was also assessed in the 16 families tested. Table 12 compares the result of this present study with the original description of the RFLP by Gitschier et al [1985A] and other more recent studies.

Gitschier et al found the allele frequency in 70 haemophiliacs to be 63% for the 0.9kb allele and 37% for the 1.2kb allele. The report does not make clear whether all haemophiliacs were unrelated. However this is a larger percentage of haemophiliacs having the 1.2kb allele than would be predicted from the results of studies in normal individuals. This is more pronounced in the present study where the allele frequency is 50% for each in 16 unrelated haemophiliacs. Thus both the initial report and the current study suggest that the 1.2kb allele is slightly more frequent in haemophiliacs than would be predicted from normal population studies. The calculated heterozygous frequency in the normal population in this study is 36.9% whereas the heterozygote frequency in 38 obligate carriers is 50%. This slight increase in linkage between the less common allele and the disease may make this particular RFLP even more useful than initially predicted.

Separating data concerning normal unrelated individuals and data from haemophiliac families in published series would help to identify whether there is a difference between the two groups.

The initial description of the association between the probe DX13 and haemophilia A [Harper et al, 1984] reported that 17 of 35 normal women tested were heterozygous for the two alleles, 9 were homozygous for the 5.8kb band and 9 homozygous for the 2.8kb band. Thus the allele frequency for each band was 50%. In this study, the 2.8kb allele was more common. 62.5% of 48X chromosomes tested shared the 2.8kb allele. This was reflected in the 16 families tested for linkage with haemophilia where the 2.8kb fragment was linked with haemophilia in 12 of the 16 families tested. Brocker-Vriends et al [1987] report a frequency of 50% for each allele in 80X chromosomes tested. Moodie et al [1988] found that 46% of 108 individuals from haemophiliac families were heterozygous but they do not give an allele frequency.

	PRESENT STUDY	GITSCHIER et al 1985A	BROCKER-VRIENDS et al 1987	PECORARA et al 1987	MOODIE et al 1988
No of X chromosomes tested	45	63	88	153	139
No with 0.9kb allele	34	-	-	-	99
% with 0.9kb allele	75.6	79	80	65	71
No with 1.2kb allele	11	-	-	-	40
% with 1.2kb allele	24.4	21	20	35	29
Comments	Non-Related Non Haemo-philiac Individuals	Non Haemo-philiac Families	?Related ?Haemo-philiac	Individuals from Haemophilia Families ?Related	Non-Related Individuals from Haemophilia Families

TABLE 12 - COMPARISON OF ALLELE FREQUENCIES OF F8A IN PRESENT STUDY COMPARED WITH PUBLISHED SERIES

	PRESENT STUDY	OBERLE et al 1985A	JANCO et al 1987	PECORARA et al 1987	MOODIE et al 1988
No of Individuals Tested	35	?	106	23	52
Heterozygote Frequency (%)	83	78	97	82	83
Comment	Obligate Carriers of Haemophilia A	Normal Individuals Families with Various X-linked Diseases	Obligate Carriers of Haemophilia A + Normal Women	Obligate Carriers of Haemophilia A	Non related Individuals

TABLE 13 - HETEROZYGOTE FREQUENCY FOR St14/Taq I RFLP IN PUBLISHED SERIES COMPARED TO PRESENT STUDY

Only 13 of the families in this present study were tested for linkage between the polymorphism and haemophilia A using probe F8B. 11 of the 13 families showed linkage with the 5kb RFLP and 2 showed linkage with the 20kb fragment. The calculated heterozygous frequency in the normal population was 31%. Antonarakis et al [1985A] first reported this RFLP and noted that the polymorphic Bg1 I site was present in differing frequencies in different ethnic groups. In Mediterraneans, 90% had the Bg1 I site giving an estimated heterozygote frequency of 18%, in Asian Indians the heterozygote frequency is less with 94% of the X chromosomes tested having the polymorphic site but in American blacks only 74% of X chromosomes had the polymorphic site giving a heterozygous frequency of 38%. The study by Moodie et al [1988] reports a heterozygous frequency of 25% in 139 unrelated X chromosomes or 31% in 115 women being investigated as members of haemophiliac kindred. The figures would suggest that the probe may be slightly more useful in Britain than in the USA.

The St14 probe reveals a polymorphic system with at least 10 allelic fragments when digested with the restriction enzyme Taq I. The initial report of this polymorphism [Oberle et al, 1985A] stated that the calculated heterozygote frequency was 78% and this is very similar to the heterozygote frequency of 83% reported here for 35 obligate carriers tested with St14 (Table 13). The initial report of the St14 probe described three separate polymorphisms with the enzyme MspI and reported a heterozygous frequency of 70% for all three. Since that initial report, there has been little further work reported using these RFLPs. In the current study, the women tested with the MspI RFLP were all non informative. However these women only came from two families and were already shown to be non informative with the St14 Taq I polymorphism. There is likely to be strong linkage disequilibrium between these two RFLPs.

V.1ii Deletions and Insertions

40 males with haemophilia A were tested. None was shown to have deletions at the site of intragenic probes. Allele sizes were as reported and there was nothing to suggest either DNA deletion or insertion as the cause of their haemophilia.

Initially, it was expected that, similar to the findings in haemophilia B, patients with an inhibitor to factor VIII would be likely to have a demonstrable deletion of the FVIII gene [Giannelli, 1983]. However, in 507 patients investigated, 19 different deletions have been identified but only 7 of these patients had a factor VIII inhibitor. One patient who apparently had deletion of the entire factor VIII gene did not develop antibodies. All deletions were associated with severe haemophilia apart from one reported case of a

5.5kb deletion in exon 22 where the patient had moderately severe haemophilia [Antonarakis et al, 1987].

Two unrelated patients have been described with insertions in exon 14 of the factor VIII gene. Both patients had severe haemophilia. These insertions appear to be what are known as L1 sequences which contain DNA which may code for a reverse transcriptase allowing the entire L1 sequence to be inserted into DNA elsewhere in the genome via an RNA intermediary [Kazazian et al, 1988].

Antonarakis et al [1987] point out that 4-5% of families tested will show a detectable deletion if probed with the whole factor VIII gene and recommend that this be done routinely when investigating for carrier detection or pre-natal diagnosis. It is unlikely that this will be done routinely in all families, however it is another approach to investigation which is available and may be particularly useful if RFLPs are not informative, if paternal DNA is unavailable or if pre-natal diagnosis would otherwise depend upon extragenic probes.

V.1iii Carrier Detection

The present study found that 51/71 females (72%) at risk of being a carrier for haemophilia A could be advised about carrier status on the basis of information obtained from at least one of the four RFLPs used. Only 41% could be advised from information obtained from intragenic probes and the rest relied upon extragenic probes.

The initial reports of the four probes all described the application of the probes to carrier detection although these reports often dealt with small numbers. Gitschier et al [1985A] mentioned carrier detection in only one female, Antonarakis et al [1985A] in five out of six families informative for either the Bcl I or the Bg1 I polymorphism although this paper gave no results for the carrier studies. Winter et al [1985] reported results of carrier detection in nine women from six families whilst Oberle et al [1985B] reported carrier detection in three women at risk of being carriers.

Subsequent reports examined larger groups of women. Early in 1986, a report appeared from Janco et al investigating families with the same four probes used in this study. They looked at "over 34" obligate carriers in 29 families. The heterozygous frequencies were similar to the present study with 92% heterozygous for St14, 37% with F8B, 35% DX13 and 65% F8A. They also looked at DNA probe combinations and in 24 obligate carriers studied there was a 100% heterozygote frequency when a combination of St14 and F8A were used. This fell to 68% when the combination was F8B and F8A. They

were also able to assign carrier status in seven female relatives but did not say how large the group of females at risk was. The conclusion of this report was that the most efficient strategy for carrier testing would be to use both St14 and F8A probes to identify heterozygosity.

The theme of identifying an efficient carrier strategy was picked up in a short report [Intrator et al, 1987] from France. In 23 families only 40% of obligate carriers were informative for F8A and 26% were informative for F8B. However there was strong linkage disequilibrium between the two probes such that there was no additional information generated by the use of F8B. A much larger study from Italy reported at the same time [Pecorara et al, 1987] investigated 61 haemophilia A families. 350 relatives as well as 61 haemophiliacs were investigated using three gene probes F8A, St14 and DX13. In obligate carriers, 30% were informative for F8A, 82% St14 and 60% DX13. Laboratory results for St14 and F8A showed 91% of obligate carriers to be heterozygous. When the three probes were combined, 23 of 24 (96%) of obligate carriers were informative. When investigating carrier detection, the families were divided into 27 families with at least two haemophiliacs in different sibships "the inherited cases" and 34 families with one or more haemophiliacs in only one sibship "the sporadic cases". Whilst this division may not be absolutely correct, it reflects the pragmatic approach which is likely to be taken when haemophiliacs and their families present to a genetics out-patient clinic. In the inherited group studied, there were 29 women at risk and all could be assigned carrier or non-carrier status from these studies. In the sporadic cases, 45 women were investigated. 6% of these families were non-informative. The studies of sporadic cases also depended upon abnormal maternal clotting studies to classify the mother as a carrier before DNA studies could be used to establish the carrier status of the daughter. It is interesting to note that in this large Italian study, the haemophiliac families appeared to be less informative with the F8A probe than in previous studies.

Brocker-Vriends et al [1987] reported a high carrier assignment rate. 52/57 women tested could be informed of their carrier status. This did not state how many women depended upon data from extragenic probes alone. A more recent report of carrier assignment in 100 potential carriers [Moodie et al, 1988] reported a 73% carrier assignment rate which is very similar to the present study. 21% of carriers depended upon data from extragenic probes.

The clinical usefulness of data relying upon extragenic probes alone has come to be questioned as it has become apparent that there is a significant crossover frequency between the extragenic RFLPs and the haemophilia A gene.

V.1iv Genetic Crossover

In this study, no evidence was found of genetic cross-over between the disease trait and the two intragenic probes, nor between the gene for haemophilia A and DX13. However the recombination rate between St14 and haemophilia A in this study was 10.7%.

At the time that extragenic or linked probes for haemophilia A were first described, it was recognised that there was at least a theoretical possibility of crossover between the polymorphic probe site and the gene for haemophilia A. The original description of DX13 [Harper et al, 1984] observed no cross-over however. Winter et al [1985] described recombination between DX13 and F8A in one woman and calculated that the recombination fraction was 5%. Peake et al [1985] quoted a recombination fraction of 8% for the same probes. Initial reports of investigations with St14 suggested that recombination was rare. Oberle et al [1985A] found no recombinants in 57 meioses between the St14 locus and the gene for factor VIII and the initial prediction by Janco et al [1986] was that it would be less than 1.5%. Peake et al [1986] estimated that it was 4.5%. However at the same time, a group from Finland reported two recombinations in 27 meioses [Lehesjoki et al, 1986] and a further recombination was reported by Driscoll et al [1986] suggesting that the recombination rate was higher than initially predicted. One of the families investigated by Lehesjoki showed recombination between St14 and FVIII and also between DX13 and FVIII suggesting that St14 and DX13 both lay on the same side of FVIII rather than flanking the haemophilia A gene. Brocker-Vriends et al [1987] also reported one recombination of St14 and haemophilia A which also showed a recombination between the DX13 and the haemophilia A locus - again supporting the hypothesis that the two probes are juxtaposed on one side of the haemophilia A gene. The recombination frequency for the two probes reported by this group was 3.9% between St14 and haemophilia A and 5% between DX13 and haemophilia A.

The position of these two probes was of some importance since flanking probes would have been more useful. Recombination would be less likely to result in recombination of both probes if they were flanking the gene of interest rather than juxtaposed on one side. However for a long time, the order of the probes in relationship to the genes at the tip of the X chromosome was disputed. Using somatic cell hybrids, Tantravahi et al [1986] placed DXS15 (DX13) and DXS52 (St14) distal to F8 giving the order centromere - F9-F8 (DXS15, DXS52) - telomere. However data from Mulligan et al [1987A] disputes this and suggests the most probable order as centromere - fra(X) - DXS15 - DXS52 - F8 - telamere. Mulligan et al suggest from their data that the recombination rate is between 3 and 5% between St14 and FVIII. This is considerably less than the present study where three recombinations were observed in 28 informative phase known meioses (10.7%)

between St14 and F8C. Clearly this is affected by the fact that two of the recombinants were in one family. It has been suggested that some families may be more predisposed to genetic recombinations in this area and until these studies are performed within each regional population, it will not be possible to say how variable the recombination rate may be. This information is important to genetic counsellors. If there does prove to be a regional or inter family variation in the recombination rate then the present system of collecting national and international data may produce misleading guidelines.

It is estimated that the recombination frequency of 1% is thought to average about 1000 kilobases of DNA [Graham et al, 1985]. Thus even a very long gene such as that coding for factor VIII which is 186kb in length is very unlikely to show recombination between the site for haemophilia A and the sequences detected by an intragenic RFLP.

V.1v Coagulation Testing

As discussed in the introduction to this study, coagulation tests have been the most important method of identifying carriers amongst women at risk prior to the introduction of recombinant DNA technology. They will continue to be important in centres where DNA probes are not available in families not informative for available probes, in mid trimester foetal blood sampling or in families where information is only available for extragenic probes. The effect of lyonisation has been known to render interpretation of results difficult since coagulation tests for potential carriers were first described [Lyon, 1962]. It is not tenable that lyonisation will cause abnormal coagulation in a normal female however the reverse situation is widely recognised and accepted as a common cause of mis-classification of females at risk. In this study, 24 women at risk were tested with coagulation studies and DNA techniques. The coagulation studies were not optimum as many women only had them done on one occasion. The current recommendations are that these are repeated at different times to elicit day to day variability in results and maximise the detection rate for carriers of haemophilia A [Rapaport et al, 1960]. Nevertheless, the results are valid as they stand although repeating tests may have improved the carrier detection rate.

13 of these women were assigned non-carrier status from the DNA probe results although only three of these were assigned from data obtained from intragenic probes. In all 13 non-carriers, the coagulation data supported the results of the DNA studies and using the chart illustrated in the paper from Prentice et al [1975] the odds were against these women being carriers. In this group of women, particularly when relying upon extragenic probes alone, coagulation data may be used to support the DNA studies and vice versa. Since lyonisation in a non-carrier will not produce abnormal coagulation, an

abnormal coagulation result which fell within the area suggesting carrier status in a woman where results of extragenic probe suggested non-carrier status would cause one to look very hard for recombination, mixed samples, non paternity or any other cause of error in DNA techniques. It would therefore seem prudent in this situation to continue to use both techniques.

11 women were assigned as carriers using DNA probes. In 8 women, the data was obtained from intragenic probes which is regarded as highly reliable. In 3 of these women where data is believed to be highly reliable, coagulation studies produced odds of carrier status of 1:16. This figure is derived from an illustration in the study of Prentice et al [1975] and 1:16 are the highest possible odds. The 3 women who were assigned as carriers using extragenic probes were assigned as non-carriers by coagulation testing in 2 cases. 1 had odds of 1:16 whereas the other was only 1:3. This data highlights two possible problems. In women assigned carrier status with intragenic probes where the coagulation data conflicts with this, one would be inclined to put more reliance on the DNA studies. One would, however, have to exclude non paternity as a cause of unreliable data and also simple laboratory error such as mislabelling of samples. The second area can be checked easily by repeating the test and paternity testing has become simpler with the introduction of hypervariable mini satellite probes [Jeffreys et al, 1985]. The difficulty increases in women assigned as carriers with extragenic probes where coagulation testing suggests non-carrier status. Simple measures such as repeating tests or excluding the possibility of non-paternity may decrease the number of women where this conflict exists but is unlikely to remove the problem. Further studies among female relatives may detect coagulation abnormalities associated with the same extragenic allele but this would not exclude cross-over between the disease locus and the gene for factor VIII:C in the women being tested.

V.1vi Prenatal Diagnosis

During the time of this study, only one woman requested early prenatal diagnosis by chorionic villus sampling as she did not wish to have a haemophiliac child. (Another patient, discussed in the Haemophilia B section had mid trimester diagnosis). The woman in this family (Pedigree 7319) had one haemophiliac brother and was an obligate carrier as she had already had a fetus diagnosed as haemophiliac in mid trimester by foetal blood sampling. This resulted in a mid trimester termination of pregnancy which the family were anxious to avoid on this occasion. The woman presented as a new referral to the Genetics Institute at eight weeks gestation which meant that chorionic villus sampling and DNA analysis had to be arranged rapidly. Whilst this particular family were both highly motivated and well informed prior to referral nonetheless some

very rapid decisions had to be made at a very emotional time for parents. By using RFLPs to detect carriers prior to pregnancy it would be hoped that they would have discussed prenatal diagnosis prior to pregnancy. That the family preferred to have early trimester diagnosis and termination if necessary rather than mid trimester did not seem surprising and indeed the assumption that women would prefer early diagnosis and termination if this is necessary has been supported by a recent study [Spencer and Cox, 1987]. Nonetheless the risk to the pregnancy is now accepted at 2-4% [Ward, 1987] which is probably twice the risk of amniocentesis even when the inevitable losses up to 16 weeks are excluded. Transcervical chorionic villus sampling is more likely to result in infection or bleeding than the transabdominal route. Both may cause Rhesus sensitisation [Jackson and Wapner, 1987]. A recent randomised study comparing transcervical chorionic villus sampling (CVS) with amniocentesis has shown a higher perinatal mortality in the CVS group with crude perinatal mortality rates of 1.3% and 0.6% respectively [Canadian Collaborative CVS-Amniocentesis Clinical Trial Group, 1989]. There is also concern that maternal contamination may confuse results. In particular techniques using PCR to amplify DNA may cause more problems with maternal contamination [Craig et al, 1989].

V.1vii Linkage between G6PD and Factor VIII Genes

It is widely accepted that the G6PD gene and the gene for FVIII lie closely together and linkage can be sought when there is an abnormality detectable in both. McCurdy in 1971 reported carrier detection for haemophilia A using an electrophoretic heterogeneity of G6PD seen in American negroes. He also suggested that the same techniques could be used for prenatal diagnosis since determination of the G6PD phenotype is possible from about 10 weeks gestation. A recent report by Filippi et al [1984] brought to a total of 58 the number of scorable siblings and non-recombinants recorded in the literature for the linkage Haemophilia A - G6PD.

In the present study, one family (Pedigree 7409) had two haemophiliac sons who were also G6PD deficient. Their sister was assigned a carrier from results obtained using the RFLP detected by St14 and was also shown to be heterozygous for G6PD deficiency. In this family, linkage is maintained between haemophilia A, G6PD deficiency and St14. It is useful to have further confirmatory evidence of carrier status when using extragenic probes. At present, the ordering of these probes in relationship to the two genes is uncertain. It would be particularly useful if St14 and G6PD flanked on either side of haemophilia A. The G6PD gene has also been sequenced [Martini et al, 1986] and it may be that intragenic RFLPs from that gene may be as useful as St14 or DX13 in carrier detection for haemophilia A.

V.1viii Linkage between Colour Blindness and Factor VIII Genes

Linkage between the genes for red/green colour blindness and haemophilia A has been long established [Haldane and Smith, 1947]. When both diseases occur in the same family, it is occasionally possible to use colour blindness as a linked marker for carrier detection in the same way as extragenic RFLPs are used although it is associated with the same problem of the possibility of genetic cross-over.

There are two genes involved in red/green colour blindness, lying adjacent to each other on the X chromosome. One codes for red pigment and the other for green pigment. They each consist of six exons but have 96% homology with each other. It is thought that this extensive homology promotes misalignment of gene sequences resulting in unequal crossing over which may cause fusion gene products or gene duplication and deletion. About 8% of men have some form of red/green colour blindness [Lancet, 1990].

Kenwrick and Gitschier [1989] have produced a physical map of Xq28 using pulsed field gel electrophoresis and have shown that the genes for red/green colour blindness lie on the opposite side of the factor VIII gene to DX13 and St14. In the family presented in this thesis with both diseases, carrier detection for haemophilia A depended upon information from the extragenic probes DX13 and St14. Any complementary information obtained by following the gene for colour blindness through the family with no evidence of genetic cross over would enhance the information from the extragenic probes alone. In this family, the gene for colour blindness appeared to derive from I1. Consequently, all his daughters will be obligate carriers of this disorder. II8 is an obligate carrier of haemophilia A with an affected son III11 who is not colour blind. Since the colour blind gene and the haemophilia A gene lie on different X chromosomes in this individual, it might be possible to use colour blindness as a linked marker. Assuming that II7 (husband of II8) is not colour blind then, in the absence of genetic cross over, a daughter inheriting the gene for colour blindness will not be a carrier of haemophilia.

The low lod score calculated between colour blindness and DX13 or St14 reflects the fact that there were only a small number of individuals in one pedigree with appropriate data. However, since lod scores can be added between pedigrees, it is data worth accumulating as the genetic distance between colour blindness and DX13 or St14 can be estimated when further pedigrees are analysed.

V.2 HAEMOPHILIA B

V.2i Allele Frequency for RFLPs

The 3 RFLPs which were used were the only 3 available at the time that the study was undertaken. The allele frequencies obtained in this study are compared to those originally published and subsequent series and are shown in Table 14.

The allele frequencies of the 3 RFLPs in this study were strikingly similar to those reported by the authors who identified them [Giannelli et al, 1984; Winship et al, 1984] although it has been suggested that allele frequencies may vary between different white ethnic groups [Hassan et al, 1985]. The relative increase in the percentage of haemophilia B families in this study showing association with the more common Xmn I polymorphism is interesting to note (10 out of 11 families) and does not reflect the frequency of this allele in the normal Scottish population which was found to be 0.68. It is however consistent with the finding that only 24% of obligate carriers from these families were heterozygous for this particular RFLP. In these small numbers it can only be speculation whether this reflects a common ancestral pool although the other genotypic differences between families would suggest that this is not the case.

These were the 3 RFLPs used to investigate families in this study. Subsequent to this other RFLPs have been described. Camerino et al [1985] described an RFLP detected using a partial cDNA probe and the enzyme MspI. The frequency of the minor allele was 0.20 with an estimated heterozygous frequency of 0.32. The MspI RFLP was found to be in strong linkage disequilibrium with the Taq I RFLP, thus limiting its usefulness. On the other hand the Bam HI polymorphism described by Hay et al [1986] gave a low estimated heterozygous frequency of 11% but showed a lack of linkage disequilibrium between it and the Taq I and Dde I polymorphic loci. A second Bam HI polymorphism was subsequently identified and found to be within 500bp 5' of the Xmn polymorphic site. Interestingly this gave a minor allelic frequency of 0.13 in American blacks but no polymorphism was identified in an American white population [Driscoll et al, 1988]. Other authors have also identified differences in allelic frequencies for RFLPs in different racial groups emphasising the importance of identifying the useful RFLPs for different populations [Reiner et al, 1990; Kojima et al, 1987; Lubahn et al, 1987].

V.2ii Carrier Detection

The thirty obligate carriers and thirty two females at risk are identified separately in the results section although they had the same investigations. This is because the

	Present Study	Gianelli et al 1984	Winship et al 1984	Hassan et al 1985	Lillicrap et al 1986	Lehesjoki et al 1990
No of X chromosomes tested	46	60	-	67	57	37
F9(VIII)/Taq I						
1.8kb	0.63	0.65	-	0.64	0.74	0.76
1.3kb	0.37	0.35		0.36	0.26	0.24
No of X chromosomes tested	60	-	72	-	48	37
F9(VIII)/Xmn I						
11.5kb	0.68	-	0.71	-	0.75	0.81
6.5kb	0.32	-	0.29	-	0.25	0.19
No. of X chromosomes tested	30	-	74	-	-	34
F9(XIII)/Dde I						
1.75kb	0.23	-	0.24	-	-	0.29
1.7kb	0.77	-	0.76	-	-	0.71
COMMENT	Unrelated Normal Scottish Individuals	Normal White Subjects	Unrelated Normal Caucasian	Normal Subjects	South Wales Population	Unrelated Finnish Individuals

TABLE 14 - COMPARISON OF ALLELE FREQUENCIES IN HAEMOPHILIA B RFLPs BETWEEN PRESENT STUDY AND OTHER PUBLISHED SERIES

information generated has different applications. With obligate carriers the applicability of these techniques is to identify those with heterozygote RFLPs who could be offered early pre-natal diagnosis following chorionic villous biopsy whereas with women at risk carrier status may be identified by RFLP analysis. Those who are identified as carriers may subsequently request pre-natal diagnosis. The relatively high heterozygote frequency recorded amongst the thirty two women at risk is fortuitous (71% were heterozygous with the F9VIII/Taq I RFLP) and might imply that there would be an increased chance of offering PND. However only 8 of these 32 women were found to be carriers and only 2/8 of women identified as carriers were tested with all 3 RFLPs (both of whom were polymorphic with at least 1 RFLP) and so this cannot be stated categorically.

DNA analysis alone permitted definitive genetic counselling of 21 of 31 females at risk (68%). Using only results of Taq I RFLP then 15 of 31 females at risk (47%) could have been counselled. So adding two further RFLPs to the investigation panel has only increased the total informativeness by 21% despite the fact that in the normal population of this area the Xmn I RFLP has a heterozygous frequency of 43% and the Dde I RFLP has a heterozygote frequency of 35% whilst in the obligate carriers tested these figures were 24% and 44% respectively. The phenomenon whereby less additional information is generated by additional RFLPs than would be anticipated by the heterozygous frequency is known as linkage dysequilibrium. In general terms this can be explained as occurring when an allelic marker is found to be close enough to another gene to be co-inherited at greater than random frequency. In the case of RFLPs this means that the various alleles associated preferentially with one another in the general population to form a frequently occurring haplotype. Thus adding a second RFLP after testing with one is likely to be less useful than would be anticipated from the calculated individual polymorphic frequencies. Interestingly Driscoll et al [1988] reported that less linkage dysequilibrium was noted between Taq I and MspI polymorphic sites in North American Negroes compared to the North American white population. Racial differences make it important for each population to ascertain their own degree of linkage dysequilibrium.

Results of large series which have been published largely reflect the experience in Glasgow. Lehesjoki et al [1990] investigated 19 of the 20 known Finnish families with haemophilia B. The allele frequencies for the three RFLPs did not differ significiantly from the original studies. Assignment of carrier status could only be achieved in 19 of 39 females tested (49%). However this figure included female relatives of sporadic cases of haemophilia B.L If these were excluded then the figure became 16 of 25 or 64% which is very close to the figure reported in the present study of 68%. Brocker-Vriends et al [1985] using only the Taq I RFLP assigned carrier status in 16 out of 19 potential

carriers from 4 kindred (82%). Lillicrap et al [1986] using the Taq I and Xmn I RFLPs assigned carrier status in 5 out of 14 potential carriers (36%) from four informative kindred. With such small numbers of informative kindred it is not possible to state whether these differences reflect true differences in these populations.

V.2iii Gene Deletions

Amongst the 31 patients from 12 families tested with RFLPs there was no evidence of absent bands or alterations of fragment size to suggest gene deletion. Large gene deletions have been described. These are commonly, although not invariably, associated with patients who have circulating factor IX antibodies known as inhibitors. Giannelli et al [1983] investigated 6 individuals with factor IX antibodies, two of whom were related, to see whether there was an association between antibody production and gene deletion. 5 of the individuals had evidence of a deletion ranging from 9kb to greater than 18kb. Interestingly two patients from the same kindred had evidence of different deletions. Since then further isolated individuals with gene deletions have been reported [Bernardi et al, 1985; Hassan et al, 1985; Peake et al, 1984] including one family investigated with three children who had haemophilia B with no evidence of inhibitor. They were found to have a relatively small deletion of 2.8kb around exon d and the surrounding flanking regions. This affected the Taq I polymorphic site and was an unexpected finding when pre-natal diagnosis was requested [Vidaud et al, 1986].

V.2iv Prenatal Diagnosis

During the course of this study only 1 woman (Ped 1756 III8) requested pre-natal diagnosis. This was a double diagnostic investigation since the woman was an obligate carrier for Haemophilia B and also had a 1 in 4 chance of having a child with cystic fibrosis. At that time in 1986 RFLPs were not available for the diagnosis of cystic fibrosis and so the diagnosis of CF was reached by a combination of amniotic fluid biochemistry and ultrasonography. CF was confirmed at necropsy. Cells obtained at amniocentesis were cultured to provide sufficient material for DNA extraction and, using the Taq I/F9(VIII) polymorphism, a male foetus without haemophilia B was predicted. Had it not been that at that time CF could only be reasonably predicted following amniocentesis then chorionic villous biopsy would have been used to obtain material for DNA extraction.

Compared to the situation with Haemophilia A the alternative method of PND by factor IX assay of fetal blood is less straightforward. In common with other Vitamin K dependent clotting factors the level of Factor IX remains low until the end of the

gestational period and into the perinatal period. This fact makes the interpretation of very low levels of factor IX obtained in mid-trimester more difficult than is the case with factor VIII [Mibashan et al, 1979]. Moreover a significant level of Factor IX is present in amniotic fluid. Any contamination of a fetal blood sample may thus falsely elevate a low level of factor IX [Thompson, 1984]. More individuals with haemophilia B have disproportionately high levels of factor IX antigen than is the case with haemophilia A. In one study over half of the haemophilia B patients had at least 10u/dl circulating factor IX antigen [Thompson, 1982].

V.3 VON WILLEBRANDS DISEASE

V.3i Allele Frequency and Linkage

The probe used in this study is an 1100bp Pst I fragment of cDNA isolated by Verweij et al [1985B]. At the time of this study, this was the only probe available to identify RFLPs. The allele frequencies of 0.64 and 0.36 found in this study corresponds closely to the original reported frequency of 0.69kb for the 9kb fragment and 0.31 for the 7.4kb fragment. A heterozygous individual may not be informative when the disease in question is autosomal dominant. Depending upon the results of the individuals partner linkage may be difficult to establish when there is limited family data and only one RFLP. The sequencing of the cDNA has enabled partial length cDNA probes from the entire length of the vWF gene to be developed. These can be used either to detect gene deletions and insertions or to recognise RFLPs. Many RFLPs have now been described [Verweij et al, 1985B; Nishino et al, 1986; Quadt et al, 1986; Bernardi et al, 1987; Iannuzzi et al, 1987; Konkle et al, 1987; Lavergne et al, 1987; Lavergne et al, 1988; Lindstedt et al, 1989A; Marchetti et al, 1989; Ewerhardt et al, 1989; Lindstedt et al, 1989B; Inbal et al, 1989]. The clinical applicability of some is very limited because of a low polymorphism frequency. Also there is strong linkage disequilibrium between many of the RFLPs making the cumulative benefit of several RFLPs less than might be anticipated.

The fact that linkage is established in this study as well as other studies supports the observation of Verweij et al, [1988], that vWD is likely to be due to a variety of intragenic defects rather than an abnormality of post translational processing.

The observation that the disease is linked to either RFLP allele in the anticipated ratios shows that neither allele is particularly linked with the disease. Also there is no evidence that all families tested have a common ancestry.

V.3ii Carrier Detection

In only two out of ten families with autosomal dominant von Willebrands disease were there informative meioses allowing the RFLP allele linked with the disease trait to be identified. The pedigrees in this study however were small. Carrier detection is a misleading term in autosomal dominant diseases where the individual with the disease trait manifests symptoms. Normally affected individuals would be identified by coagulation tests although RFLP analysis would be possible in informative families. Bernardi et al [1987] used four different RFLPs to investigate vWD and estimated that

there was a 70% chance that a subject would be informative for at least one. Combining two RFLPs in a large family with type IIa vWD increased the informativeness of the analysis despite linkage disequilibrium between the two RFLPs [Verweij et al, 1988]. Bahnak et al [1988] also demonstrated how combined data from two RFLPs was important in a large family which contained one member with severe (type III) vWD.

V.3iii Gene Deletion

There was no evidence of any gene deletion detected by this particular RFLP. Only 1 gene deletion has been described in an individual who had vWD which was not severe [Bernardi et al, 1990] and most others investigating type I or type II vWD have concluded that the disease in its mild autosomal dominant form is likely to be due to a point mutation rather than a deletion or insertion in the vast majority of patients.

Homozygous gene deletions have been described in patients with severe recessive type vWD and heterozygous deletions have been detected in their carrier relatives. Gene deletions would appear to correlate with the development of allo-antibodies in vWD. However, in one study described, 19 individuals with severe vWD were investigated, of whom only two had detectable gene deletions. These two individuals both had alloantibodies to vWF. The 17 without detectable deletions did not have antibodies. The molecular mechanism underlying severe vWD in patients without a detectable gene deletion is unknown [Shelton-Inloes et al, 1987B; Ngo et al, 1988].

V.3iv Severe Von Willebrands Disease

The one family with severe vWD studied with this RFLP had no evidence of gene deletion. The alleles were the same size as those individuals with autosomal dominant vWD or unaffected individuals. The child with severe vWD is homozygous for the 9kb fragment. Since the parents are first cousins, it is most likely that the severe vWD is due to the same defect in each chromosome. That is that she is a homozygous rather than a double heterozygote. The linkage of the disease with the 9kb fragment in this family is further confirmed by the evidence of mild vWD in the heterozygous sib and no evidence of vWD in the child homozygous for the 7.4kb fragment.

In this study it would be possible to offer PND for any further pregnancies. Furthermore in a family where consanguinous marriage is likely it may be helpful for the affected girl and heterozygous sister to have DNA testing available either for carrier detection of a related spouse or PND of any pregnancy when they themselves come to marry and conceive.

Bernardi et al [1988] investigated four unrelated individuals with type III vWD using four different RFLPs. Two of these individuals were homozygous for all four probes and the authors conclude that these two people are likely to be homozygous for a particular gene defect whereas the other two individuals who are not homozygous for the RFLPs they conclude are more likely to be double heterozygotes. The family of one individual with severe vWD were investigated by Bahnak et al [1988] using two informative RFLPs from which they were able to establish linkage and identify carriers for severe vWD. Ten affected individuals from six families were reported by Ngo et al [1988] who divided them into three groups after studies with cDNA vWF probes. The first group consisted of a family with complete homozygous deletions of the vWF gene in the four probands. Gene dosage analysis was consistent with heterozygous deletions in both asymptomatic parents and four asymptomatic siblings. The second group comprised a family in which there was a complete heterozygous deletion of the vWF gene in the proband and one asymptomatic parent, suggesting that a different type of genetic abnormality was inherited from the other parent and the affected individual was a double heterozygote. In the third group, no gene deletion could be detected. Alloantibodies developed only in the kindred with homozygous deletions.

V.3v A Further RFLP

The 15kb band present in the autoradiograph of four individuals was associated with a less densely staining 13kb constant band compared to other individuals suggesting that in some individuals the 13kb band is not constant. A polymorphism resulting in loss of the restriction enzyme site which normally produces the 13kb band results in the larger 15kb fragment being present. A personal communication with Verweij et al who first described this RFLP has confirmed this observation although in their population it was not common. Numbers of unrelated individuals studied are too small in this study to calculate allele frequencies. In only one family is it possible to observe inheritance. However the 15kb band was present in three out of eleven families investigated. A further polymorphism with the same enzyme and probe would clearly be useful in linkage studies within families. These preliminary results show this polymorphism to be present within four individuals of the thirty eight studied. Thus in this particular population it may provide useful further linkage data in a larger pedigree.

V.4 NEW DEVELOPMENTS IN MOLECULAR BIOLOGY

There can be little doubt that the developments in molecular biology have accelerated the learning about disease processes, not only in genetics and haematology but in all medical disciplines. Identifying molecular defects fundamental to the cause of a disease is becoming increasingly straightforward and commonplace. New concepts of disease aetiology such as oncogenes and tumour suppressor genes have been introduced [Cooper, 1991; Mitchell, 1991]. Improved methods for identifying minimal residual disease in malignancy using molecular techniques have been described [Neale et al, 1991].

Parallel developments in protein biochemistry and molecular biology have resulted in a rapid increase in knowledge and understanding of the pathological processes which cause the common inherited coagulation disorders. The polymerase chain reaction (PCR) which allows rapid amplification of short segments of DNA has revolutionised the field of molecular biology opening the way to subsequent techniques of improved DNA sequencing, new methods of carrier detection and point mutation identification within the field of inherited coagulation diseases [Saiki et al, 1985; Kogan et al, 1987]. Beyond this its scope is enormous, with PCR applications being identified in all areas of biological research.

V.4i Haemophilia A

The factor VIII gene is 186kb long and consists of 26 exons with 25 introns. The coding DNA is approximately 9kb with codons for a polypeptide of 2351 amino acids. The nucleotide sequences of the coding region, promoter elements and splice junctions have been determined.

Post translational modifications including glycosylation results in a mature single chain factor VIII protein of 2332 residues made up of three types of domain: A, B and C. There are three A domains with B occurring between the second and third and two C domains. The two specific thrombin cleavage sites which produce an active cofactor lie between the first two A domains, Arg 372 - Ser 373 and the carboxy terminus of the B domain Arg 1689 - Ser 1690. This second site is cleaved by thrombin activation and factor VIII exists as a two chain molecule in the circulation. Site specific mutagenesis has shown that loss of either of the thrombin binding sites affects clotting whereas the majority of the B domain which makes up half the mature protein can be removed without any effect upon blood clotting. Arg 336 - Met 337 is the cleavage site for

inactivation of activated factor VIII by activated protein C. Immunological studies using antibodies to synthetic peptides to identify presumptive epitopes have allowed some interpretation of three dimensional structures of the molecule. Epitopes for alloantibody inhibitors frequently localise to the A-2 and C-2 domains [Thompson, 1990].

Knowledge of the DNA sequence and the PCR method made examining small areas of the FVIII gene for point mutations much simpler. The sites of thrombin cleavage or protein C cleavage were early targets in the search for specific point mutations which caused haemophilia A. Gitschier et al [1988],by screening amplified DNA with discriminant hybridisation of oligonucleotides, identified 2 cleavage site mutations in 215 patients, one a nonsense mutation in the activated protein C cleavage site and the other a mutation resulting in a substitution of cysteine for arginine at amino acid 1689. It had already been predicted that the haemophiliacs with a high new mutation rate and a variable clinical presentation were likely to be associated with many different molecular defects. Kogan and Gitschier [1990] screened 228 haemophilia A patients with denaturing gradient gel electrophoresis (DGGE) and identified two further mutations around the A-2 and C-2 domains which are the neutralising anti-factor VIII antibody epitopes as well as a new polymorphism in intron 7. Rescreening the same population by making heteroduplexes between haemophiliac and normal DNA prior to electrophoresis revealed a further missense mutation. Schwaab et al [1991] identified two missense mutations in 441 unrelated haemophiliacs by investigating the thrombin cleavage sites by oligonucleotide screening, DNA amplification and direct sequencing. Other authors have also described mutations at the thrombin cleavage sites [Pattinson et al, 1990; Shima et al, 1989; Arai et al, 1990].

Gitschier et al [1985B] found that the restriction enzyme Taq I (recognition sequence TCGA) detected point mutations in the factor VIII gene of four out of 92 haemophilia A patients. Youssoufian et al [1987] provided evidence for recurrent mutations due to CG -> TG transitions at identical sites in the factor VIII gene. Further examples of recurrent mutations have since been found in five different Taq I restriction sites within exons 18, 22, 23, 24 and 26 [Antonarakis et al, 1987; Levinson et al, 1990]. One mutation has been described in exon 26 where a mutation converts a CGA (arginine) codon to a TGA (stop) codon resulting in severe haemophilia. However a C-T transition in the reverse complement strand of DNA at the same codon in a different patient results in a conversion from CGA (arginine) to CAA (glutamine) and this produces only mild haemophilia [Gitschier et al, 1986]. The CpG dinucleotide is a hotspot for mutation within the human genome because it is the preferred site for cytosine methylation. 5'methyl cytosine is prone to mutate to thymidine by deanimation. Within the factor VIII gene there are 70 CpG sites of which only 7 are Taq I restriction sites [Pattinson et

al, 1990]. PCR has allowed the screening of CpG sites which are not restriction sites to identify further mutations. 16 patients out of 793 unrelated haemophiliacs were shown to have point mutations consistent with 5'methylcytosine deamination by Pattinson et al [1990]. Millar et al [1991] identified two C -> T transitions by screening intragenic CpG sites in haemophilia A patients.

Naylor et al [1991] have recently described a method for screening the active coding sequence of the factor VIII gene, as well as its promoter and polyadenylation regions by a combination of PCR techniques and screening for mutations by the amplification mismatch detection method which uses chemical cleavage to detect mismatches. If this method proves to be sensitive in a large study of haemophilia A patients it may allow the molecular defect in the majority, if not all, of haemophilia A families to be identified.

These new methods, once developed, can also be applied to carrier detection and prenatal diagnosis. Amplification of RFLP sites by PCR allows more rapid analysis in women who are informative [Lavergne et al, 1991] although it is now appreciated that an internal control of enzyme digestion is necessary to avoid misinterpretating incomplete enzyme digestion as an absent restriction site [Sampietro et al, 1990]. PCR amplification allows carrier detection from samples other than blood, such as sputum, urine and hair follicles which is clearly useful where blood samples are difficult to obtain [Ball et al, 1990]. Methods currently being described which identify point mutations in a haemophiliac are of particular interest to the 30% of families where there is no family history and carrier detection by RFLP analysis is usually unhelpful. The point mutation identified in the affected male can then be looked for in all women at risk of being carriers and allow direct carrier detection and prenatal diagnosis.

V.4ii Haemophilia B

The shorter factor IX gene of approximately 33.5kb with 3kb of sequencing genome was sequenced fully before the factor VIII gene. The structure of the factor IX protein is described in the introduction section of this thesis. Post-translational modification consists of cleavage of the signal peptide and propeptide followed by glycosylation and modification by a vitamin K dependent carboxylase of the amino-terminal Glu residue to form the dicarboxylic Gla form which is associated with calcium binding. Activation of factor IX is calcium dependent and results in cleavage at two sites Arg 145 - Ala 146 and Arg 180 - Val 181 [Thompson, 1990].

A putative promoter region is present 5' to the transcription mutation site in the DNA. This region contains distinct nucleotide changes in patients with haemophilia B Leyden,

the condition where severe haemophilia B becomes mild after puberty [Reitsma et al, 1988].

As is the case for the factor VIII gene, further patients with partial and complete gene deletions have been described. As more have been described, the relationship between presence of factor IX inhibitor and deleted gene has been seen to be less strong than originally imagined.

CpG dinucleotides, which are relatively underrepresented in the factor IX molecule again appear to be an important mutation site. 18 mutations sites,involving CpG dinucleotides, in 45 distinct families, have been identified accounting for nearly half of the patients with haemophilia B associated with point mutation [Thompson, 1990].

Bottema et al [1990] describe a point mutation consisting of a T -> C transition at base 31311 which results in a Ile -> Thr transition. This was detected in 17% of 65 patients screened and was associated with a mild to moderate haemophilia B phenotype. Although these patients were thought to be unrelated, RFLP analysis showed that they all shared the same rare haplotype suggesting a common ancestor.

Unlike haemophilia A, where until very recently screening for point mutations has required a directed search strategy, often around known cleavage sites, the shorter length of the factor IX gene has enabled diagnosis of the molecular defects resulting in haemophilia B by direct sequencing of factor IX exons, plus intron-exon boundaries promoter and termination signal regions amplified by PCR [Green et al, 1989]. The techniques of direct detection of point mutations by mismatch analysis following chemical cleavage has also been shown to be a highly sensitive method which avoids the necessity of extensive DNA sequencing [Montandon et al, 1989].

These same methods can then be applied to carrier detection and prenatal diagnosis. Montandon et al [1989] describe carrier detection in four females where the haemophilia B was an isolated event. One case was shown to be a true new mutation but the other three showed the molecular defect in the mother of the haemophiliac and in one case his sister demonstrating the applicability of these methods in families with no family history of haemophilia B.

V.4iii Von Willebrands Disease

The structural domains of the von Willebrands factor cDNA are described in the introduction. The complete exon and intron structure of the vWF gene has now been

established by Mancuso et al [1989] and the approximate localisations for known vWF functional domains are now known. Exon 28 is the largest exon at 1.4kb and codes for the A_1 and A_2 domains which contain binding sites for platelet glycoprotein Ib, heparin and collagen. Defects resulting in type IIA vWD have been localised to domain A_2 and those resulting in type IIB vWD have been localised to domain A_1. Factor VIII binding localises to domain D' whilst the binding site for the platelet glycoprotein IIb/IIIa complex is present at the 3' end of the gene and lies at amino acid 1744-7 [Ginsburg and Bowie, 1992].

Molecular studies in von Willebrands disease have been impeded because there is a pseudogene present in chromosome 22 which has 97% homology with the central region of the vWF gene on chromosome 12 from exon 23 to exon 34 [Mancuso et al, 1991]. Studies attempting to identify deletions or point mutations in this area are severely limited by this pseudogene.

The family presenting with an individual affected by type III vWD described in this thesis had results suggesting this severe vWD was due to a double dose of an autosomal dominant disorder. It is now apparent that this is a less common pattern for type III vWD than to have parents of the proband who are completely asymptomatic [Ginsburg and Bowie, 1992]. Nichols et al [1991] demonstrated an abnormality at the level of mRNA expression from both maternal and paternal alleles in one type III vWD pedigree. All offspring inheriting both aberrant alleles were affected with type III vWD whereas both parents and five heterozygous siblings were asymptomatic and had normal vWF plasma levels.

Type IIA vWD which is particularly associated with loss of higher molecular weight multimers has been shown by a combination of protein studies and molecular biology to fall into two aetiological groups. In the first group, a point mutation results in a failure of transport of the vWF multimers causing them to be retained in the endoplasmic reticulum. The larger multimers are retained to a greater degree [Lyons et al, 1992]. In the second group a variety of point mutations result in an increased susceptibility to proteolytic cleavage between residues Tyr-842 and Met 843 [Dent et al, 1990].

Type IIB vWD is characterised by an increase in binding between the vWF molecule and platelet glycoprotein Ib. Since the binding site for GpIb has been localised to the A_1 domain of the vWF gene seven specific single amino acid substitutions and one insertion have been identified in this area. Furthermore vWF fragments expressing these identified mutations have been associated with increased platelet binding [Ginsburg and Bowie, 1992].

Mutations located within the N-terminal region of the vWF molecule interfere with the ability of the vWF molecule to bind FVIII. The identification of these mutations has helped to define a new variant of vWD associated with low levels of circulating factor VIII. This condition has caused much confusion in the past and has been described as autosomal haemophilia.

Gene deletion does not appear to be a major cause of vWD; only two partial gene deletions have been described since those identified by Shelton-Inloes et al [1987B] and Ngo et al [1988], one causing severe vWD [Peake et al, 1990A] and one causing a variant type II vWD [Bernardi et al, 1990]. The fact that vWD is a heterozygous disease caused by a variety of point mutations in the majority of cases along with the known existence of a pseudogene makes a strategy for carrier detection and prenatal diagnosis difficult. The identification of a highly informative variable number tandem repeat (VNTR) in intron 40 [Peake et al, 1990B] will improve carrier detection rates in families which are not informative for RFLPs already identified.

As yet no molecular lesion has been identified as the cause of the most common type of vWD, type I which is associated with a functional decrease in all aspects of the vWF molecule along with normal multimer distribution. This generalised decrease in protein production may not be due to an intragenic lesion but may be due to a defective promoter or initiator of RNA transcription or to a sequence of DNA which terminates transcription.

The applications of molecular biology in these disorders are not limited to diagnosis. Already limited amounts of factor VIII have been manufactured using recombinant DNA technology and in the future this may be an important source of therapeutic material. Equally important, there is a great deal of interest in and research around the possibility of gene therapy: that is of replacing the faulty gene and thereby curing the genetic disorder [Lancet, 1990; Felgner and Rhodes, 1991]. This would offer tremendous hope for carriers of genetic disease although there are as yet many technical problems to be overcome.

Molecular genetics has already transformed the investigation and management of individuals with inherited bleeding disorders. Future developments are likely to lead ultimately to both prevention and cure: an exciting prospect.

V.5 ORGANISING A HAEMOPHILIA SERVICE: PRACTICAL IMPLICATIONS

In the context of the haemophilias and von Willebrands disease, it is worth examining the practical implications of these changes. At present most haemophilia centres in the United Kingdom are run by haematologists. Haemophiliacs (and patients with von Willebrands disease) are registered at diagnosis and treatment instituted. It is usual to invite patients to attend a review clinic once or twice a year. Here they can discuss any treatment problems and also see the dentist, physiotherapist, orthopaedic surgeon or other specialist as appropriate. Genetic advice is usually given at diagnosis and then at these review clinics when requested. It is unlikely that this is adequate. In a survey published in 1986, Markova et al presented data from haemophiliacs and carriers from centres in four countries including the centre in Glasgow where this present work was carried out. Only 24% of the Scottish haemophiliacs said that they had received genetic counselling and only 78% had received information about the transmission of haemophilia. These figures compare poorly with the clinic in New York where 91% of haemophiliacs received genetic counselling and 100% had received information about disease transmission. This is not meant as an indictment of the hard pressed doctors currently running the haemophilia centre in Glasgow. Indeed it may well be that the Scottish haemophiliacs had received genetic counselling but did not recognise it as such since it took place during a routine clinic and did not involve a geneticist. However, the survey does highlight the lack of information felt by both haemophiliacs and carriers and would suggest that more time needs to be set aside for genetic counselling. A further problem with relaying information about genetic risks is that people's perception of the information may vary according to the context in which it is given and also information given to a couple who are then allowed to discuss this may have a different impact from information given to individuals [d'Ydewalle and Evers-Kiebooms, 1987]. This is very important in the context of haemophilia particularly if a couple present, seeking genetic counselling, when the woman is already pregnant and under pressure to make a decision. Interestingly, in the study by Markova et al [1986] in contrast to 44% of haemophiliacs, only 22% of carriers felt that haemophilia was no problem at all. Ten out of 37 sisters of haemophiliac men felt that there was a great degree of strain in the family caused by the disease. In this context, it is possible that the response of possible carriers of haemophilia to genetic counselling will vary depending on whether they present with their haemophiliac relative or not.

For the population at risk of inherited coagulation disorders, the benefits of improved diagnostic techniques can only be maximised by a combination of access to a molecular genetics laboratory where the appropriate tests are available along with support from

haematology, obstetric and genetics departments. At a time when the financial efficiency of all departments is being questioned, establishing a service which involves a combination of expensive and relatively labour intensive laboratory methods and genetic counselling by highly trained personnel may not seem attractive to those who administer hospital budgets. Against this however the cost of treating haemophilia should be set out. Figures for 1990 for a small haemophilia centre in Yorkshire showed that 34 men with haemophilia A were given 1.1 million units of the NHS factor VIII concentrate at 21p/unit and 150,000 units of Monoclate (factor VIII purified by monoclonal antibody) at 45p/unit. Added to this one must consider the costs of hospital attendance and admission, surgical treatment and other likely treatments. Clearly, not preventing haemophilia has financial implications over a lifetime.

The slant of this final chapter is very much aimed at families affected by haemophilia A and B rather than von Willebrand's disease where problems are rarely so severe. Nonetheless, because of this, families may not be offered genetic counselling and perhaps it should be borne in mind that they too may need support and advice prior to starting a family.

CHAPTER VI- REFERENCES

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CHAPTER VII - APPENDICES

VII.1 APPENDIX I Haemophilia A Results

Individual	F8A [kb]	F8B [kb]	DX13 [kb]	St14 [kb]	FVIIIc [iu/dl]	vWF:Ag [iu/dl]
Pedigree 2218						
I1	[0.9]		[2.8]	[2]		
I2	[1.2/0.9]		[2.8/2.8]	[3/7]		
II2	0.9		2.8	7		
II3	1.2/0.9	5/5	2.8/2.8	2/7	190	42
II5	1.2/0.9	5/5	2.8/2.8	2/7		
II7	1.2/0.9	5/5	2.8/2.8	2/3	36	43
II8	0.9		2.8	3		
II9	0.9		2.8	3		
II10	[0.9]		[5.8]			
II11	0.9/0.9		2.8/2.8	2/3		
III1	0.9	5	2.8	2		
III2	0.9	5	2.8	2		
III3	1.2	5	2.8	7	<1	
III4	1.2/0.9		2.8/2.8	7/7	130	100
III5	0.9/0.9		2.8/2.8	2/7	140	52
III6	1.2/0.9		2.8/2.8	7/7	80	125
III7	1.2	5	2.8	7	<1	
III8	0.9/0.9		5.8/2.8	2/3	82	50
III9	0.9	5	2.8	2		
III10	1.2	5	2.8	3	2	
III11	1.2/0.9		2.8/2.8	3/3		
III12	1.2	5	2.8	3	<1	
III13	0.9/0.9		5.8/2.8	3/5		
Pedigree 1065						
I1	[1.2]		[2.8]			
II1	[0.9]		[5.8]			
II2	1.2/0.9		2.8/2.8			
III1	0.9		2.8			
III2	1.2/0.9		5.8/2.8			
III4	1.2/0.9		5.8/2.8			

Individual	F8A [kb]	F8B [kb]	DX13 [kb]	St14 [kb]	FVIIIc [iu/dl]	vWF:Ag [iu/dl]
Pedigree 1065						
IV1	0.9/0.9		5.8/2.8			
IV2					38	76
IV3	1.2		2.8			
Pedigree 7319						
II1	1.2		2.8	2		
II3	1.2/0.9		5.8/2.8	2/2	82*	200*
III2	0.9		5.8	2		
III3	0.9		5.8	2		
III4	1.2/1.2					
Pedigree 7399						
I1	[1.2]	[5]	[2.8]	[8]		
II1	[0.9]	[5]	[5.8]	[2]		
II2	1.2/1.2	20/5	5.8/2.8	3/8		
II3	[1.2]	[20]	[5.8]	[3]		
II4	[1.2/1.2]	[20/5]	[5.8/2.8]	[3/8]		
II5	1.2	20	5.8	3		
II6	[0.9]		[2.8]	[6]		
II7	1.2/1.2	20/5	5.8/2.8	3/8		
III1	0.9	5	2.8			
III2	1.2/0.9	20/5	5.8/5.8	2/3	62	140
III3	[1.2]	[2.8]				
III4	1.2/1.2	20/20	5.8/5.8	3/3		
III5	1.2		2.8	8		
III6	1.2	20	5.8	3		
III7	1.2/1.2	20/5	5.8/2.8	3/8		
III8	1.2		2.8			
III9	0.9		2.8			
III10	1.2/1.2	20/20	5.8/5.8	3/3		
III11	1.2		2.8	8		
III12	1.2/0.9	20/5	5.8/2.8	3/6		
III14	1.2/0.9	5/5	2.8/2.8	6/8		
IV1	0.9	5	5.8	3		

Individual	F8A [kb]	F8B [kb]	DX13 [kb]	St14 [kb]	FVIIIc [iu/dl]	vWF:Ag [iu/dl]
Pedigree 7399						
IV2	1.2/0.9	20/5	5.8/2.8	3/7	35	72
IV3	1.2		5.8	3	<1	
IV5	1.2		5.8			
IV6	1.2/1.2	20/20	5.8/2.8		37	121
IV7	1.2		5.8	3	<1	
IV8	1.2		5.8	3		
IV9	1.2/1.2	20/20	5.8/5.8	3/3		
IV10	1.2/1.2	20/5	5.8/2.8	3/8		
IV11	1.2/1.2	20/5	5.8/2.8	3/8		
IV12	1.2	20	5.8	3		
IV13	1.2/1.2	20/5	5.8/2.8		56	105
IV14	1.2	20	5.8		<1	
V1	1.2/0.9	20/5	5.8/5.8	3/3	42	84
Pedigree 7402						
I2	[1.2/0.9]	[5/5]	[5.8/2.8]	[2/7]		
II1	0.9	5	5.8	2		
II2	1.2	5	2.8	7		
II4	1.2/0.9	5/5	5.8/2.8	2/7		
II6	1.2/0.9	5/5				
II7	[0.9]	[5]	[5.8]	[3]		
II8	1.2/1.2	20/5	2.8/2.8	5/7		
III3	1.2					
III4	1.2					
III9	1.2/0.9	20/5	5.8/2.8	3/5		
III11	0.9		5.8			
III12	1.2/0.9	20/5	5.8/2.8	3/5		
III13	0.9		5.8	8		
III14	1.2/0.9	5/5	5.8/2.8	3/7	68	50
III15	1.2	5	2.8	5		
III16	1.2	5	2.8	5		
III17	1.2	20	2.8	5		
III18	1.2/0.9	5/5	5.8/2.8	3/7		
IV4	1.2		2.8			
IV6	1.2	5	2.8	7	7	

Individual	F8A [kb]	F8B [kb]	DX13 [kb]	St14 [kb]	FVIIIc [iu/dl]	vWF:Ag [iu/dl]
Pedigree 7403						
I1	[0.9]	[5]	[2.8]	[8]		
I2	0.9/0.9	5/5	5.8/2.8	3/5		
II1	[0.9]	[5]				
II2	0.9	5	5.8	5		
II3	0.9		5.8	5		
II4	0.9/0.9	5/5	5.8/2.8	5/8		
II5	1.2		5.8	2		
II6	0.9/0.9	5/5	2.8/2.8	3/8		
III1	0.9/0.9		5.8/5.8	5/5		
III2	0.9	5	5.8	5		
III3	0.9	5	2.8	8		
III4	0.9	5	2.8	3	<1	
Pedigree 7405						
II1	0.9	2.8	5			
II2	0.9/0.9	5/5	5.8/2.8	2/3	28	44
II3	[1.2]	[5]	[5.8]	[7]		
II4	[0.9/0.9]	5/5	[5.8/2.8]	[2/3]		
III2	0.9	5	2.8	3	20	
III3	0.9	5	2.8	3	10	
III4	0.9/0.9		5.8/2.8	2/5	130	115
III6	0.9/0.9		5.8/2.8	2/5	250*	185*
III8	0.9/0.9		5.8/2.8	2/7	180	165
III9	1.2/0.9		5.8/2.8	3/7	180	82
III10	1.2		2.8			
III11	1.2/0.9	5/5	5.8/2.8	3/7	115	120
III12	0.9		2.8	6		
III13	1.2/0.9	5/5	5.8/2.8	3/7	48	48
IV1	0.9/0.9		5.8/2.8	2/7	180	180
IV2	0.9		5.8	2		
IV4	0.9	5	2.8	3	11	
IV6	1.2/0.9		2.8/2.8	3/6	130	100
IV7	0.9	5	2.8	3	6	
IV8	0.9/0.9		2.8/2.8	3/6	74	175

Individual	F8A [kb]	F8B [kb]	DX13 [kb]	St14 [kb]	FVIIIc [iu/dl]	vWF:Ag [iu/dl]
Pedigree 7406						
I2	1.2/0.9	5/5	5.8/2.8	7/7	170	106
II1	1.2	5	2.8	7	64	76
II2	0.9/0.9		5.8/2.8	3/7		
II4	1.2/0.9		5.8/2.8	7/7	40	68
II5	1.2	5	2.8	7	30	
III1	1.2/0.9	5/5	5.8/2.8	3/7		
III3	1.2/0.9	5/5	5.8/2.8	3/7		
Pedigree 7407						
I1	[0.9]	[5]	[5.8]	[3]		
I2	0.9/0.9	5/5	5.8/2.8	3/8		
II2	0.9/0.9	5/5	5.8/2.8	3/8	62	41
II3	0.9		2.8	5		
II4	0.9/0.9	5/5	5.8/5.8	3/3	19	66
II5	0.9	5	5.8	3	<1	
II6	0.9/0.9		5.8/2.8	7/8		
II7	0.9		2.8			
II8	0.9/0.9	5/5	5.8/5.8	3/3		
II9	1.2		2.8			
II10	0.9/0.9	5/5	5.8/5.8	3/3		
II11	0.9		5.8	8		
II12	0.9/0.9	5/5	5.8/2.8	3/8		
II13	1.2		2.8	8		
II14	0.9/0.9	5/5	5.8/5.8	3/3	41	50
III3	0.9		5.8			
III5	0.9	5	2.8	8		
III7	1.2/0.9		5.8/2.8	3/5		
III8	0.9/0.9		5.8/5.8	3/7	170	42
III9					56	52
III10					50	57
III11	0.9	5	5.8	3	<1	
III12	0.9/0.9		5.8/2.8			
III13	0.9/0.9		5.8/2.8			

Individual	F8A [kb]	F8B [kb]	DX13 [kb]	St14 [kb]	FVIIIc [iu/dl]	vWF:Ag [iu/dl]
Pedigree 7407						
III14	1.2/0.9		5.8/2.8		68	66
III15	1.2/0.9		5.8/2.8			
III16	1.2/0.9		5.8/2.8			
III17	0.9	5		3		
III18	0.9	5	5.8	3		
III19	0.9	5	2.8	8		
III20	0.9/0.9		5.8/2.8	8/8		
III21	0.9/0.9		5.8/2.8	8/8		
III22	1.2/0.9		5.8/2.8	3/8	68	46
Pedigree 7408						
I1	[0.9]	[5]	[5.8]	[7]		
I2	0.9/0.9	5/5	2.8/2.8	2/9	50	84
II1	0.9		5.8	1		
II2	0.9/0.9	5/5	5.8/2.8	2/7		
II4	0.9/0.9	5/5	5.8/2.8	2/7	90	88
II5	0.9	5	2.8	2	4	
II6	0.9/0.9		2.8/2.8	5/7		
II7	0.9	5	2.8	2		
III1	0.9/0.9		5.8/5.8	1/7	90	65
III2	0.9	5	5.8	7		
III3	0.9	5	5.8			
III4	0.9	5	2.8	2		
III5	0.9/0.9		2.8/2.8		33	43
Pedigree 7409						
I1	0.9		2.8	8		
I2	0.9/0.9		2.8/2.8	3/8	100	100
II1	0.9		2.8	3	1	
II2	0.9/0.9		2.8/2.8	3/8		
II3	0.9		2.8	3	1	

Individual	F8A [kb]	F8B [kb]	DX13 [kb]	St14 [kb]	FVIIIc [iu/dl]	vWF:Ag [iu/dl]
Pedigree 7410						
II1	0.9	5	2.8	2	10	
II2	1.2/0.9		2.8/2.8	7/9		
II3	1.2		2.8	3		
II4	0.9/0.9	5/5	5.8/2.8	2/5	130	120
II5	0.9		2.8	9		
II6	0.9/0.9		5.8/2.8	2/5	110	120
II7	0.9		2.8	6		
II8	0.9/0.9		5.8/2.8	2/5	66	55
II9	1.2		2.8	3		
II10	0.9/0.9		5.8/2.8	2/5	60	86
II11	0.9		5.8	3		
II12	0.9/0.9	5/5	5.8/2.8	2/5	56	50
III1	0.9		5.8	3		
III2	1.2/0.9	20/5	2.8/2.8	2/7	130	120
III3	0.9		5.8			
III4	1.2/0.9	20/5	2.8/2.8	2/7	64	74
III5	0.9		2.8		37	
III7	0.9/0.9		2.8/2.8	2/9	27	72
III8	0.9/0.9		5.8/2.8		100	100
III9	1.2/0.9		5.8/2.8	3/5	110	100
III10	1.2/0.9		5.8/2.8	3/5	100	60
III11	0.9	5	2.8	2	50	
III12	0.9	5	5.8	5		
IV1	0.9		2.8	7		
IV2	1.2		2.8	7		
IV3	1.2/0.9		5.8/2.8		140	72
Pedigree 7411						
I1	[1.2]		[5.8]	[6]		
I2	[0.9/0.9]		[5.8/2.8]	[6/7]		
II1	[0.9]		[2.8]	[2]		
II2	1.2/0.9		5.8/5.8	6/7		
II3	0.9		2.8	2		
II4	1.2/0.9	5/5	5.8/5.8	6/7		
II6	1.2/0.9		5.8/2.8	6/6		

Individual	F8A [kb]	F8B [kb]	DX13 [kb]	St14 [kb]	FVIIIc [iu/dl]	vWF:Ag [iu/dl]
Pedigree 7411						
III1	0.9		5.8	7		
III3	0.9/0.9		5.8/2.8	2/7		
III4	1.2/0.9		5.8/2.8			
III5	0.9	5	5.8	7		
III7	0.9/0.9		5.8/2.8	2/7	77	137
III8	0.9	5	5.8	7		
III9	1.2/0.9		5.8/2.8	2/6		
Pedigree 7412						
I1	1.2	20	2.8			
I2			5.8/2.8			
II2	1.2/1.2	20/20	2.8/2.8	3/5	54	85
Pedigree 7413						
II1	1.2	5	2.8	2	1	
II2	0.9/0.9		5.8/5.8	2/5		
III2	1.2/0.9	5/5	5.8/2.8	2/2		
III3	1.2/0.9	5/5	5.8/2.8	2/5	56	76
III6	1.2/0.9		5.8/2.8		94	220
III7	0.9/0.9					
IV1	1.2	5	2.8	2	2	

* = SAMPLES TAKEN WHEN WOMEN WERE PREGNANT

[ ] = RESULTS DEDUCED FROM RELATIVES' RESULTS

VII.2 APPENDIX II Haemophilia B Results

Individual	F9(VIII)/Taq I (kb)	F9(VIII)/Xmn I (kb)	F9(XIII)/Dde I (kb)	F9c (iu/dl)
Pedigree 1756				
I1	[1.3]			
I2	[1.8/1.3]			
II1	1.8/1.3			
II2	1.8			
II4	1.8/1.3			
II6	1.8/1.3			
II8	1.8			
II9	[1.3/1.3]			
II11	1.3	11.5	1.7	9
II12	1.8			
II13	1.8			
III1	1.3			
III2	1.3			
III3	1.8/1.3			
III4	1.8/1.3			
III5	1.8/1.3			
III6	1.3			6
III7	1.8/1.3			
III8	1.8/1.3	11.5/11.5	1.75/1.7	
III9	1.8/1.3	11.5/11.5	1.75/1.7	
IV8	1.3			
IV9	1.3			
IV10	1.3/1.3	11.5/11.5	1.75/1.7	
IV11	1.8			
Pedigree 1832				
I1	1.3	6.5		
I2	1.8/1.3	11.5/11.5		
II1	1.8/1.3	11.5/6.5		
II2	1.8	11.5		

Individual	F9(VIII)/Taq I (kb)	F9(VIII)/Xmn I (kb)	F9(XIII)/Dde I (kb)	F9c (iu/dl)
Pedigree 2377				
II2	1.8/1.3	11.5/11.5	1.7/1.7	
II9	1.8/1.3	11.5/11.5	1.7/1.7	
III2	1.8/1.3	11.5/6.5	1.7/1.7	106
III7	1.3			
III8	1.3/1.3	11.5/11.5	1.7/1.7	
III13	1.3/1.3	11.5/11.5	1.7/1.7	
IV1	1.3			4
IV2	1.8/1.3	11.5/11.5		120
IV3	1.3			6
IV5	1.3/1.3	11.5/11.5		80
IV6	1.3			
IV7	1.3	11.5		6
IV8	1.3			
V1	1.3			5
Pedigree 3569				
II1	1.8			
II2	1.3/1.3	11.5/6.5	1.7/1.7	
II4	1.3/1.3	11.5/6.5		
III1	1.3			
III2	1.8/1.3	11.5/11.5	1.75/1.7	
III3	1.3			
III4	1.3	6.5		
III5		11.5/6.5		
IV1	1.8			
IV2	1.3/1.3	11.5/11.5		
IV3	1.8/1.3	11.5/11.5		
IV4	1.3	11.5	1.7	8
Pedigree 4542				
III1	1.8/1.8	11.5/11.5	1.75/1.7	
III2	1.8/1.8	11.5/11.5		
III3	1.8/1.8			
III6	1.8			
III7	1.8/1.8	11.5/11.5	1.75/1.7	

Individual	F9(VIII)/Taq I (kb)	F9(VIII)/Xmn I (kb)	F9(XIII)/Dde I (kb)	F9c (iu/dl)
Pedigree 4542				
III9	1.8/1.3	11.5/6.5	1.75/1.7	
III10	1.8/1.3	11.5/6.5	1.75/1.7	
IV2	1.8	11.5		<1
IV10	1.8/1.8	11.5/11.5	1.75/1.7	
IV11	1.8	11.5	1.75	<1
IV12	1.8			
IV13	1.8/1.8			
V1			1.75/1.7	
V2	1.8			<1
V3	1.8/1.8	11.5/11.5	1.75/1.7	
Pedigree 4549				
I1	1.3	11.5		
I2	1.3/1.3	11.5/6.5	1.7/1.7	88
II1	1.3			
II2	1.3	6.5	1.7	
II4	1.3/1.3	11.5/6.5	1.75/1.7	58
II6	1.3/1.3	6.5/6.5	1.75/1.7	90
III1	1.8/1.3	11.5/6.5	1.7/1.7	82
III2	1.3/1.3			
III4	1.3			10
III5	1.8/1.3	11.5/6.5	1.75/1.7	52
III6	1.3/1.3	11.5/11.5		90
III7	1.3	6.5		
Pedigree 4551				
I1	[1.8]	[11.5]	[1.7]	
I2	1.8/1.8	11.5/11.5	1.7/1.7	
II2	1.8/1.3	11.5/11.5	1.7/1.7	
II3	1.3	6.5	1.75	
II4	1.8/1.8	11.5/11.5	1.7/1.7	
II5	1.8/1.3			
III1	1.8	11.5		15
III2	1.8	11.5		

Individual	F9(VIII)/Taq I (kb)	F9(VIII)/Xmn I (kb)	F9(XIII)/Dde I (kb)	F9c (iu/dl)
Pedigree 4551				
III3	1.8/1.3	11.5/6.5	1.75/1.7	
III4	1.8	11.5		
III5	1.8	11.5		19
Pedigree 4555				
II4	1.3/1.3	11.5/6.5	1.7/1.7	
III4	1.3	11.5	1.7	5
III6	1.8/1.3	11.5/11.5	1.7/1.7	
IV1	1.3			
IV3	1.3			8
IV4	1.8/1.3	11.5/6.5	1.7/1.7	50
IV5	1.3/1.3	11.5/11.5	1.75/1.7	
Pedigree 4559				
I1	1.8	11.5		
II1	1.3			
II2	1.8/1.3	11.5/11.5	1.75/1.7	
II3	1.8			
II4	1.8/1.3	11.5/11.5	1.7/1.7	
III1	1.8			3
III2	1.8/1.3	11.5/6.5		
III3	1.8/1.3			
III4	1.8/1.3			
Pedigree 4569				
III6	1.8	11.5		<1
IV1	1.8	11.5		
IV2	1.8/1.3	11.5/6.5	1.7/1.7	40
IV3	1.8	11.5	1.7	<1
IV7	1.8/1.3	11.5/11.5	1.7/1.7	
V1	1.8/1.3	11.5/6.5	1.7/1.7	
V3	1.3	6.5		
V4	1.3	6.5		

Individual	F9(VIII)/Taq I (kb)	F9(VIII)/Xmn I (kb)	F9(XIII)/Dde I (kb)	F9c (iu/dl)
Pedigree 5215				
I2	1.8/1.3	11.5/6.5	1.7/1.7	
II1	1.8	11.5		<1
II2	1.8	11.5		<1
II4	1.3/1.3	11.5/6.5		50-76
Pedigree 5292				
III1	1.8	11.5	1.7	
IV2			1.7	
IV3	1.8/1.8	11.5/11.5	1.75/1.7	
V2			1.7/1.7	
V4			1.7/1.7	

[] = RESULTS DEDUCED FROM RELATIVES' RESULTS

VII.3 APPENDIX III Von Willebrands Disease results

Individual	pvWF1100 RFLP (kb)	Bleeding Time (mins)	FVIII:C (iu/dl)	vWF:Ag (iu/dl)	vWF(Ricof) (iu/dl)
Pedigree 7512					
II4		>20	49	44	16
II6	9/7.4				
III3	7.4/7.4	28	75	105	25
III5	9/7.4	>20			
III7	9/9				
III8	9/9				
III9	7.4/7.4				
III10	7.4/7.4				
IV1	9/7.4	9	48	35	18
IV2	7.4/7.4				
IV3	7.4/7.4				
IV4	9/7.4	15	40	21	17
IV6		9			
Pedigree 7513					
III3	9/7.4	15			
IV1	9/7.4	9	100	82	88
IV2	9/9	5	68	38	<20
Pedigree 7514					
I1	15/9/7.4	2			
I2	9/9	11			
II2		>20	28	19	38
II3	9/7.4	3			
II4		5			
II5	15/9				
III1		15	28	27	20
III2		4	100	90	104
Pedigree 7515					
II1	9/7.4	9	100	70	41
II2	9/7.4				
III1	9/7.4	12	37	62	72
III2		4	100	90	104

Individual	pvWF1100 RFLP (kb)	Bleeding Time (mins)	FVIII:C (iu/dl)	vWF:Ag (iu/dl)	vWF(Ricof) (iu/dl)
Pedigree 7516					
II4	15/9/7.4	>20	44	15	21
III4	7.4/7.4	19	28	26	11
Pedigree 7517					
I1	9/7.4	19	30	35	14
I2	9/7.4	17	32	20	25
II1	9/9	>20	2	12	>10
II2	9/7.4	14	48	18	28
II3	7.4/7.4	5	115	72	66
Pedigree 7518					
I2	9/9				
II1	9/9	>15	58	37	40
Pedigree 7519					
II2	9/9	>30	44	20	22
III12	9/9	11	65	30	40
Pedigree 7520					
II2	9/9				
III1	9/7.4	>15	30	25	9
Pedigree 7521					
II2	9/7.4	>20	52	92	10
III1	9/7.4	>20	36		10
Pedigree 7522					
II6	9/7.4				
III6	15/9/9		12	28	50