Diagnosis, Classification and Treatment of von Willebrand Disease

by

Dr. Ioana Camelia Nitu-Whalley, MRCP (UK)

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Katharine Dormandy Haemophilia Centre and Haemostasis Unit
Department of Haematology
Royal Free and University College Medical School
Royal Free Campus
University of London
Rowland Hill Street, London NW3 2PF, United Kingdom
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Dedication

To my darling husband Simon and my beautiful daughter Adele, with love.
ABSTRACT

Von Willebrand disease (VWD) has been the subject of numerous studies and debates regarding diagnosis, classification and treatment. My research involved prospective and retrospective studies of patients with VWD registered at the Royal Free Hospital London. During the evaluation of the diagnosis of VWD we found that the von Willebrand factor ristocetin cofactor assay was superior to the ELISA assays. The study of new tests, such as the collagen binding assay and the platelet function analyser were shown to have an adjunctive role in the characterisation of patients with VWD. Another study investigated the proposed criteria for defining type 1 VWD (into 'definite' and 'possible' categories adjusted for the ABO blood group) and we showed that these criteria were too stringent as they left many individuals with bleeding histories unclassified. In another study we demonstrated that a group of patients previously diagnosed with type 1 VWD were misclassified as they were type 2 VWD as shown by phenotypes, genotypes and molecular modelling of the identified mutations. Furthermore, using molecular diagnosis and modelling a 2A/2B genotype was found in a kindred with type 2A VWD and thrombocytopenia. The role of desmopressin is well established in mild forms of type 1 VWD but there is little data on its role in severe type 1 and type 2 VWD. Our research showed that 30% of these patients can also benefit from desmopressin therapy. There are currently no guidelines on the management of elective surgery in patients with VWD and this thesis reviewed the treatment with desmopressin and clotting factor concentrates in these patients. In conclusion, this thesis brings new data for the clarification of the diagnosis of VWD, it offers new perspectives into the complex classification and, finally, it investigates the optimal therapeutic options for patients with VWD.
ACKNOWLEDGEMENTS

Over the last three years many people have supported me in different ways. First of all I would like to express my sincere gratitude to my main supervisor Pr Christine Lee, for guiding and supervising my research, for her generous support and permanent encouragement as well as for sharing her never failing enthusiasm and dynamism. I would also like to thank my second supervisor, Dr Simon Brown for his help and scientific advice.

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Finally, I would like to thank my parents and my sister for their support and help and to dedicate this thesis to my husband Simon and to my beautiful daughter Adele for their unconditional love and trust.
Publications

The following publications have derived from this thesis:


*Manuscripts in preparation:*


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<td>A</td>
<td>adenine</td>
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**Royal Free Hospital Haemophilia Centre**

**APTT** Activated partial thromboplastin time

**DDAVP** Desmopressin, 1-deamino-8-D-arginine vasopressin

**ELISA** Enzyme-linked immunosorbant assay

**FVIII** Coagulation factor VIII

**HMW** High molecular weight

**PCR** Polymerase chain reaction

**RIPA** Ristocetin induced platelet aggregation

**RFLP** Restriction fragment length polymorphism

**SAS** Statistical Analysis System
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<td>VWD</td>
<td>Von Willebrand disease</td>
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<td>VWF</td>
<td>Von Willebrand factor</td>
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<td>VWF:AC</td>
<td>Von Willebrand factor activity</td>
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<td>VWF:Ag</td>
<td>Von Willebrand factor antigen</td>
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<td>VWF:RiCo</td>
<td>Von Willebrand factor ristocetin cofactor activity</td>
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<td>bp</td>
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<td>DNA</td>
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CHAPTER 1

INTRODUCTION
1.1. GENERAL INTRODUCTION

Von Willebrand disease (VWD) is the commonest inherited bleeding disorder which was first described in 1926. Since then the knowledge and understanding of this disorder has substantially improved, however there are still many unanswered questions.

Although type 1 VWD is the commonest subtype of VWD it is also least well understood. One of the main problems regarding type 1 VWD is related to how to establish an accurate diagnosis, especially in the mild forms of the disease. The International Society of Thrombosis and Haemostasis (ISTH) von Willebrand Factor (VWF) Subcommittee have proposed several criteria of diagnosis, but these proposals have not been verified in clinical practice. Moreover, type 1 VWD has a variable phenotypic expression and a low penetrance and one of the main modifier factors of the VWF levels is the ABO blood group. To what extent the adjustment of the VWF levels to the ABO blood group has clinical relevance for the diagnosis and the decision for treatment in patients with type 1 VWD is controversial.

The diagnosis of VWD relies on the phenotypic characterisation. Of the available phenotypic assays, the measurement of VWF activity is crucial especially for defining type 2 variants. However, all the functional assays currently available have their limitations and there is no consensus on which is the most suitable method. Moreover, new assays have recently emerged and their potential value for screening and diagnosis of VWD is under evaluation.
The classification of VWD is very complex and currently it is based on the characterisation of various subtypes using the available laboratory and molecular methods. Type 2M VWD is considered a rare variant and may be misdiagnosed as type 1 VWD. In cases with an unclear phenotype the molecular genetics can provide an important tool for clarification of the type of VWD. Targeted DNA analysis has recently become useful for the characterisation of type 2 VWD, where the causative mutations tend to be circumscribed to a certain area of the VWF gene.

Regarding the therapeutic modalities in VWD, although desmopressin has been in use for over 20 years, the response to desmopressin in patients with severe type 1 and type 2 VWD is not well defined. Little data are available on the optimal management and monitoring of patients with VWD who undergo surgery as no clear guidelines exist at present.

1.2. AIMS AND OBJECTIVES

The Royal Free Hospital Haemophilia Centre (RFHHC) has one of the largest haemophilia centres in Europe with over 460 patients registered with VWD and regularly followed up. The series of studies included in this thesis were performed on patients registered at RFHHC and were designed to address the above unresolved issues regarding diagnosis, classification and treatment of VWD.
The key topics examined in this thesis include:

- Presentation of the population of patients with VWD registered at the RFHHC, and exploration of the importance of calculating various ratios between VWF and FVIII. In addition, other clotting factor deficiencies which coexist in the VWD population were also examined. All studies presented in this thesis stem from the initial step of establishing a comprehensive database of these patients with VWD, which then offered the opportunity to perform retrospective analyses and to select patients eligible for prospective studies to be performed.

- The criteria proposed by the ISTH for the definition of type 1 VWD as ‘definite’ and ‘possible’ are explored in a retrospective study with emphasis on the influence of the ABO blood group and the role of the bleeding history in diagnosis.

- Determination of the function of VWF forms a mainstay in establishing a diagnosis of the subtype of VWD. A critical comparison between two phenotypic assays measuring the functional activity of VWF (the ristocetin cofactor assay versus the ELISA activity assay) showed the limitations and benefits of these assays. The role of a new functional assay, the collagen binding assay and its utility in diagnosing various subtypes of VWD is assessed.
• A new screening test for VWD (Platelet Function Analyser, PFA-100™) is critically evaluated, the variables which might influence this test are analysed and its potential role in the monitoring of treatment is studied in patients with VWD.

• The importance of molecular diagnosis in type 2 VWD is exemplified by the study of a selected group of patients previously diagnosed as type 1 VWD in whom type 2 VWD is identified through a detailed analysis of phenotypes, genotypes and molecular modeling. Genetic analysis was also essential in the clarification of two cases with a challenging diagnosis: the first case was a patient with type 2A VWD and associated thrombocytopenia and the second case illustrates a kindred with an unusual type 2A VWD in whom an apparent compound heterozygous 2A/2B genotype was identified.

• The response to desmopressin infusion was studied in 17 patients with severe type 1 and type 2 VWD.

• The treatment patterns in VWD over a 18 years period are retrospectively assessed and the treatment with desmopressin and clotting factor concentrates in patients with VWD during 1997 was analysed. The management of elective surgery in patients with VWD over a ten year period was also reviewed.
1.3. LAYOUT OF THE THESIS

Due to the diversity of the studies and methods presented in this thesis, each chapter contains a description of the patients and a brief overview of the methods used in the study together with a detailed discussion of the results and conclusions. A more detailed presentation of the population and methods used throughout in the various studies included in the thesis is given in Chapter 3, which should be referred to in connection with each chapter. Similarly, general conclusions related to the whole thesis are presented in Chapter 10.

This thesis includes 10 chapters: Chapter 1 gives a basic introduction to the thesis presenting its aims, objectives and layout of the thesis; Chapter 2 presents an overview of the literature relating to the topics addressed in this thesis; Chapter 3 introduces the population of patients with VWD and the general methods used throughout the studies in the thesis; Chapter 4 addresses various issues related to type 1 VWD, including the criteria used to define type 1 VWD and the influence of the ABO blood group; Chapter 5 explores the problems related to the classification of VWD focusing on the assessment of phenotypic laboratory tests; Chapter 6 and Chapter 7 underline the importance of molecular biology in the diagnosis of type 2 VWD; Chapter 8 is dedicated to the PFA-100™ testing; Chapter 9 analyses issues related to the treatment of VWD. Finally, in Chapter 10 the discussion and general conclusions related to the whole thesis are presented and issues that warrant further studies are highlighted.
CHAPTER 2

OVERVIEW OF VON WILLEBRAND DISEASE
2.1. HISTORICAL PERSPECTIVE

Von Willebrand disease (VWD) was first described in 1926 by Dr Erik von Willebrand (Figure 2.1) who identified a hereditary bleeding disorder in 23 out of 66 members of a family from the Åland archipelago in the Gulf of Bothnia situated between Sweden and Finland (von Willebrand 1926).

![Figure 2.1. Dr Erik von Willebrand](image)

The proband, a 5 year old girl called Hjordis S., suffered from severe mucosal bleeding and later died of uncontrolled haemorrhage during her fourth menstrual period at the age of 13 (Figure 2.2). All but one of her 11 siblings had bleeding symptoms like both parents, who were third cousins. Four of her sisters had died of uncontrollable bleeding in
early childhood. Family members were found to have prolonged bleeding times but normal coagulation times and platelet counts. Clot retraction in the proband was normal. Von Willebrand recognised the autosomal dominant pattern of this disorder and called it 'hereditary pseudohemophilia'. He attributed the disease to abnormalities in the vessel wall, as well as to a functional disorder of platelets.

![Family Tree Diagram]

**Figure 2.2.** The original family tree of Family S from Fölgö.

At the beginning of the 1950s it was shown that the level of factor VIII (FVIII), at that time called antihemophilic globulin or antihemophilic factor, was low, not only in patients with haemophilia A, but also in males and females suffering from a severe inherited bleeding disorder similar to that described by von Willebrand disease. Nilsson et al. also described 10 families in Sweden with a severe bleeding disorder characterised
by a FVIII deficiency and a prolonged bleeding time (Nilsson 1957). They found that administration of plasma fraction I-O, containing both FVIII and von Willebrand factor (VWF), corrected both the prolonged bleeding time and the FVIII levels of individuals with VWD (Nilsson 1957). Similarly, plasma fraction I-O prepared from an individual with hemophilia was found to decrease the BT and to improve the FVIII levels in VWD individuals (Nilsson 1959). Cornu et al further observed that when individuals with VWD were transfused with plasma from individuals with haemophilia A, there was a paradoxical and sustained rise in the FVIII level (Cornu 1963). This pattern was quite distinct from the abrupt rise and fall of FVIII level obtained when a individual with haemophilia A was transfused with plasma from a normal individual or a VWD patient (Cornu 1963, Larieu 1968). This difference in response to plasma infusion was often used to distinguish VWD and haemophilia A.

In 1971 Howard and Firkin developed a method, the ristocetin co-factor test, using the antibiotic ristocetin to induce platelet aggregation in platelet rich plasma from normal individuals, but not from the majority of VWD patients (Howard 1971). Later, it was shown that the platelet aggregation induced by ristocetin and the platelet adhesion to subendothelium are dependent on VWF (Weiss 1974). When immunological assays (Zimmerman 1971) became available for measuring the von Willebrand factor antigen referred to as FVIII-related antigen (FVIII-RAg) until the mid 1980s, it was possible to verify patients with decreased levels of FVIII-RAg, in addition to the decreased FVIII level, making the diagnosis of VWD became easier. In 1980, a method for analysing the multimeric structure of VWF in plasma was developed (Ruggeri and Zimmerman 1981)
and an abnormal pattern of multimers was shown in some cases. Subsequently, based on the different multimeric patterns, various forms were classified as subtypes of the disease.

In 1984 the FVIII cDNA was cloned (Toole 1984) followed by cloning of VWF one year later (Ginsburg 1985, Lynch 1985, Sadler 1985, Verweij 1985). The advent of the polymerase chain reaction (PCR) has led to the precise characterisation of many of the molecular defects in individuals with VWD. In 1994 a revised classification of VWD defined by the presence of a gene defect of the locus of VWF and essentially based on phenotypic characteristics was established (Sadler 1994).

VWF plays two main roles in the primary haemostasis and blood coagulation: firstly, in the adhesion and aggregation of the platelets at the site of vascular injury with subsequent formation of the platelet plug and secondly, as a carrier protein for factor VIII with stabilisation of the FVIII in the circulation and its protection against degradation by factor Xa and activated protein C (Weiss 1977). In order to better understand the multiple roles of VWF in haemostasis and the pathogenesis of VWD, a brief overview of the synthesis, structure and function of the VWF and of the VWF gene are presented.
2.2. BIOSYNTHESIS, STRUCTURE AND FUNCTION OF VON WILLEBRAND FACTOR

The biosynthesis of VWF is extremely complex. VWF is synthesised in the endothelial cells and the megakaryocytes as a precursor molecule known as pro-pre-VWF which is a 2813 amino acid polypeptide. It consists of a 22 amino acid residue signal peptide, a large 741 amino acid residue propeptide (also known as VWF Ag II) and the mature VWF subunit of 2050 amino acid. Following translation of the mRNA, the signal peptide is removed, and the remaining pro-VWF undergoes an initial glycosylation and extensive post-translational processing to produce the multimeric VWF. Figure 2.3. demonstrates the genetic and biochemical structure of the propeptide-mature VWF monomer.

Figure 2.3. Schema of genetic and biochemical structure of von Willebrand factor.
In the rough reticulum endoplasmic the pro-VWF undergoes dimerisation by formation of disulphide bonds between the carboxy-terminal ends resulting in a dimer (protomer) which is made up of two subunits with a total molecular weight (MW) of 550 KD. The dimers are transported to Golgi and post Golgi compartments where further cysteine residues in the protomer yields a series of oligomers by formation of disulfide bonds between the amino-terminal parts. The oligomers further aggregate to form multimers of varying sizes. Meanwhile, the propeptide (841 amino acid) is removed from all the mature protein (2050 amino acid). Thus, the mature VWF is a heterogenous collection of a series of multimers, from a minimum of two to a maximum of 50-100 subunits. In addition to the disulfide bond formation, a post-translational modification includes the addition of 12 N-linked and 10 O-linked carbohydrates which probably have a direct role in maintaining the integrity of the multimeric structure. Mature VWF is stored in Weibel–Palade bodies of endothelial cells and α–granules of platelets (Wagner 1990).

A characteristic feature of VWF is the high cysteine content, 169 out of 2050 residues. These cysteines are essential for the linkage of subunits into higher order structures and conformation of functional domains (Azuma 1993). The propeptide (VWF antigen II) and mature subunit of VWF are almost entirely composed of four repeating domains, designated A through D (Shelton-Inloes 1986), arranged from amino to carboxy-terminal in the following order: D1-D2-D3- D'-A1-A2-A3-D4-B1-B2-B3-C1-C2 (Figure 2.3).
Functional domains of VWF

i. Multimerisation domain

The VWF is a multimeric glycoprotein and the large forms of multimers are crucial for promoting the haemostatic plug. Both D domains in the propeptide have been shown to be necessary for multimers formation and further storage of VWF protein (Journet 1993).

ii. FVIII binding site

The binding site for FVIII is located within the first 272 amino acid residues of the N-terminal of the mature VWF subunit (Foster 1987). An important role in the binding of FVIII has been attributed to the arginine residues present in the binding region and also to the conformation of the VWF (Layet 1992). Apparently, multimerisation of the VWF protein does not affect the binding to FVIII and the protective effect of VWF is not related to the size of the multimers (Fischer 1999).

iii. Binding sites to platelets and damaged subendothelium

VWF works as a ‘bridge’ by linking platelets to damaged blood vessel walls (platelet adhesion) or to platelets themselves (platelet aggregation) under conditions of high shear stress (Figure 2.4). The sequence of events appears to be the following: binding of VWF to components of the subendothelium (mainly collagen) which causes conformational changes of the protein and promotes the VWF binding to platelet receptor glycoprotein GpIb (platelet adhesion). In vitro, ristocetin and botrocetin can induce conformational changes which allow interaction of VWF to GpIb/IX/V complex without the
subendothelium (Andrews 1989) and in vivo, the conformational changes can be induced by high shear stress (Ikeda 1991). Once the VWF is bound to GPIb complex, this induces transduction of an intra-platelet signal leading to the expression of a functional integrin glycoprotein GPIIb/IIIa complex on the platelet membrane. Binding of VWF to GPIIb/IIIa receptor induces irreversible platelet adhesion, platelet spreading and platelet aggregation (Mazurier 1996).

Figure 2.4. Functions of circulating VWF

Platelet activation leads to the release of the large multimers of VWF from the α-granules, which contribute to platelet adhesion to collagen, platelet aggregation and
thrombus formation (Moake 1988). Importantly, platelet aggregation and collagen binding are strictly dependent on the degree of multimerisation as only the HMW forms of VWF are capable of initiating these interactions (Fischer 1999). In addition, these functions of VWF are dependent on the presence of a series of binding sites for specific receptors. Two fragments in the A1 and A3 domain have been identified to be important for the collagen binding. GPIb binding sites on VWF are located within the A1 disulfide loop, between Cys509-Cys695 residues. The A1 domain also contains binding sites for the snake-venom protein botrocetin, heparin and sulfatides. Under basal conditions there is little interaction between VWF and GPIb receptor and only immobilization on a thrombogenic surface such as collagen induces conformational changes within the VWF molecule which permit the binding to GPIb to occur. In addition high shear forces may act on the conformation of the VWF but platelet activation is not required in this process. The specific binding site for GPIIb/IIIa is the sequence Arg-Gly-Asp (RGD) which corresponds to the residues 1744-1746 at the carboxy-terminal domain C1.

The complex relationship between VWF structure and function has been better understood with the knowledge of the three-dimensional structure of the VWF protein. To date, the crystal structure of the two homologous VWF domains A1 and A3 have been solved. Both domains have a typical $\alpha/\beta$ fold consisting of a central $\beta$-sheet flanked by $\alpha$-helices on each side. The A1 domain was crystalised in complex with the Fab fragment of the functional blocking antibody NMC-4 and this provides information on the possible location of the GPIb binding site (Celikel 1998).
2.3. VON WILLEBRAND FACTOR GENE

The VWF gene is located on the short arm of chromosome 12 and spans approximately 178 kilobase (kb) of genomic DNA. It contains 52 exons and 51 introns of which exon 28 is exceptionally large at 1379 nucleotides (nt). The transcribed 8.7 Kb VWF mRNA is expressed only by the vascular endothelial cells and the bone marrow megakaryocytes. Analysis of VWF gene is complicated by the existence of a partial, unprocessed pseudogene at chromosome 22. The pseudogene is 21-29 kb in length and corresponds to exons 23-34 of VWF gene. The pseudogene has 97% homology with the authentic gene. The sequence divergencies between the real gene and the pseudogene can be used to design oligonucleotide primers for selective PCR amplification of VWF gene segments for the detection of mutations.

**VWF Database Nomenclature**

The standard nomenclature for VWF gene mutations and polymorphisms has been recently updated and published on behalf of the ISTH SSC Subcommittee on VWF (Goodeve 2001). Nucleotide are numbered according to their position within the VWF mRNA (or complementary DNA cDNA) starting with the "A" of the initiator "ATG" codon as +1. Amino acids (aa) numbering should be from the start of translation with the initiator ATG methionine as the +1 position with sequential numbering of aa throughout VWF.
However, throughout this thesis the numbering of the nucleotides and amino acids is based on the previous recommendations as follows: nucleotides in the VWF (cDNA) are numbered from the major transcription cap site which is located 250 nucleotides before the first nucleotide in the ATG codon for the initiating methionine. Amino acid positions are numbered from the initiator methionine as +1, for the residues in the propeptide and from serine 764 (N-terminal residue of processed VWF) as +1 for residues in the mature VWF subunit (Ginsburg and Sadler 1993). This preferred nomenclature has been used throughout this thesis because it was the recommended one at the beginning of the thesis and this was maintained for consistency and familiarity reasons. Moreover, the majority of current published literature still uses the old nomenclature. To convert to the new nomenclature, 763 should be added to the amino acid position in the previous numbering of residues in the mature VWF subunit. Single letter amino acid code is used throughout the thesis.

An updated database for VWD mutations and polymorphisms is available on website (accessible on the internet at http://mmg2.im.med.umich.edu/vWF) and frequent referrals to the database are made throughout this thesis. At present over 200 mutations are registered in the VWF database.
2.4. CLASSIFICATION AND DIAGNOSIS OF VWD

2.4.1 Classification and molecular pathology of VWD

VWD is a result of quantitative and qualitative abnormalities in VWF. Three major categories are recognised and summarised in Table 2.1.

Table 2.1. A revised classification of VWD (adapted from Sadler and Gralnick 1994).

1. All VWD is caused by mutations at VWF locus.
2. a) Type 1 refers to partial quantitative deficiency of VWF.
   b) Type 2 refers to qualitative deficiency of VWF.
   c) Type 3 refers to a complete absence of VWF.
3. Type 2A VWD refers to qualitative variants with decreased platelet-dependent function that is associated with the absence of intermediate and high molecular weight VWF multimers.
4. Type 2B VWD refers to qualitative variants with increased affinity to platelet glycoprotein GPIb.
5. Type 2M VWD refers to qualitative variants with decreased platelet-dependent function that is not caused by the absence of high molecular weight VWF multimers.
6. Type 2N VWD refers to qualitative variants with markedly decreased affinity for factor VIII.
7. When recognised a mixed phenotype caused by compound heterozygosity is indicated by separate classification of each allele separated by a slash (/).
Type 1 VWD refers to partial quantitative deficiencies of VWF. Type 2 VWD includes qualitative abnormalities of VWF structure and function and it is further subdivided into four variants (2A, 2B, 2M and 2N) based on the phenotype. Type 3 VWD refers to the total absence of VWF from the circulation. These categories correspond to distinct pathophysiological mechanisms and are intended to correlate with distinct clinical features and therapeutic modalities. A characterisation of each subtype of VWD with an overview of the underlying molecular defects is summarised.

**Types of VWD**

**i. Type 1 VWD**

In type 1 VWD there is a decreased levels of normal circulating VWF. Hence, the VWF multimers have a normal distribution and the function of the VWF is also normal. This type is the commonest form of the disease. Based on the plasma and platelet VWF levels several subgroups of VWD type 1 have been recognised: platelet normal, where the VWF levels are decreased in plasma but normal in the platelets, 'platelet low' where the VWF levels are decreased in both plasma and platelets and ‘platelet discordant’ where the ristocetin cofactor activities and the HMW multimers are reduced in both plasma and platelets but the levels of VWF are normal in platelets. As in the ‘platelet discordant’ subtype qualitative abnormalities of VWF are present this subgroup is best classified as type 2 defect. This subclassification is not routinely used but it is of clinical relevance in predicting the response to desmopressin which correlates positively with the platelet VWF level (Mannucci 1985). Thus, patients with platelet ‘low’ and ‘discordant’ are
usually unresponsive to DDAVP and when measured the bleeding time, which is
dependent on the platelet content of VWF, is not corrected after DDAVP.

Type 1 VWD exhibits a significant phenotypic heterogeneity, even within the same
family. This large variability can be attributed to various factors, such as the effects of
the blood group O (Gill 1987, Orstavik 1989), estrogen (Triplett 1991), stress (Rickles
1976) and by recently described polymorphisms in the promotor region of VWF which
can lead to variation in the VWF antigen circulating levels (Keightley 1999, Harvey
2000). It has been estimated that 30% of the variance of VWF antigen levels are due to
the ABO blood type (Orstavik 1989). The relationship between ABO blood type and type
1 VWD and its influence on the bleeding manifestation and the impact on classification
are discussed in detail in Chapter 4.

While gene deletions, missense or nonsense mutations all can cause reduced levels of
VWF, the genetic defect causing the VWF mutation in the majority of patients with type
1 VWD is not known. Type 1 VWD is most commonly inherited by an autosomal
dominant mode but recessive inheritance has been described. Two distinct groups of type
1 VWD are recognised. One group represents individuals who are heterozygous carriers
of a type 3 defect (they have a null allele i.e. silent allele which does not express at
mRNA level). The phenotype in the carrier of a null allele is very mild or asymptomatic,
the VWF levels are mildly reduced or normal and it has a very low penetrance. The
genetic defects described in these heterozygous type 1 individuals is similar to those
found in type 3 VWD (mainly deletions, frameshifts and nonsense mutations) so both
type 1 and type 3 VWD probably represent the spectrum ends of the same disease. The diagnosis of mild forms of type 1 VWD is difficult and this is explored in *Chapter 4*.

The second group of patients with type 1 VWD has an autosomal dominant inheritance with very high penetrance, very low levels of VWF and a moderate to severe symptomatology. The underlying molecular mechanism represents an abnormal allele which inhibits the function of a coinherited normal allele and leads to the formation of abnormal subunits of VWF. Those dimers which contain abnormal subunits are retained in the endoplasmic reticulum and they are not released in the circulation leading to markedly reduced levels of VWF in plasma (the dominant negative effect of the abnormal allele on the normal allele). Mutations in the D3 domain which lead to the loss of the disulphide bridge between the monomers have been associated with this distinct severe phenotype (Eikenboom 1996).

Traditionally, VWD is thought to result from defects in the VWF gene. However, a defect in another gene than VWF gene could potentially account for a VWD phenotype. Linkage analysis in the inbred mouse strain RIII/J, an animal model for type 1 VWD, has shown that the low VWF levels in these mice is not due to a defect at the VWF locus, but rather to a novel locus on mouse chromosome 11 (Nichols 1995, Mohlke 1996).

### ii. Type 2A VWD

Type 2A VWD is characterised by the absence of intermediate and large forms of VWF from plasma caused by a defective VWF–dependent platelet interaction. Also, in type 2A
there is a characteristic increase in the small satellite band of each multimer and an accumulation of a distinct 176 KDa proteolytic fragment of the mature VWF monomer which can be visualised on reducing gels (Ruggeri and Zimmerman 1981). A large variation in the satellite bands and the triplet structure of the multimers is observed across various subtypes incorporated in 2A VWD classification, and some are the result of proteolytic degradation (Zimmerman 1986).

The inheritance of type 2A VWD is both dominant and recessive. The dominant type 2A VWD accounts for the majority of type 2 VWD (Holmberg 1992). At least 24 missense mutations and one small in-frame deletions are known to cause dominant type 2A VWD (Ginsburg 1994). The majority of these mutations are situated within a short segment of the A2 domain (between G742 to E875) and only a few in the A1 domain. The molecular characterisation of type 2A VWD will be described in detail in Chapter 7.

iii. Type 2B VWD

Type 2B VWD is characterised by an increased affinity of the mutant VWF for platelets. This causes spontaneous binding of large VWF multimers to platelets and subsequent rapid clearance of the VWF HMW multimers-platelets complex from the circulation. The inheritance is dominant. Thrombocytopenia can be present and may be intermittent and can be exacerbated by pregnancy, stress, surgery or DDAVP. Whilst the HMW multimers are absent from plasma, the platelet VWF multimers are normal (Ruggeri 1980). The increased binding of VWF to platelets can be reproduced in vitro by adding ristocetin to platelet rich plasma and an enhanced response is observed at small doses of
ristocetin (increased RIPA). At least 21 mutations have been described and they are confined to the A1 domain which contains the GPIb binding site. The most frequent mutations associated with type 2B VWD are R543W, R545W, V553M and R578Q, which account for 90% of all cases. Rare variants of type 2B VWD with normal distribution of plasma VWF multimers and enhanced RIPA have also been described (Holmberg 1986, Weiss 1986).

iv. Type 2M VWD

Type 2M VWD (M for multimers) refers to variants of VWD with defective VWF-dependent platelet adhesion that is associated with normal or nearly normal multimers. To date, mutations described in association with type 2M VWD are localised in the C-terminal part of the C509-C695 disulphide loop of the A1 domain and impair binding to GPIb. Among the well characterised mutations causing type 2M VWD are the G561S substitution (Rabinowitz 1992), a deletion of 11 amino acid at R629 (type 2M :Milwaukee-1) (Mancuso 1996), and more recently the del K654 (Hilbert 2000). The substitutions found at the residue R611 are contentious as to the correct classification and this will be further explored in Chapter 6. Type 2M Vicenza represents a subgroup of patients who exhibit ultra large multimers (‘supranormal’) (Mannucci 1989). Candidate mutations for type 2M Vicenza have been recently identified in the D3 domain (Schneppenheim 2000).
**v. Type 2N VWD**

Type 2N VWD ('N' from Normandy) is a qualitative variant of VWD which was first described in 1989 (Nishino 1989). This variant is characterised by a defect in the VWF molecule which results in a reduced capacity to bind FVIII caused by mutations in the FVIII binding domain of VWF (Mazurier 1992). Typically the FVIII levels are reduced but the plasma and platelet VWF levels are normal with normal patterns of plasma and platelet multimers. The functional interaction between VWF and platelets is also normal. Symptomatically type 2N VWD is very similar to mild haemophilia A, and patients with 2N VWD can be misdiagnosed as sufferers or carriers of haemophilia A. However, the inheritance of the two conditions is different (2N VWD is autosomal recessive and haemophilia A is an X-linked disorder).

A reduced FVIII binding to VWF can also be caused by mutations affecting the VWF binding domain of FVIII molecule which give rise to mild/moderate haemophilia A. The FVIII regions involved in binding to VWF are the amino terminal part of the A3 domain and the C2 domains of FVIII (Saenko 1994). Recently mutations in the C1 domain of FVIII have also been associated with reduce FVIII binding to VWF (Jacquemin 2000) leading to moderate haemophilia A.

The FVIII binding site is found within the mature VWF subunit from amino acid 1-272 of the N terminal and is encoded by exons 18-23 of the gene (Foster 1987; Takahashi 1987). Layet at al suggested that the sequence between amino acid 272 and 911 was also important for either binding of FVIII or its protection from activated protein C (Layet
To date, six mutations in the FVIII binding region of VWF have been reported: R19W and H54Q, E24K, T28M, R53W and R91Q. 75% of all mutations are accounted for by the R91Q, the R53W and T28M substitutions. The most common of these is the R91Q mutation encoded by exon 20 (Mazurier 1992).

vi. Type 3 VWD

Type 3 VWD includes patients with a total lack of VWF who present with severe, potentially life-threatening bleedings. It is inherited as an autosomal recessive trait and it is seen only in patients who are homozygous for the same mutant allele or compound heterozygous for two different alleles. The most common type 3 mutation reported in the northern Europe is a frameshift mutation in exon 18 resulting from a cytosine deletion (Zhang 1992). A recent study of a multiethnic group of patients with type 3 VWD reported a large number of mutations arising randomly within the entire VWF gene and often responsible for null alleles (Baronciani 2000). The presence of gene deletions in both alleles seem to predispose to the formation of alloantibodies which can be found in approximately 10% of individuals with type 3 VWD (Shelton-Inloes, 1987).

vii. Platelet-type (Pseudo) Von Willebrand disease

Pseudo-VWD is not a genetic defect of VWF, but presents with similar symptoms and initial laboratory findings suggestive of type 2B VWD. The defect is caused by a mutation in the VWF receptor on platelets, GPIb, which results in spontaneous binding to plasma VWF and clearance of large multimers and platelets from the blood.
2.4.2. EPIDEMIOLOGY OF VWD

VWD is the most common inherited bleeding disorder in humans, with a reported prevalence between 0.1% and 3%, depending on the disease definition (Ginsburg 1999). The majority of individuals identified in these prevalence studies appear to have type 1 VWD (Rodeghiero 1987, Werner 1993) however, it is likely that many individuals who are asymptomatic remain undiagnosed. The correct estimation of the prevalence is difficult as VWD has a variable penetrance and expression. Furthermore several variables can affect the level of circulating VWF and also the diagnostic criteria are often limited and difficult. In clinical terms, only one in ten families have clinically relevant disease and only 10-20 cases/million require specific treatment (Holmberg 1985).

Of all VWD, type 1 comprises about 70-80%. Type 3 VWD has an estimated prevalence of 0.1 to 3 per million population (Weiss 1982). Type 2 accounts for about 20% of all VWD (Ruggeri 1991) with type 2A the most common subtype (15% of all VWD) and type 2B accounting for 5% of VWD cases (Ginsburg 1992). Other variants of VWD are rare with only a few case reports, although the prevalence of type 2N may be substantial (Eikenboom 1993). Recently, type 2M and 2M Vicenza have been reported with a higher frequency than previously thought (Zieger 1997, Schneppenheim 2000).

2.4.3. CLINICAL MANIFESTATIONS OF VWD

Because VWF is essential for the formation of the platelet plug, the pattern of bleeding observed in VWD is muco-cutaneous and the most frequent bleeding manifestations are
epistaxis, heavy periods, bleeding after dental extraction and easy bruising (Silwer 1973). Gastrointestinal bleeding are not frequent but can be very serious and sometimes underlying angiodysplasia can be present (Ramsay 1976, Fressinaud 1993). In many cases the symptoms are mild to moderate and sometimes the bleeding tendency manifests itself only when the individual is exposed to a haemostatic challenge, such as surgery. In the severe type 3 forms intra-articular and deep muscle bleedings similar to those observed in patients with severe haemophilia A are observed. The symptoms may vary among family members and also over time in the same individual.

Some of the most important clinical issues in VWD are related to women. Menorrhagia is a very common symptom and in a recent study VWD was diagnosed in 13% of women presenting with menorrhagia (Kadir 1998). Other important issues related to women with VWD are corpus luteum bleeding, pregnancy, delivery and postpartum bleeding problems.

2.5. LABORATORY DIAGNOSIS OF VON WILLEBRAND DISEASE

The laboratory diagnosis in VWD relies on phenotypic tests. These can be divided into screening tests, diagnostic tests and discriminatory tests. DNA analysis is only a complementary tool which is not as yet a routine diagnostic method.
2.5.1 Screening tests for VWD

i. **Bleeding time**

Bleeding time (BT) represents a screening test for primary haemostasis reflecting of the ability to form the platelet plug and offers an initial assessment of VWF and the platelet interaction with the subendothelium. The main limitations of the BT are discomfort to the individual, poor reproducibility and lack of sensitivity.

The current method used to measure the BT is the modified Ivy technique where an incision is made on the forearm using a disposable device while venous pressure is maintained at 40 mmHg with a blood pressure cuff. The normal time for cessation of bleeding is BT is between 8-10 minutes.

ii. **Closure time by Platelet Function Analyser (PFA-100™)**

The closure time as measured by PFA-100™ is a new global screening test for primary haemostasis. As with the BT it is not specific for VWD but it is considered superior to BT in terms of sensitivity, reproducibility and ease of execution (Fressinaud 1998). The role of the PFA testing in the VWD patients is further discussed in *Chapter 8*.

iii. **Platelet count**

Platelet count is usually normal in VWD, except in type 2B VWD where it can be low.
iv. **APTT (activated partial thromboplastin time)**

This is a screening test used for determination of abnormalities in the classical intrinsic pathway of the coagulation cascade. A prolonged APTT may be due to a variety of inherited or acquired coagulopathy. In VWD the APTT is usually prolonged but it can be normal if the FVIII levels are normal.

### 2.5.2. Specific diagnostic tests

i. **Von Willebrand factor antigen (VWF:Ag)**

VWF:Ag is quantitated by using a polyclonal rabbit anti-VWF antibody, by either Laurell rocket electroimmunoassay or by an enzyme linked immunoabsorbant assay (ELISA).

ii. **Factor VIII coagulant activity (FVIII:C)**

FVIII:C is usually determined by one-stage APPT based clotting assay or by using a chromogenic assay.

iii. **Ristocetin cofactor activity (VWF:RiCo)**

The negatively charged antibiotic ristocetin induces the agglutination of platelets in the presence of VWF by inducing the binding of VWF to the GpIb receptor on the platelets. The test is performed on an aggregometer by adding ristocetin to a mixture of control platelets which are either fresh washed or formalin-fixed and the patient platelet-poor
plasma (Macfarlane 1975). The VWF:RiCo assay reflects the function of VWF by measuring the ability of VWF to bind to the GPIb platelet receptor.

More recently an ELISA-based method became available for assessment of the ability of VWF to bind to GPIb (Murdoch 1997) by using a monoclonal antibody directed against the GPIb binding site in the VWF-A1 domain. The ELISA activity is considered as a replacement for the VWF:RiCo, but findings described in this thesis suggest that these two tests are not interchangeable (Chapter 5).

Another VWF functional assay is the collagen binding assay (VWF:CBA) which can assess the ability of VWF to bind to various types of collagen (Favolorolo 1993) and which will be described in detail in Chapter 5.

2.5.3 Discriminating tests of VWD type and subtype

i. Ristocetin induced platelet aggregation (RIPA)

Several doses of ristocetin (0.2-1.5 mg/ml) are added to platelet-rich plasma in order to determine the lowest concentration of ristocetin which is able to induce platelet aggregation via the binding of VWF to GPIb receptor. An enhanced RIPA (platelet aggregation obtained by adding very low doses of ristocetin < 0.6mg.ml) is indicative of an increased binding of VWF to GPIb receptor and characteristic of type 2B VWD or 'platelet type' VWD.
ii. VWF multimers analysis

The multimer composition of VWF from plasma and platelets can be determined by agarose gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) (Ruggeri 1981). A variety of techniques are used for the detection of multimers, such as I\textsubscript{125}–labelled antibodies to VWF and visualisation by autoradiography. Certain multimeric patterns are characteristic of various types of VWD, as illustrated in Figure 2.5.

**Figure 2.5.** Plasma VWF multimeric patterns
The proteolytic degradation of the plasma VWF is indicated by the occurrence of the triplet pattern, made visible by high resolution gels (Ruggeri 1981, Fischer 1988). The intermediate (central) band in each triplet represents multiple numbers of VWF dimers (Fischer 1988) and the faster and slower migrating bands of the triplet have too many or too few subunit structures.

iii. FVIII binding assay

This assay measures the functional ability of VWF to bind to factor VIII which is estimated using a chromogenic or an ELISA method (Nishino 1989, Mazurier 1990). A decreased affinity of VWF to FVIII is characteristic for type 2N VWD.

iv. Botrocetin induced binding assay to GPIb

Botrocetin is a snake venom derived from Bothrops jararaca which can stimulate the VWF binding to platelets in a similar fashion to ristocetin. However, ristocetin and botrocetin have distinct binding sites on the VWF A1 domain. The discrepancy between ristocetin and botrocetin induced VWF binding to platelets appears to be useful in defining type 2M VWD.

2.5.3. MOLECULAR DIAGNOSIS OF VWD

Due to the large size of VWF gene (178 kb) most molecular defects have not yet been identified. For type 1 and type 3 the defect can be scattered throughout the gene and the
screening of the whole gene remains difficult. However, for type 2 VWD the molecular
defects tend to be localised in certain exons corresponding to the functional domains of
the protein (for type 2A, 2B and 2M exon 28 and for type 2N exons 18-23).

Linkage analysis techniques are less frequently used but gene tracking by analysis of
restriction fragment length polymorphisms (RFLPs) or variable number of tandem
repeats (VNTR) can be used. Informative VNTRs have been located in intron 40 (Peake
1990) and have been successfully used in prenatal diagnosis, in carrier detection in
recessive forms of VWD and in families with type 1 VWD where the diagnosis may be
phenotypically uncertain.

2.6. TREATMENT OF VON WILLEBRAND DISEASE

The aim of therapy in patients with VWD is to correct the dual defect of haemostasis,
namely the prolonged bleeding time due to the platelet adhesive defect and the abnormal
coaagulation due to low levels of FVIII. There are two treatments of choice for VWD:
desmopressin (DDAVP) and replacement therapy with blood products (Mannucci 1998).

i. Desmopressin

DDAVP is the treatment of choice for type 1 VWD and certain type 2 VWD, but it is
ineffective in type 3 patients. The response to DDAVP is variable and it should be
assessed on an individual basis. Administration of DDAVP and the response to treatment
in different types of VWD is addressed in Chapter 9.
\textit{ii. Transfusional therapies}

For patients unresponsive to DDAVP replacement therapy with blood products containing FVIII and VWF is available. FVIII-VWF may be infused as fresh frozen plasma, cryoprecipitate or plasma derived virus-inactivated clotting factor concentrates. All forms of currently available replacement therapy carry some risk of viral transmission (Mannucci 1992). The concentrates commonly used in VWD are intermediate-purity (FVIII 1-5 IU/mg of total protein) and high-purity (50-250 IU/mg). These concentrates have been successfully used for the management of VWD patients despite inconsistent effects on the bleeding time (Rodeghiero 1988). Moreover, there are several limitations connected to the plasma-derived VWF concentrates, such as proteolytic degradation during the manufacturing process, variation in multimer composition, reduced number of high molecular weight multimers, but some of these limitations can be overcome by the recombinant VWF product (Fischer 1999). A very high purity plasma derived VWF concentrate (rich in VWF and low in FVIII) is also available (Burnouf-Radosevich 1992) and has been efficaciously used in type 3 VWD patients (Meriane 1993). Platelet concentrates due to their content of VWF are also useful and have been shown to correct the bleeding time. Therapy with clotting factor concentrates in VWD is largely empirical and is often determined by the response to prior treatment. For surgical intervention the optimal treatment and monitoring of the response to treatment is not clear and this topic is addressed in detail in \textit{Chapter 9}.
iii. Adjunctive therapy

Adjunctive therapy for VWD includes antifibrinolytics and oestrogens. Antifibrinolytics prevent the lysis of the newly formed clot by saturating sites on plasminogen and thus making it unavailable to attach to the fibrin. The most commonly used antifibrinolytics are epsilon aminocaproic acid and tranexamic acid, which can be used alone or as adjuncts for muco-cutaneous types of bleeding. Antifibrinolytics are contraindicated in upper urinary tract bleeding (due to the risk of ureteric colic) and in prothrombotic states.

Oestrogen increase FVIII/VWF and partially correct the bleeding time (Alperin 1982) via a direct effect on the endothelial cells (Harrison 1984). In the form of combined oral contraceptive they represent a useful treatment for menorrhagia.
CHAPTER 3

PATIENTS AND GENERAL METHODS
3.1. PATIENTS

3.1.1. CHARACTERISTICS OF THE POPULATION WITH VWD

REGISTERED AT THE RFHHC - ESTABLISHING A PATIENTS' DATABASE

At the outset of this thesis a database containing the most important characteristics of the population of patients registered with VWD at the RFHHC was established. All studies described in this thesis have stemmed from this database: selected patients were entered in several prospective studies and the available data on this population was also analysed retrospectively.

Each individual medical record was reviewed and relevant data on each patient was collected. All data was entered into a computer program (Microsoft Excel 97). The data from each patient included the following: sex, age, diagnosis of type of VWD, prothrombin time, activated thromboplastin time (APPT), bleeding time, VWF:Ag, VWF:AC or VWF:RiCo, FVIII:C, RIPA, plasma multimers analysis, details of the family history, the bleeding history, the treatment received and any relevant additional information (for example the presence of additional diagnosis, such as infection with HCV or additional clotting factor deficiencies). When available, the values for the VWF and FVIII:C levels were taken as the median of at least two determinations performed on separate occasion and the date when the tests were performed were also recorded.
The database was constantly updated until 1\textsuperscript{st} June 2000 and as more information regarding the type of VWD became available through the studies described in this thesis, the patients were continuously reclassified accordingly.

\textit{Description of the VWD population registered at the RFHHC}

By 1\textsuperscript{st} June 2000 a total of 463 individuals were registered with VWD at the RFHHC. The distribution of the types of VWD, as defined by the revised classification (Sadler 1994) is illustrated in Figure 3.1.

\begin{center}
\textbf{Classification of VWD population RFHHC}
\end{center}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure3_1}
\caption{Classification of patients with VWD registered at the RFHHC}
\end{figure}
In this population, 63% of all patients were female with the sex distribution of the male 172 patients and female 291 patients. The age ranges were 1-82 years, with a mean ±SD of 27 ±18 years.

Of the laboratory tests, data on the VWF:Ag, VWF:AC and FVIII:C levels and the bleeding time values are presented for each type of VWD in Table 3.1.

<table>
<thead>
<tr>
<th></th>
<th>Type 1</th>
<th>Type 2A</th>
<th>Type 2B</th>
<th>Type 2M</th>
<th>Type 2N</th>
<th>Type3</th>
</tr>
</thead>
<tbody>
<tr>
<td>No patients</td>
<td>463</td>
<td>367</td>
<td>23</td>
<td>14</td>
<td>38</td>
<td>3</td>
</tr>
<tr>
<td>VWF:Ag</td>
<td>41.8±17.3</td>
<td>35.9±13.3</td>
<td>50.7±16.4</td>
<td>28±14.6</td>
<td>44.3±36.6</td>
<td>2.6±2.7</td>
</tr>
<tr>
<td>VWF:AC</td>
<td>35.7±14.8</td>
<td>13.1±8.7</td>
<td>26.7±11.0</td>
<td>13.6±8.5</td>
<td>30.0±16.6</td>
<td>1.0±0.8</td>
</tr>
<tr>
<td>FVIII:C</td>
<td>59.5±23</td>
<td>43.4±15.1</td>
<td>53.6±21.3</td>
<td>45.8±18.6</td>
<td>10±3.6</td>
<td>6.1±6.3</td>
</tr>
<tr>
<td>BT</td>
<td>9.3±4.3</td>
<td>11.1±2.9</td>
<td>9.1±5.3</td>
<td>9.1±3.4</td>
<td>6</td>
<td>14.3±5.1</td>
</tr>
<tr>
<td>N</td>
<td>202</td>
<td>4</td>
<td>6</td>
<td>14</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1. Laboratory data on the patients with VWD registered at FRHHC. N = number of patients in whom the BT (min) was available. Values are given as mean ±SD.

3.1.2. VWF:AC/VWF:Ag and FVIII:C/VWF:Ag ratios in patients with VWD

The available data enabled the calculation of the VWF:AC/VWF:Ag and FVIII:C/VWF:Ag ratios, as recently it has been suggested that these ratios could be indicative of the type and sometimes of the underlying genetic mutation in patients with VWD.

VWF:AC/VWF:Ag ratio
Over the past few years it has become apparent that the use of the VWF:AC/VWF:Ag ratio represents an initial step in trying to discriminate between type 1 and type 2 VWD. As type 2 VWD is characterised by a functional defect in VWF, the VWF:AC is generally lower than the VWF:Ag leading to a discrepant ratio of less than 0.7 (Mancuso 1996, Federici 1997, Hillery 1998, Federici 2000). In contrast in type 1 VWD the ratio is characteristically concordant (> 0.7). In this population the mean±SD of the VWF:AC/VWF:Ag ratios for each subtype of VWD are presented in Table 3.2:

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Type 1</th>
<th>Type 2A</th>
<th>Type 2B</th>
<th>Type 2M</th>
<th>Type 2N</th>
<th>Type 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>VWF:AC</td>
<td>0.86±0.16</td>
<td>0.36±0.17</td>
<td>0.53±0.16</td>
<td>0.49±0.22</td>
<td>0.78±0.24</td>
<td>0.70±0.38</td>
</tr>
<tr>
<td>Ranges</td>
<td>0.14-1.5</td>
<td>0.08-0.69</td>
<td>0.16-0.83</td>
<td>0.14-1.33</td>
<td>0.57-1.05</td>
<td>0.1-1</td>
</tr>
</tbody>
</table>

Table 3.2. The VWF:AC/VWF:Ag ratio (as mean±SD and ranges) for patients with different types of VWD.

In the population of type 1 VWD patients registered at the RFHHC, the mean ratio VWF:AC/VWF:Ag was concordant. However, a wide range of ratios was noticed, including some patients with clearly discordant ratios. Starting from these observations, a group of patients originally classified as type 1 VWD with discrepant ratios in whom a type 2 VWD was suspected were selected for further analysis which is presented in detail in Chapter 6. Further testing showed that at least half of these patients were misclassified and they were in fact type 2 VWD.
In all patients classified as type 2 VWD the VWF:AC/VWF:Ag ratio was discordant and the lowest ratios were found in patients with type 2A VWD. This is in keeping with recent reports were the VWF:RCo/VWF:Ag ratio was found to be <0.3 in type 2A VWD but < 0.7 in type 2B and 2M (Federici 2000).

The importance of the VWF:AC/VWF:Ag ratio cannot be underestimated as it provides a simple and rapid assessment of whether the VWF defect present in a patient is quantitative or qualitative. This initial demarcation between quantitative versus qualitative abnormalities has profound clinical implications as will be discussed in Chapter 6.

**FVIII:C/VWF:Ag ratio**

Previous studies on normal controls have shown that, as expected, the normal FVIII:C/VWF:Ag ratio is near unity (between 0.6 to 1.6, Blomback 1980). In one study, the mean ratio of FVIII:C/VWF:AC and FVIII:C/VWF:Ag in normal individuals were 1.04 and 0.90, respectively (Eikenboom 1998).

In several mutation reports it has been shown that an increased ratio of FVIII to VWF suggests the presence of a carrier of a null allele (gene deletions, frameshift mutations, nonsense mutations and defects of mRNA expression) (Peake 1990, Standen 1990, Eikenboom 1993, Schenppenheim 1994, Zhang 1995, Eikenboom 1998). The ‘carriers’ have usually normal FVIII and lower VWF levels in plasma (mean value 22- 46 IU/dl) and the FVIII:C/VWF:Ag ratio is above 1.6 (usually
above 2) (Blomback 1992). Thus, a large group of individuals with type 1 VWD are 'carriers' and heterozygous for a quantitative mutation present on one allele (mutant/wt). If both parents are 'carriers' of VWD they can transmit the defective allele to their children who could be affected by severe type 3 VWD passed down through a recessive inheritance mode. Hence, type 3 VWD is a homozygous or compound heterozygous form of type 1 VWD. Clinically, the 'carriers' of VWD are very mildly affected or even asymptomatic (mild type 1 VWD).

This phenotypic feature of higher ratios of FVIII:C/VWF:Ag may be of use in identifying possible carriers of mutant VWF alleles. In the RFHHC VWD population 130/367 (35%) had the FVIII:C/VWF:Ag ratio > 1.6 (ranges 1.6-12.4) which suggests that they are possible carriers of VWD. Clinically this group of patients have less severe symptoms.

Some individuals with type 1 VWD have been shown to be compound heterozygous with a different mutation on each allele (mutation1/mutation 2) (Eikenboom 1992). In general one mutation gives rise to a null allele (with a defective expression at the RNA level) and the other mutation is usually a missense mutation, such as a type 2N mutation (Eikenboom 1993, Zhang 1993). In these situations the FVIII:C/VWF:Ag ratio is less than 1, between 0.4-0.7. Phenotypically these individuals are affected by severe bleeding symptoms (severe type 1 VWD).
At the RFHHC 42/367 (11%) patients with type 1 VWD had a FVIII:C/VWF:Ag ratio less than 1 and 10/367 (4%) had a ratio between 0.4-0.7. This group of patients are probably compound heterozygous for type 1/type 2 (frequently type 2N) VWD and indeed genetic analysis has shown the compound heterozygous state in several individuals who have been analysed. In general these patients had a more severe bleeding history than the patients with higher ratios.

In individuals who are heterozygous for type 2N VWD (wt/2N), the FVIII:C/VWF:Ag ratio is generally 1. In the RFHHC population with type 1 VWD 20/367 (5%) had a FVIII:C/VWF:Ag ratio of 1, suggesting that they may be heterozygous for type 2N VWD.

The homozygous patients for type 2N mutation (2N/2N) will have a very low FVIII:C/VWF:Ag ratio of < 0.2 due to the very low FVIII:C levels present in plasma (Eikenboom 1993, Zhang 1993), whereas the compound heterozygous patients with type 2N VWD have less discrepant ratio (FVIII/VWF:Ag between 0.4-0.7) Only the homozygous and compound heterozygous patients with type 2N VWD have associated bleeding problems and the heterozygous type 2N individuals tend to be asymptomatic (Mazurier 1992). At the RFHHC three patients were classified as type 2N VWD and the FVIII:C/VWF:Ag ratios were 0.1, 0.3 and 0.4 respectively. All three patients have a significant bleeding history. The FVIII binding assay (as it will be described in chapter 3) was suggestive of a homozygous pattern only in the patient with the ratio < 0.2. Genetic
analysis on these patients is not available at the present time, but the relationships between the phenotypic assay and the clinical picture are in keeping with the FVIII:C/VWF:Ag ratio.

The calculation of the ratios between FVIII:C/VWF:Ag is a simple and very informative tool which can suggest the more likely underlying genetic mechanism in patients with type 1 and type 2N VWD. These ratios merit further genotypic and phenotypic investigation and they can have a potential role as an initial step in diagnosis of VWD subtype.

3.1.3. PREVALENCE OF OTHER ASSOCIATED CLOTTING FACTOR DEFICIENCIES IN PATIENTS WITH VON WILLEBRAND DISEASE

VWD has been reported to occur in individuals who also have factor XI (FXI) or factor XII (FXII) deficiency. As little data is available on this topic, the RFHHC database offered the opportunity to study the prevalence of other clotting factor deficiencies in patients with VWD. At the time of performing this study, a total of 435 patients were registered with VWD and regularly followed-up at the RFHHC. A retrospective analysis showed that only 125/435 (28%) patients had a prolonged APTT (> 38 sec). Among this subgroup, seven (5%) patients were found to have associated other clotting factors deficiencies: two had a partial FXII deficiency (35 IU/dl and 42 IU/dl, respectively, NR: 50-150 IU/dl)) and five had partial FXI deficiency (median FXI 60 IU/dl, range 44-68
IU/dl, NR: 70-150 IU/dl). In a further four patients the prolonged APTT was accounted for by the presence of inhibitors: lupus antibodies (two patients), acquired VWF inhibitors (one patient) and alloantibodies to VWF (one patient). In the remaining patients there was no evidence of other associated clotting factors deficiencies and the prolonged APTT was due to decreased FVIII levels in the context of VWD.

In patients with VWD and a family history of other clotting factor deficiencies the respective factors were investigated despite a normal APTT. In those patients it was found that 10 additional patients had an associated partial FXI deficiency (median FXI 61 IU/dl, range 41-70 IU/dl) and two patients had an associated mild factor IX (FIX) deficiency (29 IU/dl and 35 IU/dl, respectively; NR: 50-150 IU/dl). Thus, in total, 19/435 (4%) patients with VWD were found to have an associated clotting factor deficiency and all 19 patients with combined deficiencies had type 1 VWD.

According to these findings, FXII deficiency is rare among patients with VWD (0.4%). This prevalence is much lower than the recently reported prevalence of 10% of FXII deficiency in a population of 270 patients with VWD compared to 1.5% found in healthy blood donors (Bux-Gewehr 2000). The findings of this study are also against the recommendations made by Bux-Gewehr et al. (Bux-Gewehr 2000) who suggested that FXII level should be determined in every diagnostic work-up of patients with VWD with a prolonged APTT, particularly when the APTT is used to monitor therapy. Moreover, using the APTT to monitor therapy is probably suboptimal and the determination of
FVIII and/or VWF activity levels is usually required. In addition to its low prevalence, the association of FXII deficiency does not apparently influence the course of VWD (Bux-Gewehr 2000).

Other clotting factors deficiencies involved in the intrinsic coagulation pathway were found with a similar (FIX) or higher (FXI) frequency than factor XII deficiency. Regarding factor XI, the high prevalence in this cohort from the RFHHC also reflects the ethnic background of the catchment area with a large Ashkenazi Jewish community. In contrast to factor XII, associated FIX and FXI deficiencies did influence the type and severity of the bleeding pattern and more importantly the management of the affected individuals.

In conclusion, this study showed that when finding a prolonged APTT in a patient with VWD, investigation for factor XII deficiency is probably not a priority, as it is rare and without clinical significance, in contrast to other combined deficiencies with FIX or FXI which should be carefully ruled out.

3.2. GENERAL METHODS

3.2.1 COAGULATION ASSAYS

An overview of the specific coagulation investigations for VWF analysis as performed in the RFHHC laboratory is described. For all coagulation tests venous whole blood was
collected into 0.106M sodium citrate (Sarstedt Monovette 9NC/3ml) tubes. After centrifugation at 2000g for 10 minutes at 4°C, samples were aliquoted into Sarstedt cryo tubes and stored at -70°C until further testing.

**Chemicals and Reagents**

Chemicals were supplied by BDH Chemical Ltd., and Sigma Chemical Company unless otherwise stated.

**Common reagents for ‘in-house’ ELISA methods**

A common methodology using the sandwich ELISA principle was adopted for a number of assays. The following buffers were used:

- **0.05M Bicarbonate buffer** pH 9.6
  
  1.59gm Na₂CO₃, 2.93gm 0.15M, NaHCO₃ 0.035M in 1 litre distilled water

- **0.155M High salt wash buffer with TWEEN (HSBT)** pH 7.4
  
  Na₂H₂PO₄ 0.3125M, Na₂HPO₄ 0.0935M, NaCl 1.25M

- **Tag and Sample Dilution buffer**
  
  15gm PEG 8000 in 500 ml HSBT
0.1M Substrate buffer  pH 5.0

7.3gm citric acid, 23.87gm Na₂HPO₄·12 H₂O in 1 litre distilled water

For all ELISA, NUNC maxisorb microtitre plates (Life Technologies Ltd., Paisley, Scotland) were used. The optical density readings were determined by a ‘Titertek plate reader’ (ICN, Flow Biomedicals LTD, Bucks). Each dilution of plasma was assayed in duplicate. In-house 20 normal pool (calibrated against the 7th British standard, NIBSC, Potters Bar, UK) was used as a standard.

a) FVIII coagulation assay (FVIII:C)

In-house twenty normal pool (20NP) (calibrated against international reference plasma for FVIII) was used as a standard in all assays. Three point APTT based, one-stage assays were performed on the ACL 3000R (IL Ltd., Warrington, UK). Patient platelet poor plasma (PPP) was diluted in 1:10, 1:20 and 1:40 in Owren’s Buffered Saline and compared to a three-point standard curve derived from pooled normal plasma. The FVIII deficient plasma was obtained from Diage (Diagnostic Reagents Ltd., Thames, Oxon).

b) ELISA for von Willebrand antigen (VWF:Ag)

Polyclonal anti-VWF:Ag (Dako Ltd., Bucks) was diluted 1/1000 in bicarbonate buffer. 100 μl of diluted coat antibody was added to each well of a microtitre plate. The plate was sealed and left at 4°C overnight. A seven-point standard curve (125-6.25 IU/dl) was
prepared using normal pool, initially diluted 1/80 (125 IU/dl). Test plasmas and controls (20NP and Baxter Dade abnormal control plasma “trol P”) were diluted to give a VWF:Ag concentration within the linear range of the standard curve (1/100 and 1/200). The coated plate was washed five times with HSBT, using a plate washer (Well Wash 5, Stacking Microtitre Plate washer, Denley), then inverted and blotted gently on absorbent paper. Test, control or standard curve dilutions were added in 100 μl volumes to the plate. The plate was sealed and incubated on a plate shaker (400-500 oscillations /min) (Amersham International plc., Bucks) for one hour at room temperature. At the end of incubation the plate was washed a further five times, after which 100 μl of diluted (1/8000) horseradish peroxidase conjugated anti-VWF antibody (Dako) was added to each well of the plate. The plate was again incubated for one hour on a plate shaker at room temperature. Just before the end of incubation the substrate solution was prepared by dissolving one 10 mg ortho-phenyline-diamine (OPD) tablet in 15 ml substrate buffer. The plate was then washed for five times. Immediately before the next step, 7 μl of 30% hydrogen peroxide was added to the substrate solution. 100 μl of substrate solution was then added to each well at time intervals (approx. one second). After ten min the reaction was stopped by addition of 100 μl 1.5M sulphuric acid to each well at the same interval. The absorbance of each well of the plate was then read within 30 min at 492 nm. The plate reader software calibrated a VWF:Ag standard curve (optical density against IU/dl on a semi-log scale) and then calculated the mean test and control results from the curve (Ceijka 1982).

c) ELISA for von Willebrand factor activity (VWF:AC)
The method is essentially the same as described for VWF:Ag with the following exceptions: coat antibody: 1/1000 (RFF-VIII:R/1 monoclonal antibody specific for Gplb-IX-V binding site of VWF); standard: initial dilution 1:20 (+125 IU/dl) and tests and controls: 1:25 and 1:50.

d) VWF Ristocetin cofactor activity (VWF:RiCo)

- **Preparation of fresh washed platelets**

40-60 mls of whole blood was collected into 0.134M dipotassium EDTA (10:1 whole blood to anticoagulant). The samples were centrifuged at 150-200 g. The platelet rich plasma (PRP) was aspirated into a round-bottomed plastic universal and the total volume level marked. The PRP was spun at 1500-2000 g (2500-300 rpm) for 15-20 min. The platelet poor plasma (PPP) was then discarded. The platelets were washed in 20 ml of EDTA–citrate saline (citrate saline: 1 part 31.3g/l tri-sodium citrate to 9 parts 0.9% saline; EDTA-Citrate saline: 1 vol 0.134 K$_2$EDTA to 9 vol citrate saline), by gentle resuspension. The platelet suspension was next centrifugated at 1500-2000 g for 15-20 min. After pelleting the EDTA-citrate saline supernatant was removed and the platelets washed again. This process of washing was repeated for a total of four times. After the final centrifugation, the EDTA-citrate saline was removed and the platelet pellet gently resuspended in citrate-saline to the original PRP volume. The platelet suspension was left for 30 min. By this stage the platelets were free of VWF. This was confirmed on a Payton platelet aggregometer connected to a pen recorder by adding 100 µl citrate-saline to 400 µl platelet suspension. This was considered zero aggregation. 100 % was calibrated as 500 µl of citrate-saline. 5 µl of ristocetin (to 1.0 mg/ml) was added and the recorder run
at 1 cm/min. The washed platelets were considered suitable if no aggregation was larger than 5 divisions over 2 min.

- **VWF:RiCo assay**

VWF:RiCo assay was measured by a Payton platelet aggregometer using fresh washed platelets from a normal donor (McFarlane 1975). As platelets aggregate the OD of the PRP falls, and this can be measured. Doubling dilutions of the 20 NP was prepared 1/2 or 1/32 for use as a standard curve. 100% aggregation for the sample was calibrated as 400 µl platelet suspension + 100 µl diluted plasma. 5 µl ristocetin was added to the zero cuvette and the aggregation recorded for 2 min. Patients plasma were tested similarly at two dilutions that fall within the aggregation range of the standard curve. The standard curve is plotted on a log-linear paper (2 cycles) with aggregation in divisions on the linear scale and u/dl on the log scale. The patient VWF:RiCo was read off the standard curve.

e) **Von Willebrand factor Collagen binding assay (VWF:CBA)**

VWF:CBA was measured using Type III collagen (Human placenta type X acid soluble, Sigma Chemicals, Poole, Dorset, UK) as previously described by (Brown & Boask 1986). Briefly 25mg of Type III collagen was dissolved in 20mls of 50mM acetic acid, and then diluted to a final concentration of 4µg/ml. ELISA plates were coated with 100µl of 4µg/ml Type III collagen in coating buffer (0.14M NaCl, 0.006M Na₂HPO₄, 0.002M NaH₂PO₄, 0.03M KCl pH 7.3). The coated plates were sealed and left at room temperature for 24 hours, and then placed at 4°C for a further 48 hours prior to use.
Patient and control plasma was diluted (1/200 and 1/400 in 3% w/v PEG dilution buffer) and aliquoted. The ELISA plates were incubated and washed as described as for the VWF:Ag assay. Patient and control values were compared to a 20 normal pool standard curve. A value of 100u/dl was designated for the collagen binding of the 20 normal pool plasma.

f) Ristocetin - induced platelet aggregation (RIPA)

The RIPA was measured by platelet aggregometry. PRP and PPP were prepared as described above. 500 µl of PPP is calibrated as the 100 % response and 500 µl of the PRP as the zero response. 450 µl of PRP was placed in the cuvette with a metal flea, and ristocetin was added. Testing was performed over a range of ristocetin concentration from 0.5 mg/ml to 1.5 mg/ml. Increased platelet aggregation at low ristocetin concentrations (0.25-0.75 mg/ml) is characteristic of type 2B VWD.

g) Analysis of the plasma multimeric composition of VWF

The multimeric composition of VWF was assessed by discontinuous SDS-agarose gel electrophoresis (Ruggeri and Zimmerman 1981). VWF multimers are detected by use of labelled antibody and an appropriate detection system. This protocol describes the use of I\(^{125}\) -labelled polyclonal anti-VWF for detection.

The agarose gel casting apparatus was prepared by placing a 1 mm U-shaped spacer on a clean glass plate (130×250 mm). A second glass plate is placed and aligned on the spacer. The casting apparatus is fixed in position by bulldog clamps. The casting apparatus is
pre-warmed at 70°C. The 1.2% separating gel is prepared by dissolving 0.6g agarose (Biorad, Hemel Hampstead, UK) in 50 ml of separating buffer (Tris 0.375 M, 1% SDS, pH 8.8). The gel was poured into the glass plates and allowed to solidify. The 0.8% stacking gel was prepared by dissolving 0.16 g in 20 ml stacking buffer (Tris 0.125 M, 0.1% SDS, pH 6.8). 12 mm of the separating gel was cut and the casting apparatus re-assembled. The stacking gel was then poured. Ten well were cut in the solid agarose slab using an 8 mm × 3 mm punch.

The samples were prepared by diluting 10 ul of plasma in dilution buffer (10 mM Tris, pH 8.0, 1 mM EDTA, Urea 8 M, SDS 5 %). To a final concentration of 0.01 to 0.1 u/dl. The SDS agarose gel was placed on a horizontal slab electrophoresis unit cooled to 10 C. 3 MM chromatography paper strips were used as electrode wicks. These were wetted in the electrophoresis buffer (Tris 0.05 M, Glycine 0.384 M, SDS 0.1%, pH 8.35). Thirty μl of diluted sample were loaded onto the agarose gel. A constant current of 6 mA is applied and the samples electrophoresed for 16 hr.

After electrophoresis, the gel was placed on the hydrophilic side of a gel-bond sheet (Flowgen, Lynn Lane, UK). The gel was fixed for 15 min in a 20% propanol, 5% acetic acid solution. The gel was then rinsed in distilled water for 15 min. The gel was then blocked by immersion in a 1% -globulin in Tris buffered saline (TBS) (Tris 50mM, NaCl 150 mM, pH 7.4) for 20 min. The gel was rinsed in TBS and then blocked in a solution containing 10% rabbit serum (Sigma, Poole, Dorset) in TBS for 20 min. After a wash in TBS the gel was immersed containing 1 [g l \(^{125}\)-labelled polyclonal antibody (Dako) (1
MBq). The radioiodinated polyclonal antibody was prepared prior to analysis by Medical Physics Department, St George’s Hospital, London. The gel was incubated for 3-4 hs, then washed three times for a total of one hour in 3% saline and then three times in 0.9% saline for 1 hr. The gel is placed in a dry-oven overnight. The dried gel was then exposed to X-ray film overnight at -70°C. The film was developed using an automatic Fugi film developer.

h) FVIII binding assay

The FVIII binding assay as used in the RFHHC laboratory based on the method described by Nesbitt et al (Nesbitt 1996). The assay is based on the immobilisation of FVIII:C complex on a microtitre plate, coated with a mAb raised against the ristocetin cofactor/GPIb (VWF:RiCof) binding site of VWF. Endogenous FVIII is removed by overnight incubation with a high ionic strength buffer. The washed VWF remaining bound to the mAb is then incubated with a recombinanat preparation of FVIII (rFVIII) at physiological concentration in the presence of Ca$^{2+}$. The amount of rFVIII bound to the immobilised VWF is measured using a chromogenic assay substrate for FVIII activity and detected at 405 nm. The method is for 1×96 well microtitre plate (10 samples, in duplicate). The results are plotted as a curve of FVIII activity (U/dl) versus the dilution. A known homozygous control and a normal pooled plasma control are used. Patients with no binding to FVIII give a flat curve similar to the homozygous pattern. Heterozygous samples show a reduction in FVIII binding and give an intermediate curve (Figure 3.2).
Figure 3.2. Factor VIII binding assay in type 2N VWD

Binding of plasma VWF from a patient with type 2N VWD homozygous (filled circle) shows no binding, a patient with type 2N VWD heterozygous (open circle) shows intermediate binding as compared to a normal pool plasma (open rectangle) which shows full binding.
3.2.2 MOLECULAR BIOLOGY TECHNOLOGY

a) Reagents for molecular biology analysis

- **Chemical reagents**

  Common chemical reagents were supplied by BDH Chemical Ltd., and Sigma Chemical with the exception of the Qiaquick PCR purification/gel extraction supplied by Qiagen.

- **Enzymes**

  Polymerase Chain Reaction (PCR) - BioTaq polymerase (5 U/µl) by Bioline, London, UK.

  Restriction endonucleases (4-20 U/µl) supplied by Boehringer Mannheim/ Promega/ New England Biolab.

- **Nucleotides for PCR analysis**

  2'-deoxynucleoside 5'- triphosphates (dATP, dTTP, dCTP, dGTP) by Promega

- **PCR methods**

  Taq Polymerase Buffer (10x)

  670 mM Tris-HCl, pH 8.8, 166 mM (NH₄)₂SO₄; 67 mM MgCl₂; 1.7 mg/ml serum albumin (BSA) and 100 mM β-mercaptoethanol. Stored in 0.5 ml aliquots at -20 °C.

  BSA and β-mercaptoethanol are added just before the use.
• **General Stock Solutions**

Sucrose loading buffer (6×) 40% sucrose; 10 mg/ml xylene cyanol and 10 mg/ml bromophenol blue.

TBE (×10): 90 mM Trizma base; 90 mM boric acid and 1 mM EDTA, pH 8.2.

**b) Extraction of genomic DNA**

Genomic DNA was extracted from the buffy coats (white blood cells) using the Qiagen extraction kit according to the manufacturer instructions.

c) **Techniques using the polymerase chain reaction (PCR)**

PCR was performed with 50-100 ng of genomic DNA. The DNA was amplified in a 50 μl mixture containing: 5 μl ×10 BioTaq polymerase buffer, 5 μl 5 mM dNTPs; 2.5μl primer a (10 pmol/μl); 2.5 μl primer b (10 pmol/μl) and 2.5 μl of BioTaq polymerase. This mixture was topped with 50 μl of mineral oil. 30-40 cycles of PCR (93°C/30 sec; 50-55°C/ 30 sec; 72°C/ 30 sec) were performed in a Perkin Elmer/Cetus thermal cycler. These were preceded by 5 min incubation at 94°C and followed by a 10 min incubation at 72°C. Each PCR experiment included a ‘no template’ control.

d) **Analysis and purification of PCR products**

PCR products (generally 5 μl) were analysed by electrophoresis through 1-3% ethidium stained agarose mini-gel and photographed using an UVP video recording equipment.
PCR products were purified by one of two methods. In the absence of any faint aberrant bands PCR products were purified by use of the Qiagen PCR purification kit. In the presence of faint aberrant bands the PCR product was subjected to electrophoretic separation on 1% ethidium-stained agarose prior to purification, the required band was excised and purified using the Qiagen Qiaquick gel extraction method.

e) Sequence analysis of PCR products

The PCR products were sequenced by automated sequencing technology. This was performed by cycle-sequencing using Big Dye technology (fluorescent labelled dNTPs) using the ABI 310 genetic analyser or by infra-red technology (MWG-Biotech, Milton Keynes).

Acknowledgements for the performing of assays

The coagulation assays were in general performed by the laboratory staff at the RFHHC. I have participated in performing some of the coagulation assays in particular the VWF:RiCo assay. I have also performed several of the molecular biology assays with kind help and support from Dr V Jenkins, Mrs G Mellors and Dr S Enayat who are gratefully acknowledged.
CHAPTER 4

4.1 INTRODUCTION

The diagnosis of type 1 VWD can be problematic, especially in the mild forms. The difficulties of diagnosis are partly due to a multitude of factors which can interfere with the diagnosis of type 1 VWD, such as the incomplete penetrance and variable expression of the disease, with females exhibiting greater variability than males (Blomback 1992, Werner 1994). Moreover, the VWF levels show very broad and variable normal ranges, with large variabilities within the same family and also in the same individual on different occasions. Several modifier factors either linked or non-linked to the VWF gene are recognised which have an important influence on the phenotype of the disease. However, the relative contribution of genetic and environmental factors to VWF expression have not yet been clearly identified. For example, it has recently been shown that genotypic variation at the VWF locus, such as the presence of amino acids polymorphisms in the promotor region of VWF gene influence plasma VWF levels (Keightley 1999). Amongst the factors extrinsic to the VWF locus that can affect the VWF concentration in plasma are physical and psychological stress, diurnal variation, age, sex, race, hormones and the ABO blood group (Abildgaard 1980; Alperin 1982; Gill 1987, Rickles 1976). Blood type may account for as much as 30% of the genetic variance of VWF levels within a pedigree (Orstavik 1985). Previous studies have shown that individuals with blood group O have lower levels of plasma VWF than individuals with blood group A, B or AB. The mean von Willebrand factor antigen (VWF:Ag) level is 25% lower for persons of blood group O compared to other blood types and one likely explanation is
related to the presence of glycosyltransferases (Rodeghiero 1987; Gill 1987; Yamamato 1990). Other factors which may contribute to this variability are compound heterozygosity status (Eikenboom 1993, Peerlinck 1992, Siguret 1994, Zhang 1993) and the secretor locus (Orstavik 1989). Due to this complexity, it is not surprising that one of the main difficulties is to distinguish between the mild forms of type 1 VWD and normal subjects with blood group O.

At present the diagnostic criteria of type 1 VWD are based on low levels of structurally normal VWF, evidence of a significant mucocutaneous bleeding history and a family history of type 1 VWD or demonstration of an appropriate causative mutation. Strict diagnostic criteria for type 1 VWD imply decreased levels of VWF ristocetin cofactor activity (VWF:RiCo) and VWF:Ag (less than 2 SD below ABO type adjusted population mean, on two determinations), a significant mucocutaneous bleeding history (requiring at least two bleeding symptoms in the absence of a blood transfusion history, or one symptom requiring treatment with blood transfusion, or one symptom recurring on at least three distinct occasions) and a positive family history (which includes either at least one first degree relative, or at least two second degree relatives with both a personal history of bleeding and laboratory values compatible with type 1 VWD (Batlle 1997).

Because the association between low levels of VWF and bleeding history can be coincidental, and not causative, a definitive diagnosis of VWD requires that their co-
inheritance is demonstrated. Thus, it appears that a ‘definite’ type 1 VWD diagnosis needs documentation of low levels of VWF, the presence of a bleeding history and inheritance (family history or appropriate VWF mutation). The diagnosis of type 1 VWD remains only ‘possible’ in patients with laboratory parameters compatible with type 1 VWD (low levels of normal VWF) and who have either a significant muco-cutaneous bleeding history or a positive family history.

Starting with the above considerations, it was of interest to analyse how these specific diagnostic parameters and their relationship with the blood group had influenced the decision to diagnose an individual as affected with type 1 VWD, as no such studies are present in the literature. Thus, a retrospective study was initiated analysing the patients previously diagnosed as type 1 VWD and included in the population database at the RFHHC (Chapter 3) focusing on the blood group, the bleeding history, the family history and the laboratory aspects. Consequently, this population was reclassified into two categories as ‘definite’ and ‘possible’ type 1 VWD. For each category the role of the bleeding history was analysed and compared between the groups of patients. Another important question addressed in this study was to what extent the stratification of patients by the ABO blood group and the adjustment of the VWF levels for the specific blood group had a significant utility in the clinical diagnosis and management of patients with type 1 VWD.
4.2 MATERIALS AND METHODS

4.2.1 Patients Selection

A total of 270 patients previously diagnosed with type 1 VWD and registered at the RFHHC up to December 1997 were analysed. For each patient the medical records and data including VWF levels, blood group type, personal bleeding history and family history were reviewed. Twenty four patients were excluded from the study as their blood group was not recorded.

4.2.2 Coagulation assays

The VWF:Ag and VWF:AC assays were measured by the in-house ELISA as described in Chapter 3. The reference ranges for both tests in the laboratory were 50-150 IU/dl. Epidemiological studies have shown that the VWF functional assay is the most sensitive screening test for type 1 VWD (Rodeghiero 1990). As the criteria for diagnosing type 1 VWD were not available in a standard protocol, historically a diagnosis of type 1 VWD has been accepted at the RFHHC if the VWF activity levels were less than 50 IU/dl, irrespective of blood group or age.

For the purpose of this retrospective analysis, a lower limit of normal of 35 IU/dl for blood group O and 50 IU/dl for blood group non-O was assumed. The cut-off value of 35 IU/dl for blood group O is the traditionally accepted value in literature (Gill 1987). Ideally, the lower limit of reference ranges should be calculated directly in the local normal population for both O and non-O subjects and age adjusted, but this was beyond the scope of this study.
The age of this population ranged from 1 to 93 years, and although it is known that the VWF levels increase with age (Gill 1987), an age variable was not included in the retrospective analysis.

### 4.2.3 Bleeding history

A bleeding history was considered positive if the subject presented with at least two bleeding symptoms or one symptom recurring on at least two occasions, as documented in the medical records.

Data on the bleeding symptoms were incomplete with respect to the site, severity and frequency of bleeding. Therefore, a sentinel bleeding symptom which characterised the different subgroups of patients had to be defined. Hence, the bleeding events were classified broadly into muco-cutaneous and non-mucosal bleeding.

Muco-cutaneous bleeding included spontaneous mucosal bleeding (nose bleeds, easy spontaneous bruising and haematoma, bleeding from oral cavity and gingival bleeding, menorrhagia), traumatic bleeding (prolonged bleeding after cuts and wounds and excessive bruising and haematoma post trauma) and excessive bleeding after mucosal surgery (tooth extraction or oral surgery and ENT surgery). Non-mucosal bleeding included spontaneous deep bleeding (gastro-intestinal haemorrhage, bleeding post partum and bleeding into muscle or joints) and bleeding post abdominal surgery.
Another classification of the bleeding symptoms was into bleeding requiring any form of specialised treatment (such as desmopressin and/or clotting factor concentrates) and bleeding episodes not requiring treatment. In the category 'bleeding requiring treatment' any form of treatment administered either preventive (e.g. for surgical procedures) or for treatment of a spontaneous / traumatic bleed was included. The category 'bleeding not requiring treatment' included patients who had a positive bleeding history (with or without a haemostatic challenge) but no previous treatment was documented.

4.2.4 Family history

A positive family history of VWD was accepted if there was at least one first degree family member recorded in the notes who was registered with a diagnosis of type 1 VWD based on a personal bleeding history, laboratory diagnosis or both. For a few patients, the relatives were registered elsewhere, so their coagulation parameters were not always available. In these rare cases, a confirmation of the specific diagnosis of VWD based on the bleeding history was sought from the index patient.

4.2.5 Statistics

Parametric statistics were used to compare the bleeding histories between blood group O and non O putative VWD patients. Comparisons of non categorical values were made using the Chi-square test.
4.3. RESULTS

The study included 246 patients with a documented blood group who were registered with type 1 VWD on the basis of a minimum requirement of having levels of VWF:AC and VWF:Ag < 50 IU/dl.

This population was initially classified according to blood group: 173/246 (70%) patients had blood group O and 73/246 (30%) patients had blood group non-O. Further analysis was done on the two categories based on the blood group.

i. Patients with type 1 VWD and blood group O (n = 173)

For the 173 individuals blood group O the diagnosis was re-evaluated based on the VWF:AC values adjusted for ABO blood group (normal range 35-150 IU/dl). Of the 173 patients, 71 had VWF:AC < 35 IU/dl and 102 patients had VWF:AC levels between 35 and 50 IU/dl (Figure 4.1).

a) Individuals blood group O and VWF:AC between 35 and 50 IU/dl (n = 102)

Of the 102 individuals who did not have low VWF:AC levels when adjusted for their blood group (i.e. normal levels for the non-O blood group), 45/102 (44%) would otherwise be eligible for a ‘definite’ type 1 VWD diagnosis and 53/102 (52%) would qualify for a ‘possible’ type 1 VWD diagnosis, 18/102 (18 %) on the basis of positive FH and 35/102 (34 %) on the basis of positive BH. Four individuals in this group had neither a family history nor a bleeding history (Table 4.1).
Figure 4.1. Classification of patients with type 1 VWD according to blood group and VWF:AC levels.

Table 4.1. Classification of previously diagnosed type 1 VWD into 'possible' and 'definite' type 1 VWD categories and stratification by blood group type.

<table>
<thead>
<tr>
<th>Blood group</th>
<th>'possible'</th>
<th>'possible'</th>
<th>'definite'</th>
<th>No FH, no BH</th>
<th>Number patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>O VWF Ac &lt; 35 U/dl</td>
<td>12 (17%)</td>
<td>15 (21%)</td>
<td>40 (56%)</td>
<td>4 (6%)</td>
<td>71</td>
</tr>
<tr>
<td>O VWF Ac 35-49 U/dl</td>
<td>18 (18%)</td>
<td>35 (34%)</td>
<td>45 (44%)</td>
<td>4 (4%)</td>
<td>102</td>
</tr>
<tr>
<td>nonO VWF Ac &lt; 50 U/dl</td>
<td>14 (19%)</td>
<td>10 (14%)</td>
<td>48 (66%)</td>
<td>1 (1%)</td>
<td>73</td>
</tr>
<tr>
<td>Total</td>
<td>44 (18%)</td>
<td>60 (24%)</td>
<td>133 (54%)</td>
<td>9 (4%)</td>
<td>246</td>
</tr>
</tbody>
</table>

FH- family history; BH- personal bleeding history
b) *Individuals blood group O and VWF:AC < 35 IU/dl (n = 71)*

Seventy one patients with blood group O had VWF:AC levels < 35 IU/dl (i.e. low levels for blood group O), but only 40/71 (56%) could be categorised as ‘definite’ type 1 VWD. Of the remainder, 27/71 (38%) were ‘possible’ type 1 VWD: 12/71 (17%) had a documented family history and 15/71 (21%) had a recorded bleeding history. Four individuals had neither a bleeding nor a family history demonstrated (Table 4.1).

**ii. Patients with type 1 VWD and blood group non-O (n = 73)**

Seventy three patients with blood group non-O had VWF:AC levels < 50 IU/dl. Within this group, 48/73 (66%) patients were classified as ‘definite’ type 1 VWD, having both a personal and a family bleeding history. 14/73 (19%) patients had a positive family history and 10/73 (14%) patients demonstrated a significant bleeding history. Thus, 24/73 (33%) patients blood group non-O were ‘possible’ type 1 VWD. One individual had no bleeding or family history demonstrated (Table 4.1).

**iii. Diagnosis of ‘possible’ and ‘definite’ VWD**

Of the original 246 patients previously diagnosed as type 1 VWD, 133/246 (54%) fulfilled all the criteria for a ‘definite’ type 1 VWD diagnosis and 104/246 (42%) had only ‘possible’ type 1 VWD: 44/246 patients (18%) had a positive family history alone and 60/246 (24%) patients had a personal bleeding history.
Of note, 14 individuals, four of whom were asymptomatic, had VWF:AC levels between 5 and 10 IU/dl and did not fulfil the criteria for a ‘definite’ type 1 VWD.

**iv. Assessment of the bleeding history**

A direct comparison between individuals who experienced any kind of bleeding episodes *versus* individuals with no bleeding symptoms showed very similar results among different categories of patients stratified by blood group (p= 0.959, Table 4.2).

<table>
<thead>
<tr>
<th></th>
<th>No bleeding history</th>
<th>Bleeding + treatment</th>
<th>Bleeding + no treatment</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood grp O, VWFAc &lt; 35 U/dl</td>
<td>16 (23%)</td>
<td>36 (51%)</td>
<td>19 (27%)</td>
<td>71</td>
</tr>
<tr>
<td>Blood grp O VWFAc 35 - 49U/dl</td>
<td>22 (22%)</td>
<td>45 (44%)</td>
<td>35 (34%)</td>
<td>102</td>
</tr>
<tr>
<td>Blood grp nonO VWFAc &lt; 50 U/dl</td>
<td>15 (21%)</td>
<td>43 (59%)</td>
<td>15 (21%)</td>
<td>73</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>53 (22%)</td>
<td>124 (50%)</td>
<td>69 (28%)</td>
<td>246</td>
</tr>
</tbody>
</table>

**Table 4.2.**

Classification of different subgroups of type 1 VWD patients according to the presence of bleeding episodes that did or did not require specialised treatment.
Requirements for specialised treatment for bleeding symptoms in the different subgroups of patients is shown in Table 4.2. There were similar requirements for treatment in the blood group O and blood group non-O patients (p = 0.303). In total, out of the 246 patients type 1 VWD, only 124/246 (50%) had required specialised treatment for a bleeding event.

The comparison between different groups of patients who experienced mucosal, non-mucosal or both types of bleeding is presented in Table 4.3. Similar patterns of mucosal bleeding were observed in all groups of patients (p = 0.164). Thus, a similar clinical pattern of bleeding symptoms was found in both blood group O and blood group non-O patients, irrespective of the VWF levels.

<table>
<thead>
<tr>
<th></th>
<th>No bleeding</th>
<th>Mucosal bleeding</th>
<th>Non mucosal bleeding</th>
<th>Mucosal and non mucosal bleeding</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood grp O VWFAc &lt; 35 U/dl</td>
<td>16 (23%)</td>
<td>53 (75%)</td>
<td>1 (1%)</td>
<td>1 (1%)</td>
<td>71</td>
</tr>
<tr>
<td>Blood grp O VWFAc 35-49 U/dl</td>
<td>22 (22%)</td>
<td>71 (70%)</td>
<td>0</td>
<td>9 (9%)</td>
<td>102</td>
</tr>
<tr>
<td>Blood grp non O VWFAc &lt; 50 U/dl</td>
<td>15 (21%)</td>
<td>54 (74%)</td>
<td>0</td>
<td>4 (5%)</td>
<td>73</td>
</tr>
<tr>
<td>Total</td>
<td>53 (22%)</td>
<td>178 (72%)</td>
<td>1 (0.4%)</td>
<td>14 (7%)</td>
<td>246</td>
</tr>
</tbody>
</table>

Table 4.3.

Classification of different subgroups of patients with type 1 VWD according to the type of bleeding pattern (mucosal, non mucosal or both).
4.4. DISCUSSION

Type 1 VWD comprises a heterogenous group of patients in whom the clinical diagnosis is often difficult because of a considerable intra and inter-individual phenotypic variation. Traditionally, low levels of VWF due to modifying influences were not included in the diagnosis of type 1 VWD. Thus, the demarcation between mild type 1 VWD and normal poses a diagnostic dilemma.

In this study a population of 246 individuals who had been previously diagnosed as type 1 VWD were reviewed and the criteria used for diagnosis were analysed. In accordance with other studies, the majority of this group of patients (70%) were blood group O in comparison with the expected frequency of 45% blood group O in the normal population (Gill 1987).

Only 59% of this cohort had low VWF levels when adjusted for their blood group. By definition, only these patients would have been eligible for a diagnosis of VWD and could be further classified into ‘definite’ and ‘possible’ type 1 VWD (36% exhibited all the criteria for a ‘definite’ type 1 VWD diagnosis and 21% of the population qualified for a ‘possible’ type 1 VWD diagnosis). In 2% of the studied population the diagnosis was unacceptable as it was made solely on the detection of low levels of VWF, without the presence of a bleeding or family history.
In this cohort, it emerged that 41% had blood group O and ‘normal levels’ of VWF:AC for their blood group (between 35–50 IU/dl). On reclassification, this group is considered as ‘not VWD’ as the levels of VWF are within the normal range for blood group O and the possibility of an alternative diagnosis in the presence of bleeding symptoms is implied. However, this group represents an indeterminate one, where the VWF level reduction comprises a mix of ‘genetic’ and ‘non-genetic’ VWD causes.

The ABO locus affects the level of plasma VWF and the combination of a VWF mutation and blood type O may be associated with bleeding symptoms. Blood group O alone could cause low levels of plasma VWF and suggest a VWD diagnosis, without the presence of a mutation at the VWF locus. Bleeding symptoms and low levels of VWF are individually common and may occur together by chance, so 0.5% of all classified VWD patients are made on a chance combination alone (Rodeghiero 1987). It is estimated that about 20% of normal subjects experience excessive bleeding in a lifetime (Miller 1979).

In this series of patients, a large number of individuals with VWF levels ranged between 35 and 50 IU/dl and blood group O did not have definitive VWD, but the majority (approx. 80%) had bleeding symptoms. Moreover, this subgroup of patients had a similar bleeding pattern (type of bleeding and requirement of treatment) as the other groups of patients with blood group non-O and VWF levels between 35 and 50 IU/dl. Although the sample size differed and the kind and severity of bleeding symptoms among
different subgroups were not analysed, it was obvious that similar clinical manifestations in individuals with similar levels of VWF, irrespective of their blood group were present. These results suggest that the use of ABO adjusted ranges for VWF levels might not be essential for diagnosis, because bleeding symptoms may depend on the absolute value of VWF levels regardless of the ABO type. Asymptomatic patients who have borderline normal values for VWF should be regarded with caution, as many of them might have not had a haemostatic challenge to manifest a bleeding tendency. In these doubtful cases, patients should be reassured and the investigations repeated. However, when the VWD diagnosis can neither be confirmed nor excluded and the risk of bleeding is unknown, empirical treatment is recommended (Nishino 1997).

A recent study has analysed the association between the phenotype of bleeding and low VWF and genetic markers at the VWF locus and has found that there was a lack of association in many families with mild type 1 VWD (Castaman 1999). Consequently, as the present classification defines VWD as a genetic disease caused by mutations at the VWF locus (Sadler 1994), it implies that families in which there is no association between phenotype and VWF genotype by definition do not have VWD. Hence, an important question is whether individuals with low VWF and bleeding histories but no demonstrable inheritance really manifest the disease or just have a bleeding diathesis associated with mild reduction in VWF secondary to other causes, such as blood group O.
Other investigators have also encountered similar problems with the definition and classification of type 1 VWD. Dean et al. (Dean 1997) reported difficulties in classifying a high percentage of paediatric cases and Ingerslev & Gursel (Ingerslev 1999) exemplified with family studies which posed a diagnostic dilemmas. Fressinaud et al. (Fressinaud 1998) described two patients blood group O, VWF:AC > 35 IU/dl and positive bleeding history as ‘borderline normal subjects’, as they could not be diagnosed using the highly sensitive PFA-100™. In a recent ongoing study looking at the effect of adjusting the VWF levels for the blood group, about 30 % of patients with menorrhagia had subnormal VWF levels, but nearly half did not fit the ABO-adjusted laboratory criteria for VWD. Similar to the findings presented in this study, such patients had similar bleeding features and warranted consideration for similar therapies as the VWD patients (Kouides 1999).

It is thought that individuals with blood group O have lower levels of plasma VWF because there is a different release and/or clearance rate of the VWF. Thus, the carbohydrate groups present only in blood group non-O individuals, may result in a prolonged release and/or increase half-life of plasma VWF. Moreover, it has been shown that the platelet VWF (Rodeghiero 1992) and also the pro-peptide (VWF:Ag II) (de Romeuf 2000) which both reflect the intracellular synthesis of VWF, is similar in O and non-O blood groups, suggesting that it is a clearance rather than a synthetic effect.
In summary, this retrospective clinical study underlined the practical difficulties in the diagnosis of type 1 VWD. Full criteria for a ‘definite’ type 1 VWD diagnosis (low levels of VWF for the blood group, bleeding history and inheritance) were found in only a third of patients as these criteria are very stringent and difficult to meet. Of the three parameters, the bleeding history was of prime importance in the clinical decision to diagnose and treat type 1 VWD, which suggests that the distinction between different blood groups and separate normal ranges for VWF should not influence the diagnosis in symptomatic individuals.

It is of interest to study if the ABO blood groups have any effect on other tests, especially on the new tests such as the PFA-100™, which is being increasingly used to diagnose VWD and whether adjustment to the blood group is important. This idea is explored further in Chapter 8 where the influence of the ABO blood group on the PFA-100™ is investigated.
CHAPTER 5

ISSUES RELATED TO TYPE 2 VON WILLEBRAND DISEASE - COMPARATIVE ASSESSMENTS OF DIFFERENT ASSAYS TO MEASURE THE VON WILLEBRAND FACTOR ACTIVITY
The diagnosis and classification of VWD relies on phenotypic characterisation. This commonly involves determinations of plasma factor VIII level, the VWF:Ag and assessment of the functional ability of VWF to bind platelets, as determined by the ristocetin co-factor agglutination activity (VWF:RiCo). Of all the phenotypic assays, the measuring of the VWF functional activity seems to be the best assay to diagnose VWD (Rodeghiero 1992). Two alternatives to the VWF:RiCo are now available, the ELISA based assay for measuring the VWF activity and the collagen binding assays (Favolorola, 1997). However, which test is the most suitable for assessing the function of VWF is controversial. Few studies have compared the different tests and their utility in recognising the different subtypes of qualitative variants of VWD. This chapter will address the above issues from a critical viewpoint.

5.1 A CRITICAL COMPARISON BETWEEN THE VWF:RICO AND FUNCTIONAL VWF:ELISA IN THE DIAGNOSIS OF TYPE 2 VWD

5.1.1 INTRODUCTION

Classically, the functional activity of VWF has been measured by the ristocetin co-factor activity (VWF:RiCo) assay (Macfarlane 1975). Ristocetin is a positively charged antibiotic that promotes the interaction between VWF and platelet GPIb-IX-V. The ristocetin mediated platelet agglutination is dependent of the HMW multimers of VWF, thus reflecting the functional activity of VWF. The assay is usually performed on an aggregometer on freshly washed or commercial lyophilized normal platelets (either
formalin or paraformaldehyde-fixed). As discussed in *Chapter 3*, a presumptive diagnosis of type 2 VWD is initially suggested by analysing the VWF:RiCo/VWF:Ag ratio: a concordant VWF:RiCo/VWF:Ag ratio of ≥ 0.7 suggests type 1 VWD, whereas a discrepant ratio (< 0.7) is characteristic for type 2 VWD, except for type 2N (Federici 1998). The VWF:RiCo assay is a sensitive test for VWD but it is fraught by many problems, such as poor reproducibility, large inter-assay and inter-laboratory variability, it is laborious and time-consuming. Trying to overcome the methodological inconsistencies associated with the VWF:RiCo assay, a VWF activity (VWF:AC) based on an ELISA method has been described as an alternative to the VWF:RiCo (Murdoch 1997). This assay is based on the ability of the monoclonal antibody RFF-VIII:R/1 to recognise and bind to an epitope on the VWF involved in its binding to GpIb-IX (Chand, 1986). The binding of VWF to the platelet GPIb binding site is of prime importance in haemostasis as it leads to the initial platelet adhesion.

Many laboratories within the UK are using a commercially available kit to determine the VWF functional activity which involves an ELISA method. Manufactured originally by Porton Cambridge and now by Shield Diagnostics, the kit uses a monoclonal antibody (purified murine anti-VWF IgG) that captures the plasma VWF activity and the complex is detected by horseradish peroxidase labelled anti-human VWF. Early studies comparing the performance of VWF activity ELISA test with the VWF:RiCo assay have shown a good correlation between the two tests, proposing the ELISA method as an elegant alternative to VWF:RiCo. Moreover, the ELISA test seemed to be able to discriminate
between type 1 and type 2 VWD based on the ratio between the VWF activity/antigen. However, a recently undertaken NEQAS survey has revealed contradictory results, showing that the activity levels detected by the ELISA were significantly higher than by the conventional VWF:RiCo assay, thus the diagnosis of the variant VWD was potentially missed (Preston 1998).

Clearly, there is no consensus of opinion as to which assay should be employed to better reflect the function of the VWF and to offer a simple and efficient method capable to diagnose and to distinguish between different subtypes of VWD. The aim of this chapter was to analyse a group of patients previously diagnosed with various subtypes of VWD and to compare the three widely used methods for VWF activity: the classic VWF:RiCo assay, the mAb based ELISA assay (in-house ELISA) and the VWF activity using a commercially available kit provided by the Shield Diagnostics (Shield ELISA).

5.1.2 METHODS

Patients

A group of 30 patients (from 17 kindreds) previously registered with type 1 VWD at the RHHHC were included in the analysis. Their selection was based on detection of a discrepant VWF:AC/VWF:Ag ratio (<0.7) and an apparent normal plasma VWF multimers as identified form the patient database (described in Chapter 3). This group of patients is the same group described in Chapter 6 in whom mutation analysis was
performed and led to their reclassification as type 2 VWD. The numbering of the patients and kindreds was kept the same in both studies for consistency.

**Laboratory Assays**

The VWF:RiCo assay was performed with fresh, washed platelets using an aggregometer (Macfarlane 1975). The VWF:AC assay was performed by ELISA using the Harlan Sera-Lab monoclonal antibody (mAb) mouse anti-human VWF MAS 533 and a commercial kit (Shield Diagnostics, now Stago Diagnostica, Dundee, UK). For details of methodologies, see *Chapter 3*.

**Statistics**

To compare two methods, the Bland and Altman plot for assessing agreement between two methods of clinical measurement was used as in this context the use of correlation coefficients would have been inappropriate (Bland 1986). In order to find out by how much a method is likely to differ from the other and to see if the two methods are interchangeable, a plot of the difference between the methods against their mean was calculated.

**5.1.3 RESULTS**

The VWF:RiCo, ‘in-house’ ELISA and Shield ELISA assays for assessing the VWF function were performed on the same samples obtained from 27 of the 30 patients (Table 5.1).
Table 5.1. Laboratory characteristics of the 17 kindreds with a discrepant VWF:Activity/VWF:antigen ratios.

<table>
<thead>
<tr>
<th>Kindred</th>
<th>Blood group</th>
<th>Bleeding time (min)</th>
<th>FVIII:C (lU/dl)</th>
<th>VWF:Ag (lU/dl)</th>
<th>VWF:RiCo (lU/dl)</th>
<th>RiCo/Ag</th>
<th>In-house VWF:AC (lU/dl)</th>
<th>In-house Ac/Ag</th>
<th>Shield VWF:AC (lU/dl)</th>
<th>Shield Ac/Ag</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal range</td>
<td>&lt;8</td>
<td>50-150</td>
<td>50-150</td>
<td>50-150</td>
<td>1</td>
<td>50-150</td>
<td>1</td>
<td>50-150</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>A pos</td>
<td>20</td>
<td>165</td>
<td>76</td>
<td>20</td>
<td>0.26</td>
<td>45</td>
<td>0.59</td>
<td>92</td>
<td>1.21</td>
</tr>
<tr>
<td>2-1</td>
<td>O pos</td>
<td>&gt;20</td>
<td>38</td>
<td>23</td>
<td>&lt;5</td>
<td>&lt;0.22</td>
<td>14</td>
<td>0.61</td>
<td>25</td>
<td>1.09</td>
</tr>
<tr>
<td>2-2</td>
<td>O pos</td>
<td>ND</td>
<td>25</td>
<td>13</td>
<td>&lt;5</td>
<td>&lt;0.38</td>
<td>8</td>
<td>0.62</td>
<td>14</td>
<td>1.08</td>
</tr>
<tr>
<td>2-3</td>
<td>O pos</td>
<td>ND</td>
<td>22</td>
<td>11</td>
<td>&lt;5</td>
<td>&lt;0.45</td>
<td>8</td>
<td>0.73</td>
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<td>1.64</td>
</tr>
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<td>8</td>
<td>68</td>
<td>65</td>
<td>14</td>
<td>0.21</td>
<td>46</td>
<td>0.71</td>
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<td>0.97</td>
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<td>4</td>
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<td>ND</td>
<td>33</td>
<td>16</td>
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</table>

ND= not done.
Obvious differences were found in VWF:AC when measured by the two ELISA methods and the VWF:RiCo assay. In contrast to the VWF:RiCo / VWF:Ag ratios, all 30 of which were less than 0.7, only 7 of 27 patients (1, 2-1, 2-2, 6, 10-1, 11 and 16-2) now had ratios of less than 0.7 on re-testing using the ‘in-house’ ELISA assay. This was surprising as 11 patients previously identified with a ratio of less than 0.7 were initially diagnosed by this method (2-1, 2-2, 2-3; 5-1, 5-2, 5-3, 12-1, 12-2, 15-1, 15-3, 17). The only difference in the assay was that the original analysis employed the original ascites source of functional mAb RFF-VIII:R/1, whereas the present one used a commercial preparation of this mAb (Harlan Sera-Lab, Loughborough, UK). The measurement of VWF:AC using the commercial kit (Shield ELISA) detected only one disproportionately reduced VWF activity compared to the VWF:Ag level (patient 6). All other ratios were greater than 0.7 in a range between 0.78 and 2.06. Due to the presence of both normal VWF:Ag and VWF:AC levels, the use of the commercial kit would have resulted in patients 1, 3, 15-3 and possibly 15-2 being excluded as having VWD.

The different assays were compared by assessing the agreement between methods (Bland 1986). A plot of the difference between the ‘in-house’ ELISA and the VWF:RiCo assay against their average showed from the differences from the horizontal axis that, at levels of less than 25 IU/dl as detected by the ELISA method, the two assays cannot be compared (Figure 5.1a). However, at VWF activity levels higher than 25 IU/dl, the two methods might be in agreement with a constant difference of about 25 IU/dl between the two sets of values, however the sample was too small to be certain of this agreement.
Figure 5.1. Bland and Altman plots to assess the agreement between the different assays.
The data from 27 patients also demonstrated that the Shield ELISA gave divergent results when compared to the VWF:RiCo assay (Figure 5.1b), where the VWF:AC levels measured by the commercial assay were increasingly higher than the VWF:RiCo levels, with increasing differences at increasing VWF:RiCo levels.

5.1.4 DISCUSSION

In this study the VWF binding capacity to GpIb was reassessed using the ristocetin cofactor activity assay and critically compared to the in-house and also a commercial ELISA assay based on a mAb directed against the GpIb binding site on VWF.

Gross differences were found between the VWF:RiCo and ELISA values, where the ELISA always gave higher values (Figure 5.1) and resulted in more normal VWF:AC/VWF:Ag ratios (Table 5.1). The VWF:RiCo assay was clearly able to diagnose and differentiate between type 1 and type 2 VWD. The values obtained by the VWF:RiCo assay were generally very low, and patients with qualitative variants showed a discrepant ratio VWF:RiCo/VWF:Ag. The mAb based ELISA assay gave much higher results than the VWF:RiCo assay analysed in the same sample for all patients, irrespective of their subtype of VWD. Most of the VWF:AC/VWF:Ag ratios were concordant, making it difficult to identify the qualitative variants of VWD.
Although the data analysed were relatively small, it appeared that, at lower VWF:AC values, the in-house ELISA assays were not in agreement with the VWF:RiCo assays. Furthermore, the commercial ELISA assays showed no correlation with the VWF:RiCo assays and would have left some patients to be considered normal. This means that the ELISA methods are not sensitive to qualitative variants, and their usefulness in diagnosing type 2 VWD is questionable.

The differences between these tests are not surprising, as the basis of the tests are completely different. The VWF:RiCo assay is a functional test, where ristocetin is a positively charged antibiotic that induces the binding of VWF to platelets. Ristocetin binds to the active-site crevice of the VWF-A1 domain which is defined by the crossover point of the central beta sheet and flanking alpha helices (Jenkins 1998; Cruz 2000). Thus, in patients with various subtypes of VWD the qualitative deficiency in VWF would be reflected in the VWF:RiCo assay, irrespective of whether the defect relates to decrease or increased affinity for the GPIb binding site or the absence of the HMW multimers. In contrast, the antibody-based ELISA assay only recognises a functional epitope on VWF-A1 domain that is involved in binding to GPIb (Chand 1986). If mutations in the VWF-A1 domain lead to an alteration of function but no change in the epitope recognised by the antibody, the ELISA assays will not detect them. The Shield assay gave even higher results than the in-house ELISA methods and the Shield VWF:AC/VWF:Ag ratio was not informative in differentiating the type of VWD (the ratio was $>4$ in the majority of
patients). It appears that the commercially available kit for VWF activity assay was insensitive and not very accurate for the VWD diagnosis.

In conclusion, this study had shown that despite its considerable inter-laboratory and inter-assay variability (Favaloro 1997), the VWF:RiCo assay was sensitive to qualitative variations. By consensus, the standard and most discriminatory assay of VWF function clearly remains VWF:RiCo, although alternatives such as VWF collagen binding assay have been proposed and will be discussed in the next section (section 5.2) The mAb-based ELISA assays were shown to be insensitive to most of the qualitative variants investigated. A recently-described ELISA method based on a recombinant GPIb fragment is expected to be of value (Vanhoorelbeke 2000).
5.2 A CRITICAL COMPARISON BETWEEN THE VWF:RICO AND THE VWF:CBA IN THE DIAGNOSIS OF TYPE 2 VWD

5.2.1 INTRODUCTION

As shown above, the VWF:RiCo assay although the best method to assess the qualitative abnormalities of VWF has several limitations such as sensitivity, reproducibility and inter-laboratory variability (Favaloro 1999). As a consequence there has been a renewed interest in the VWF-collagen binding assay (VWF:CBA) as a measure of VWF function (Brown & Bosak, 1986; Favaloro 1990 and Favaloro 1993; Favaloro & Koutts, 1997; Fischer 1998; Favaloro 2000). The VWF:CBA is based on the ability of VWF to bind collagen, primarily by the A3 and A1 domains, and by its ability to bind preferentially to HMW VWF multimers. The assay has been shown to be sensitive in the discrimination of type 1 and types 2A and 2B VWD (Favaloro 1997; Wermes 1998) where VWF:CBA/VWF:Ag ratios appear to be significantly lower in the latter two groups.

In this study the VWF:CBA was used in parallel with the VWF:RCo assay to test previously characterised patients with type 2A and a group of patients with type 2M VWD. The majority of these patients have been included in the study discussed in Chapter 6 where the diagnosis of type 2M VWD was based on finding a discriminant VWF:RiCo/VWF:Ag ratio (<0.7), the presence of HMW multimers and in half of them, a genetic mutation compatible with type 2M disease (Chapter 6). This study demonstrates that the VWF:CBA assay is insensitive to the functional defect present in the 2M VWD.
5.2.2. METHODS

Patients and Controls

The patient group consisted of 32 patients with VWD registered at the RFHHC and selected from the database (Chapter 3). This group consisted of 6 patients with type 2A VWD, and 25 patients from 14 kindred with 2M VWD (analysed in Chapter 6) and a single patient with type 2B VWD. The control group consisted of 22 healthy volunteers. Informed consent was given by all participants.

Coagulation assays

Venous whole blood was collected into 0.106M sodium citrate (Sarstedt Monovette 9NC/3ml tubes). After centrifugation at 2000g for 10 minutes at 4°C, samples were aliquoted into Sarstedt cryo tubes and stored at -70°C until further testing. VWF:Ag was measured by the in-house ELISA, the VWF:RiCof was measured using an aggregometer and fresh washed platelets and the VWF:CBA was measured by an ELISA as detailed in Chapter 3.

Statistical Analysis

The single patient with type 2B VWD was not included in statistical analysis. The Mann-Whitney non-parametric t-test was used for comparing the patient groups and controls.
5.2.3. RESULTS

Controls

The normal controls gave comparable results for VWF:Ag (median 101 IU/dl, range 60-165 IU/dl), VWF:RiCo (median 95 IU/dl, range 64-169 IU/dl) and VWF:CBA (median 99 IU/dl, range 66-156 IU/dl) and the VWF:RiCo/VWF:Ag and VWF:CBA/VWF:Ag ratios were concordant (Table 5.2).

Table 5.2. Laboratory data in the control group (shown as range and median).

<table>
<thead>
<tr>
<th>VWF :Ag IU/dl</th>
<th>VWF:RiCO IU/dl</th>
<th>RiCO/VWF:Ag ratio</th>
<th>VWF:CBA IU/dl</th>
<th>VWF:CBA/VWF:Ag ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>60 - 165</td>
<td>64 - 169</td>
<td>0.8 - 1.1</td>
<td>0.9 - 1.6</td>
</tr>
<tr>
<td>Median</td>
<td>101</td>
<td>95</td>
<td>1.0</td>
<td>99</td>
</tr>
</tbody>
</table>

Type 2A and 2B VWD

The individual laboratory data obtained for patients with type 2A and 2B VWD is presented in Table 5.3. In patients with type 2A VWD, whilst the VWF:Ag was below normal, both the VWF:RiCo and VWF:CBA were similarly reduced compared to the normal group (p=0.026, and p=0.004 respectively). In all patients with type 2A and type 2B both the VWF:RiCo/VWF:Ag and VWF:CBA/VWF:Ag ratios were discordant (< 0.7). (Table 5.3) with no significant difference between the two ratios (the median VWF:RiCo/VWF:Ag ratio was 0.45 and the median VWF:CBA/VWF:Ag ratio was 0.1).
Moreover, both ratios were significantly reduced as compared to the normal control group VWF:RiCo/VWF:Ag ratio (median 1.1) (p=0.002).

Table 5.3. Laboratory data in patients with type 2A and 2B VWD.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>VWF:Ag IU/dl</th>
<th>VWF:RiCO ratio</th>
<th>VWF:RiCO/VWF:Ag ratio</th>
<th>CBA IU/dl</th>
<th>VWF:CBA/VWF:Ag ratio</th>
</tr>
</thead>
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<td></td>
<td></td>
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<td>&lt;0.1</td>
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</tr>
<tr>
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<td>&lt;0.1</td>
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<td>&lt;0.1</td>
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<tr>
<td>30</td>
<td>25</td>
<td>9</td>
<td>0.4</td>
<td>13</td>
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</tr>
<tr>
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<td>14</td>
<td>7</td>
<td>0.5</td>
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<td>0.4</td>
</tr>
<tr>
<td>Type 2B</td>
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<td>32</td>
<td>17</td>
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</table>

Range 8 – 43 <5- 18 <0.1 – 0.6 1–13 0.1 – 0.5
Median 22.5 <5 0.45 1 0.1

Type 2M VWD

The individual laboratory data obtained for patients with type 2M VWD is presented in Table 5.4. In the patients with type 2M VWD no significant difference was shown between the VWF:CBA (median 35 U/dl) and VWF:Ag (median 26.5 IU/dl) (p=0.156). As expected all the VWF:RiCo were below normal and a significant difference was obtained between the VWF:RiCo and VWF:Ag (p=<0.0001).
Table 5.4. Laboratory data in patients with type 2M VWD.

<table>
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<tr>
<th>Patient number</th>
<th>VWF:Ag IU/dl</th>
<th>VWF:RCo IU/dl</th>
<th>VWF:RCo/Ag Ratio</th>
<th>CBA U/dl</th>
<th>VWF:CBA/VWF:Ag Ratio</th>
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<td>0.3</td>
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<td>10</td>
<td>0.4</td>
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<td>1.7</td>
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</tbody>
</table>

Range: 8-76 <5-30 <0.1-0.6 6-66 0.7-2.5
Median: 26.5 5 0.3 35 1.1

*VWF:CBA within the normal range.
There was also a significant difference between the VWF:RiCo and VWF:CBA (p = 0.0001). In contrast to the VWF:RiCo/VWF:Ag ratios which were disproportionately reduced in all patients with type 2M, the VWF:CBA/VWF:Ag ratios were concordant in almost all patients with type 2M VWD (Table 5.4). Three patients (2, 4 and 10) displayed VWF:CBA/VWF:Ag ratios of 0.7, and another 4 patients (3, 8, 9, 22) displayed ratios of 0.8 (Table 5.4). Both these values are slightly lower than the lowest value in the control group of 0.9, but are not dramatically reduced. There was no significant difference between the VWF:CBA/VWF:Ag ratios in patients with type 2M VWD (median 1.1) when compared with the normal control group (median 1.1, p = 0.315).

A graphic representation of the VWF:CBA/VWF:Ag ratios (Figure 5.2) and the VWF:RiCo/VWF:Ag ratios (Figure 5.3) illustrates the differences observed between these ratios in type 2A, type 2M and the controls groups.

5.2.4. DISCUSSION

Due to the poor reproducibility and considerable inter-assay and inter-laboratory variability of the VWF:RiCo assay there has been renewed interest in alternative assays for the measuring VWF function, in particular the use of the VWF:CBA assay (Favaloro 1993; Fischer 1998; Favaloro 1999, Favaloro 2000b). Collagen types and source have been previously shown to be important variables in the performance of the VWF:CBA. In this study a group of 32 patients with previously characterised VWD and 22 controls were tested for VWF:CBA using type III collagen from human placenta, which has previously been shown to be sensitive to the loss of HMW multimers (Favaloro 2000a).
Figure 5.3. Scatter plot showing VWF:CBA/VWF:Ag (CBA/Ag) ratio for type 2A and 2M VWD patients compared to the control group.

Figure 5.4. Scatter plot showing VWF:RiCo/VWF:Ag (RCo/Ag) ratio for patients with type 2A and 2M VWD compared to the control group.
The VWF:Ag and VWF:RiCo assays were also tested in the same sample to allow a comparison between these assays. All three assays (VWF:Ag, VWF:RiCo and VWF:CBA) were within the normal range in the control group reflecting the presence of full binding of VWF to GpIb-V-IX and VWF binding to human type III collagen, and thus normal levels of fully functional HMW VWF.

All patients with type 2 VWD displayed disproportionately reduce VWF:RiCo/VWF:Ag ratios and these patients would have been recognised as having a qualitative defect on the basis. This was an expected finding, as the VWF:RiCo assay, together with multimer and RIPA analysis, were originally used to identify and to classify these patients as detailed in Chapter 6.

Patients with type 2A VWD and the patient with type 2B VWD also displayed a disproportionately reduced VWF:CBA/VWF:Ag ratio. Thus, using the VWF:CBA these patients would have been correctly assigned as type 2 VWD with a defective VWF function. However, in contrast none of the type 2M patients displayed an obviously reduced VWF:CBA/VWF:Ag ratio, as the ratio was either borderline or concordant. The use of the VWF:CBA as a replacement for the VWF:RiCo assay would have led to these patients with type 2M VWD as being classified as type 1 VWD, or in the cases of patients 10, 19, 20, 21 and 22 as normal (Table 5.4 asterisked).
These data suggest that the VWF:CBA has been shown to detect qualitative defects associated with non-specific loss of HMW multimers as present in type 2A VWD (where there is decreased synthesis or an increased proteolysis leading to the loss of HMW multimers) and in type 2B VWD (where increased clearance of HMW multimers occurs as a result of increased reactivity of platelets to VWF). However in the presence of HMW multimers which define type 2M VWD, the VWF:CBA is purely a reflection of the VWF:Ag, as no differences between the VWF:CBA/VWF:Ag ratios in controls and type 2M patients were observed. This is an important observation as not many cases of type 2M VWD are described which is likely to be due to under-diagnosis of this subtype and commonly mistaken for type 1 VWD as demonstrated in Chapter 6 (Sadler 1994; Hilbert 2000).

Despite the recognised limitations of the VWF:RiCo assay, based on these findings, it appears that the VWF:CBA should not be considered a replacement for the VWF:RiCo assay. The two assays are not interchangeable as they measure different functions of the VWF. Thus, as discussed in subchapter 5.1, VWF:RiCo assay measures the ability of VWF to interact via GpIb to platelets on addition of ristocetin, whereas the VWF:CBA is a direct reflection of the presence of HMW multimers and ability of VWF to bind to collagen. Due to the multifunctional nature of VWF no single functional assay could reflect the complete functional integrity of the VWF protein. The use of more than one functional test allows a fuller characterisation of the abnormalities present in VWD. Although shown to be insensitive to type 2M VWD variants, the use of the VWF:CBA in
combination with the VWF:RiCo assay may allow a more powerful and complete approach to the diagnosis of qualitative variants.

In conclusion the VWF:CBA is a sensitive test in the diagnosis of type 2A and 2B VWD but is of limited use in discriminating type 2M VWD. The VWF:CBA therefore should not be considered a replacement for the VWF:RiCo assay, but rather as another useful diagnostic test in the profile of VWD testing, enhancing the ability to determine functional variants in VWD.
CHAPTER 6

IDENTIFICATION OF TYPE 2 VON WILLEBRAND DISEASE IN PREVIOUSLY DIAGNOSED TYPE 1 PATIENTS: ASSESSMENT OF THE PHENOTYPES, GENOTYPES AND MOLECULAR MODELLING
6.1. INTRODUCTION

Minimum requirements to investigate a presumptive diagnosis of type 2 VWD should include the analysis of the VWF:RiCo/VWF:Ag ratio, the plasma VWF multimers and the ristocetin-induced platelet agglutination (RIPA). In type 1 VWD the VWF activity levels are proportionately reduced to the VWF:Ag levels whereas in type 2 VWD (except type 2N) the ratio of VWF:AC / VWF:Ag is characteristically discrepant with values of less than 0.7 (Federici 1998; Federici 2000).

In patients with type 2A VWD who lack the HMW and intermediate molecular weight plasma multimers, the diagnosis is relatively straightforward. The main difficulties arise in differentiating between type 1 and type 2M VWD as both subtypes are defined by the presence of normal plasma multimers. Furthermore, the assays available to detect the presence of an abnormal VWF protein which is characteristic for type 2M VWD are not very sensitive. It is estimated that the sensitivity of the standard laboratory tests is approximately 60% and it is also known that plasma VWF levels are subjected to huge variations (Miller 1979). Distinct regions of the VWF-A1 domain have been implicated in the interaction with ristocetin and botrocetin and the discrepancy between ristocetin and botrocetin induced VWF binding to platelets appears to be useful in defining certain type 2M VWD forms (Brinkhous 1983; Sugimoto 1991). Although multimer analysis plays a crucial role in differentiating the subtypes of VWD, the recognition patterns are not always well defined and specific for a certain subtype. Occasionally only a slight
decrease in the HMW multimers can result in a significantly decrease in function and the issuing phenotype is difficult to classify (Hilbert 1995).

In the patients with type 1 VWD registered at the RFHHC and included in the patient database described in Chapter 3, an analysis of the ratio between VWF:AC/VWF:Ag showed that in some of these patients classified as classical dominant type 1 VWD, the ratio was discrepant. This observation suggested the possibility that some of these patients could be misclassified and they could have type 2 VWD. The hypothesis was that, based on previously reported 'normal' VWF multimeric pattern, this subgroup of patients with discrepant ratios might include patients with type 2M VWD. Consequently, a group of 111 patients originally diagnosed with type 1 VWD was re-analysed and of them a subgroup was identified who required reclassification as type 2 VWD based on repeat phenotype and genotypic data. The molecular graphics modelling provided explanations of the possible functional effects of the mutations identified in this study.

6.2. MATERIALS AND METHODS

Patient selection

As of 1st January 1998, 317 patients were registered with type 1 VWD at the RFHHC as included in the patients’ database (Chapter 3). The patients' records showed that full phenotypic data including VWF GPIb binding activities (VWF:Activity), VWF:Ag levels and VWF multimers were available for 111 patients. The VWF:Activity had been determined by one of two tests based on either the VWF:RiCo method or the in-house ELISA method (VWF:AC) (see Chapter 3). In all 111 patients multimeric pattern had
been reported as showing the presence of all VWF plasma multimers. For this group of patients, retrospective data on the VWF:Activity and VWF:Ag levels had been collected in order to calculate the VWF:Activity / VWF:Ag ratios which are plotted in Figure 6.1.

![VWF:Ac / VWF:Ag Ratio in Type 1 VWD](chart)

**Figure 6.1.** Scattergram illustrating the VWF:AC/VWF:Ag ratio in 111 patients previously diagnosed as type 1 VWD.

The median ratio for this group of 111 patients was 0.87 with ranges between 0.14 and 1.52. However, the study was restricted to those patients who presented with a discrepant VWF:AC/VWF:Ag ratio of less than 0.7, as this is the 'cut-off' value suggested in the literature (Federici 1998, Federici 2000). Of these patients, a subgroup of 30
individuals (from 17 unrelated families) who showed apparently normal VWF multimers and a ratio VWF:Activity / VWF:Ag of < 0.7 was selected. This group of 30 patients (with the exception of kindred 4) were reviewed at the RFHHC and informed consent for the study was obtained. From each patient a detailed bleeding history, family history and blood samples to perform the phenotypes and genotypes investigations were obtained.

**Phenotype analysis**

The VWF:Ag, FVIII:C, VWF:RiCo and plasma VWF multimers were performed as described in *Chapter 3*. These tests were repeated on fresh samples for 29/30 patients analysed.

The multimeric analysis of plasma VWF:Ag was performed using SDS-agarose gel electrophoresis at gel concentrations of 1.2%, 1.4% and 1.8% and the visualisation of the multimeric bands by autoradiography using $^{125}$I labelled polyclonal anti-VWF antibody (Dako Ltd, Bucks, UK) (Enayat 1999). The relative proportion of each multimer band was evaluated by scanning the autoradiograph on a GS 700 densitometer (Bio-Rad, Hemel Hempstead, UK). The high molecular weight (HMW) multimers were defined as those with a mass larger than the ninth band, and expressed as a percentage of the total when compared to normal pooled plasma.

**Genetic analysis**

Genomic DNA was extracted from peripheral blood leukocytes using the QIAmp DNA extraction kit (Qiagen Ltd., Crawley, West Sussex, UK). A 936 bp fragment of the 5'
region of the exon 28 that encoded for the VWF-A1 domain was amplified by polymerase chain reaction (PCR). The primer sequences (Inbal 1993) were as follows:

5'-TGGGAATATGGAAASGTGCATTG-3' (primer 226)
5'-CCGATCCTTCCAGGACGAASC-3' (primer 227a)

The PCR products were purified using the QIAmp DNA purification kit (Qiagen Ltd.). Both the forward and reverse strands were directly sequenced by automated sequencing technology using either an infra-red detection system (MWG Biotech, Milton Keynes, UK) or a fluorescent detection system (ABI 377, PE Biosystem, Warrington, UK). The candidate mutations were confirmed by either restriction analysis or by manual sequencing (manual sequencing was performed by Dr PV Jenkins at the RFHHC).

**In-vitro expression work and platelet binding assays of the recombinant VWF**

Functional expression studies of mutations were part of a collaborative project with the French INSERM Network on Molecular Abnormalities in von Willebrand disease coordinated by Pr D. Meyer (INSERM U.143 – Le Kremlin Bicêtre, France) and the experiments were performed by Dr L Hilbert and Dr C. Mazurier (LFB Lille, France). A brief overview of the techniques used is presented, then the characteristics of the mutated recombinant VWF (rVWF) are reported.

**Site-directed mutagenesis**

The vector containing the V516F mutation (F516pSVVWF), was constructed by site-directed mutagenesis using the commercial Transformer™ Site-Directed Mutagenesis Kit
(Clontech Laboratories, Palo Alto, USA). This method simultaneously anneals two phosphorylated primers to one strand of the denatured double-strand plasmid. One primer (mutagenesis primer) introduces the desired mutation, the second primer (selection primer) mutates a unique restriction site of the plasmid for the purpose of selection. The G3835T (numbering is from A from ATG as nt +1) substitution was inserted, by this method, in the pUC-Bgl II vector and the unique restriction site Ssp I was destroyed in the selection primer Sspl/0. The pUC-Bgl II vector was constructed by inserting at the BamHI polycloning site of the commercial pUC19 vector the Bgl II fragment of VWF (nt 2141-6836). After DNA elongation and ligation, the mixture of mutated and nonmutated DNAs was digested with Ssp I and transformed into a mutS E. coli strain defective in mismatch repair, according to the manufacturer's instructions (Clontech). Plasmid DNA was prepared from the mixed bacterial population using a Qiagen tip 20 (Qiagen, Germany). DNA was then digested again with Ssp I. Since the mutated DNA lacked the Ssp I restriction site, it is resistant to digestion whereas the parental DNA, sensitive to digestion, is linearized and thus becomes at least 100 times less efficient in transformation of bacterial cells. A final transformation with the digested DNA was performed to recover the desired mutated plasmid. As the Val516Phe mutation induces the destruction of a restriction site Asp I, DNA from some clones was amplified by PCR and digested with the Asp I restriction enzyme. Two mutated Phe516 pUCBgl II clones were then selected and further subcloned into pSVVWFA (Hilbert 1995) by transferring the Not I-Afl II fragment from the mutated pUC-Bgl II clone in the final vector pSVVWFA digested in parallel with the two enzymes. The Not I-Afl II sequence
of the final vector Phe516pSVVWFA was sequenced to confirm the absence of other unwanted mutations.

**Cell culture and transient transfection**

Expression of normal and mutated vectors was performed in COS-7 cells, an African green monkey kidney cell line. These cells have been permanently transformed with origin-defective SV40 virus, which has integrated into COS cell chromosome DNA. SV40 origin-containing plasmid (as pSVVWFA) replicates in the COS cell and produces to relatively high level but over a short period of time the protein which cDNA has been inserted in the plasmid.

Cos-7 cells were grown in DMEM containing L-Glutamine, penicillin, streptomycin, fungizone and 10% (V/V) bovine serum. These cells were transfected with normal or mutated plasmids using the diethylaminoethyl dextran method, as previously described (Hilbert 1994). On the day before transfection, 1-1.5 x 10^6 cells were seeded in 75 cm^2 flasks. The subconfluent cells were transfected with 25 μg of plasmid DNA per culture flask in 3.8 ml Tris-Saline buffer (Hilbert 1994). After 1 hour of incubation at 37°C, the transfection medium was removed and the cells were incubated for 4 hours in 8 ml of medium containing 100 μmol/l chloroquine. The cells were then washed twice with Tris-Saline buffer and left to recover for 40 hours in 10 ml of medium. The cells were then cultured for 72 hours in 8 ml of medium. Conditioned media containing normal rVWF (WTrVWF) or mutated rVWF (F516rVWF) were collected after 72 hours of expression in the presence of benzamidine (10mM) and phenylmethansulfonylfluoride (1 mM).
The rVWF multimers were electrophoresed in 1.5% agarose gels in the presence of 0.1% SDS and visualized with alkaline phosphatase-conjugated immunopurified anti-VWF polyclonal antibodies (Hilbert 1994). Ristocetin or botrocetin-dependent platelet binding assays were carried out as previously described (Hilbert 1994).

**Location of mutation sites in the VWF-A1 crystal structure**

Molecular modeling of mutations were performed as part of a collaborative project with Pr SJ Perkins and Dr PV Jenkins in the Department of Biochemistry and Molecular Biology Royal Free Hospital London. Mutation sites were located in two VWF-A1 crystal structures for the free domain and its complex with the GPIbα-binding inhibitory antibody NMC-4 at resolutions of 2.2 Å and 2.3 Å respectively (Celikel 1998; Emsley 1998) (Protein Data Bank codes: 1auq and 1oak). Protein structures were visualised using INSIGHT II (MSI, San Diego, USA) on Silicon Graphics Workstations equipped with Crystal Eyes stereo glasses. Secondary structures and side chain solvent accessibilities were calculated using DSSP and COMPARER (Jenkins 1998). The secondary structure labelling follows that of (Emsley 1998; Jenkins 1998) in which α-helix A2 is atypically not found in the VWF-A1 domain.
6.3. RESULTS

Phenotype Analysis

The phenotype and genotype results for the 17 kindred are presented in Table 6.1.

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<th>Kindred</th>
<th>Blood group</th>
<th>Bleeding time (min)</th>
<th>FVIII:C (IU/dl)</th>
<th>VWF:Ag (IU/dl)</th>
<th>VWF:RiCo (IU/dl)</th>
<th>VWF:RiCo/VWF:Ag ratio</th>
<th>Mutation (heterozygous)</th>
<th>Amino-acid substitution</th>
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<tr>
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<td>38</td>
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<td>R611L</td>
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<tr>
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</table>

Normal range < 8 50-150 50-150 50-150 1

Table 6.1. Phenotypic and genotypic data on the 17 kindred with a discrepant VWF:RiCo/VWF:Ag ratio. ND= not done; NI= not identified
The repeat analyses confirmed the presence of reduced VWF levels and activity as measured by VWF:Ag and VWF:RiCo. The ratio of VWF:RiCo / VWF:Ag was less than 0.7 in each affected patient (range < 0.13 - 0.58). A wide range of VWF:Ag was observed (8-76 IU/dl). In patients 1 and 14, the VWF:Ag levels were normal. The majority of patients had VWF:RiCo levels of less than 20 IU/dl (range < 5 - 41 IU/dl). Patient 14 had a VWF:RiCo of 41 IU/dl and VWF:Ag of 70 IU/dl with a normal BT, so this patient has borderline normal VWF levels. Retrospective analysis showed that RIPA assays were performed in 26/30 (87%) patients and an absent or decreased responsiveness to low dose ristocetin with reduced agglutination at 1.5 mg/ml was reported in all cases.

**Genetic and structural analysis of the mutations identified**

The genetic analysis of the 17 kindred revealed eight heterozygous mutations in nine kindred. Four were novel candidate mutations, while four were previously described mutations associated with type 2 VWD (Table 6.1). In the remaining eight kindred, no mutation within the 5’ part of exon 28 of the VWF gene was identified.

**Genetic and structural analysis of the four novel candidate mutations**

- **V516F mutation**

Kindred 1 was found to be heterozygous for G4085T substitution (V516F mutation). The affected woman had a significant bleeding history, including life-long easy bruising, epistaxis, severe menorrhagia and post surgical bleeding. The mutation results in the
substitution of an aliphatic residue by a larger aromatic one. The substitution does not affect the expression of protein as demonstrated by the normal VWF:Ag (Table 6.1). The VWF-A1 crystal structure has a central β-sheet that is flanked by α-helices A4, A5 and A6 above it and α-helices A3, A1 and A7 below it (Figures 6.2a and 6.2b). Inspection showed that V516 is buried at the centre of the VWF-A1 domain in the first β-strand BA of the central β-sheet. Its side chain is pointed above the β-sheet towards about four residues on the buried face of α-helix A4 (Figure 6.2c), which is itself close to the GPIb receptor binding surface and residues involved with ristocetin binding (Celikel 1998; Emsley 1998). V516 is well conserved in VWF from 28 mammalian species, only being occasionally replaced by a smaller Ala residue (Jenkins 1998). It is deduced that the substitution is capable of disrupting the internal packing of side chains in the VWF-A1 domain to affect residues involved with functional binding in the structural region corresponding to type 2M mutations (Figures 6.2a and 6.2b) (Jenkins 1998; Cruz 2000).
Figure 6.2.
Location of the four novel candidate mutations (yellow) and the four previously-described mutations (purple) in the crystal structure of the VWF-A1 domain (PDB code 1auq).
(a) and (b) correspond to two orthogonal perspectives rotated by 90° about the vertical axis. The protein mainchain backbone is represented by a ribbon. The N- and C-termini are denoted by N and C respectively. The central β-sheet is seen edge-on in both views. The six α-helices A1, A3, A4, A5, A6 and A7 are labelled in (b). The mutated residues are identified by spheres at their α-carbon atom. The type 2B mutations are shown in green, while the type 2M mutations are shown in blue.
(c) The internal packing of the sidechain of V516 on β-strand BA against four residues at the buried face of α-helix A4, viewed in the same orientation as in (a). The sidechains of T601, I605 and F606 are in the closest proximity to that of V516.
(d) The involvement of R552 in salt bridges with D514 and D610, and that of R611 in hydrogen bonds with the mainchain O atoms of Q548 and W550, viewed in the same orientation as in (a).
To demonstrate that the candidate mutation identified is responsible for the patient's phenotype, normal and mutated VWF molecules were transiently expressed in Cos-7 cells. F516rVWF displayed a slight decrease in the HMW multimers although they were present. The ability of mutated rVWF to bind to formalin-fixed platelets in the presence of agonists was further tested. Wild type rVWF induced ristocetin dose-dependent binding to platelets with maximal binding reached at 1 mg/ml. In contrast, F516rVWF was almost unable to bind to ristocetin irrespective of the concentration of ristocetin (0 to 1 mg/ml). This capacity was dramatically decreased in the presence of botrocetin (0 to 0.25 μg/ml). The platelet binding experiments of the hybrid rVWF (resulting from cotransfection experiments of wild type and mutated plasmids, which is a direct reflection of in-vivo patient VWF composed of both normal and mutated subunits present in the heterozygous state) are currently in progress.

- **R636C mutation**

Kindred 3 were heterozygous for a C4445T substitution (R636C mutation). This mutation occurred in an individual with a history of recurrent epistaxis. The mutation CGC to TGC is located at a codon containing a previously described polymorphism G4446 to A (CGC to CAC) that results in the mutation R636H. This polymorphism has an incidence of 1.5% in the general population (Cooney 1991). Scanning mutagenesis experiments have shown that R636 is located in an important site for binding to GPIb as it selectively decrease the botrocetin induced binding to GPIb (Matsushita 2000) and play a direct role in the GPIb binding (Vasudevan 2000). Even though R636 is well conserved in 28 mammalian VWF (Jenkins 1998), the mutation occurs at the surface of the VWF-A1
domain in the GPIb receptor binding site region, and is well positioned to disrupt GPIb binding as for type 2M mutations (Figures 6.2a and 6.2b).

In vitro expression studies of the function of the recombinant VWF containing the R636C mutation are currently in progress as part of a collaborative project with the French INSERM Network on Molecular Abnormalities in von Willebrand disease.

- **I653T mutation**
  Kindred 5 were heterozygous for a T4497C substitution (I653T mutation). This kindred had a dominant family history of VWD spanning three generations. The clinical symptoms included easy bruising, epistaxis and bleeding post dental extraction. I653 is fully conserved in 28 mammalian VWF (Jenkins 1998). It is buried at the C-terminus of β-strand BE near the active site cleft of the domain, between α-helices A6 and A7 (Figure 6.2b) and its side chain points towards the GPIb and ristocetin binding sites. The substitution of a hydrophobic residue by a polar one is likely to affect the local folding of the VWF-A1 domain close to these binding sites, i.e. in the region corresponding to type 2M mutations (Jenkins 1998).

- **Y558D mutation**
  Kindred 8 were heterozygous for a T4211G substitution (Y558D mutation). This kindred also had a dominant family history of VWD with a moderate bleeding history. The presence of the mutation was confirmed in the two affected members, whereas the
unaffected members did not inherit the affected allele. Y558 is buried at the C-terminus of β-strand BB close to the active site cleft, with its sidechain pointing above the central β-sheet in proximity to α-helix A4 (Figure 6.2a and 6.2b). It is conserved in 27 of 28 mammalian VWF (Jenkins 1998; Matsushita 2000). This mutation has potential to disrupt the active site cleft through the replacement of a hydrophobic residue by an acidic charged one with high solvent affinity.

The three candidate mutations V516F, R636C and I653T are associated with reduced VWF-platelet interaction as suggested by normal plasma VWF multimers and a reduced VWF:RiCo as compared to the VWF:Ag. In contrast to the five other mutations of Table 6.1, these three candidate mutations were associated with normal or mildly reduced VWF:Ag and they are likely to reduce directly or indirectly the ability of the VWF-A1 domain to interact with the GPIb-IX-V complex. Therefore, these three candidate mutations are clinically associated with type 2M VWD in accord with the molecular graphic views of Figure 6.2.

**Genetic and structural analysis of the four previously described mutations**

Three previously described heterozygous mutations involving the same residue were found in kindred 2 (R611L), kindred 4 (R611C), and kindred 6 and 7 (R611H) (Hilbert 1995; Meyer 1997). A previously described heterozygous mutation R552C was found in kindred 9 (Meyer 1997; Zhang 1995).
Restriction enzyme digestion

The nucleotide substitutions present at residue R611 resulted in the abolition of a *Bsa OI* restriction site. Restriction of the normal PCR sequence resulted in three fragments of 110 bp, 426 bp and 400 bp length. The presence of substitutions at nucleotides 4370 or 4371 resulted in the abolition of one of the restriction sites leading to only two fragments of 510 bp and 426 bp. The digestion pattern with *BsaOI* enzyme confirmed that the kindreds with mutations at residue R611 were heterozygous for these mutations (Figure 6.3).
Restriction enzyme analysis using *Bsa O1*

**Normal Sequence**

936bp → 426bp → 110bp → 400bp

**Mutated Sequence : R611 to H/L/C**

936bp → 426bp → 510bp

**Digestion pattern with the BsaO1 restriction enzyme**

Lane 2,4,17,18 = kindred 2,4,17,18 respectively; M = marker (100bp ladder)
All five kindreds in whom four previously identified mutations were found had a family history of VWD, mild bleeding symptoms and similar reduced VWF:Ag levels between 11 and 25 IU/dl. These four mutations can be considered as a single group as in molecular views they are all located near the N-terminus of the VWF-A1 domain on the opposite side from the active site cleft. The two residues are at the N-terminus of β-strand BB (R552) and on the loop between the alpha-helix A4 and β-strand BD (Arg611). In the crystal structures (Celikel 1998; Emsley 1998), the sidechain of R552 forms salt bridges with D514 and D610 above the central beta-sheet, and that of R611 forms hydrogen bonds with the mainchain carbonyls of Q548 and W550 to stabilise the VWF-A1 domain (Figure 6.2d). These interactions stabilise the beta-sheet and loop residues Q548-R552 relative to the upper part of the VWF-A1 structure. Both R and D residues are fully conserved in the 28 mammalian VWF sequences with only one conservative substitution (Jenkins 1998).

**Multimeric analysis of VWF**

The plasma VWF multimer analyses were repeated in the nine kindred in whom mutations were found. The multimer patterns for kindred 1, 2, 3, 4 and 5 showed that high, intermediate and low molecular weight multimers were present. The triplet configuration was normal on high resolution (1.8%) gels. Densitometric scanning confirmed that the percentage of the protomers and the high molecular weight (>9 mers) multimers were similar to normal pooled plasma controls. The gels for kindred 6, 7 and 8 showed a slight decrease in the HMW multimers when these were compared with normal
plasma (Figure 6.4). When the HMW multimers were expressed as a percentage of the total multimers, kindred 6 showed 20% vs 33% normal, kindred 7 showed 12% vs 34% normal and kindred 8 showed 29% vs 41% normal control. In kindred 9 the presence of both HMW and ultra large molecular weight multimers ('supranormal') were visually observed and resembled type 2M Vicenza phenotype (Mannucci 1988) (Figure 6.4).
Figure 6.4.

Autoradiograph of the plasma VWF multimers from a normal individual (Lane 1), patient 8-1 showing a decrease of the HMW multimers (Lane 2: type 2 unclassified VWD), and patient 9-2 showing “supranormal” multimers (Lane 3: type 2M Vicenza). On the left of the multimer gels are the densitometric traces from these samples in the same order from left (Lane 1) to right (Lane 3). Samples were electrophoresed on 1.4% agarose gels and the direction of the electrophoresis is from top to bottom.
6.4. DISCUSSION

A group of 30 patients (17 kindreds) was initially diagnosed as type 1 VWD on the basis of a moderate bleeding history, an autosomal dominant family history, reduced levels of VWF, non-enhanced RIPA and a multimeric pattern labelled as 'normal'. However, these patients had discrepant VWF:AC / VWF:Ag ratio, which is an indirect reflection of an impaired interaction between the VWF and platelet GPIb and suggestive of a qualitative defect of VWF. Consequently, the hypothesis of the study was that these patients may have a functional type 2 VWF defect, in particular type 2M VWD based on the reported normal multimeric pattern.

Whereas over 40 type 2A VWD mutations (http://mmg2.im.med.umich.edu/vWF) have been reported at present only a few mutations inducing type 2M VWD have been identified. Three of these mutations are missense mutations responsible for the peculiar type B VWD phenotype characterized by normal botrocetin-induced binding to platelets but absence of binding in the presence of ristocetin: G561S (Rabinowitz et al 1992), F606I and I662F (Hillery et al 1998). The other two mutations are deletions responsible for decreased ristocetin-and botrocetin-induced binding to platelets (deletion of a lysine within a four lysine residue repeat (Hilbert 2000) and a 11 aa deletion (Mancuso 1996).

Reappraisal of standard phenotypic data, together with a search for mutations in the VWF-A1 domain, molecular modelling and expression work, demonstrated that at least half of the kindreds included in the RFHHC study had qualitative abnormalities, as
mutations associated with reduced binding to GPIb were identified in nine of them. In the remaining eight kindred, the defect is still likely to be qualitative as indicated by the phenotype analysis, however the location of the underlying mutation could be outside the VWF-A1 domain, and further analysis of the VWF gene is required. Another possible cause why mutations have not been identified in the 5’ part of exon 28 in these eight kindred could be attributed to the primers which have been used for the PCR analysis in this study. As will be shown in Chapter 7 the use of primer 226 can lead to failure of amplification of an allele which contains a polymorphism in the region recognised by the primer (Eikenboom 1994). Hence it is possible that in some of these kindred who may harbour a mutation on an allele which also contains the same polymorphism common to the primer, the DNA sequence appears normal due to failure of amplification of the respective allele. In order to circumvent this problem, the 5’ part of exon 28 from the eight kindred in whom no mutations were identified will be re-amplified using a different set of primers.

The reclassification of VWD type was based on the phenotype and it was complemented by genetic analysis of the 5’ part of exon 28 of the VWF gene, which is the region that corresponds to the VWF-A1 domain responsible for the GPIb-IX-V interaction. The four novel candidate mutations (V516F, Y558D, R636C and I653T) occurred in four kindreds with mild bleeding histories. Mutations R636C and I653T led to a normal multimeric pattern suggesting a type 2M phenotype.
The V516F mutation led to a normal plasma VWF multimeric pattern however, the recombinant product containing this mutation showed a slight reduction in the HMW multimers. Studies of platelet binding to plasma VWF demonstrated that the underlying defect in the kindred with V516F mutation was a qualitative abnormality, specifically the reduced ability of VWF to interact with platelets. The expression of the V516F recombinant VWF demonstrated that the mutation is the cause of the underlying bleeding disorder in this kindred.

Of note, in parallel with the expression work on the V516F mutation, the French group have also analysed another mutation occurring at the same position but leading to a different amino acids substitution, V516I (Hilbert 2001). In contrast to V516F, the multimeric structure of the I516rVWF was normal and it showed a decreased capacity to bind platelets in the presence of ristocetin but normal botrocetin induced binding. Therefore, the patient with V516I mutation displays a type 2M (previously type B) phenotype. In contrast, the F516rVWF showed a decreased in both ristocetin and botrocetin induced platelet binding activities and a small decrease in the HMW multimers. These characteristics of the recombinant product with V516F mutation argue against a classical type 2M VWD and are more in keeping with 2A VWD phenotype, hence the V516F mutation remains at present ‘type 2 VWD unclassified’. The two novel mutations identified at position 516 in the mature VWF in the A1 domain leading to the substitution of two different amino acids showed different functional characteristics of the recombinant product. These observations confirmed the importance not only of the
position of the amino acids but also of the nature of the amino acids change involved in binding of VWF to GPIb platelet receptor.

The difficulties of accurately classifying the variant of VWD underline the current limitations of the existing classification of the VWD (Sadler 1994). The revised classification system was designed to allow a simple approach to the separation of major VWD variants and is entirely based on the phenotypic characterisation of the subtypes. Apart from the first definition in the classification which states that 'All VWD is caused by mutations at the VWF locus' – which is now coming under review as more data suggest that defects in genes other than the VWF gene may result in VWD (Nichols 1995; Mohlke 1996), the molecular characterisation is not part of the diagnosis of VWD subtypes. Moreover, the identification of a mutation alone cannot lead to a prediction of its effect as this requires an understanding between the association of phenotype and genotype. Expression work can confirm the causative nature of a mutation and furthermore the study of the mutation site on the three-dimensional structure of VWF domains can provide a rational explanation of the effects of different mutations. However, because of the very complex relationship between the structure and function of VWF, the present classification of VWD which is entirely based on phenotypic criteria, cannot allow all variants described to strictly fit into a certain type of disease, hence an increasing number of mutations, such as the V516F remain 'type 2 VWD unclassified'.

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Mutation Y558D was associated with a slight decrease in the plasma HMW multimers. As the multimeric pattern present in the kindred with Y558 mutation is distinct from the classical type 2M phenotype (normal multimers) or type 2A phenotype (absence of HMW and intermediate forms of multimers) the kindred is probably best identified as 'type 2 VWD unclassified' until further in vitro expression studies are performed.

The four previously known mutations at R552 and R611 possess a type 2 phenotype whose subtype 2A or 2M is again unresolved. However, there is agreement that these mutations cause a qualitative variant of VWD, by generating a loss of function in VWF with decreased binding to platelet GPIb.

The R611 mutation has been previously reported on multiple occasions (http://mmg2.im.med.umich.edu/vWF). It occurs at a CpG dinucleotide which is a hotspot for mutations in the human genome (Barker 1984). Initially this mutation was described as a type 2B-like mutation because it was found in patients with DDAVP-induced thrombocytopenia, enhanced RIPA and a relative decrease in HMW multimers (Castaman 1995). However, the type 2B-like pattern has not been confirmed by subsequent studies. Hilbert et al (Hilbert 1995) demonstrated that mutant recombinant VWF C611 and H611 reproduced a low affinity for platelet GPIb in plasma and expression in Cos7 cells exhibited an abnormal multimeric pattern with moderate decrease in HMW and an increase in low MW multimers in plasma and platelets. They demonstrated that the R611 mutations lack ristocetin induced platelet binding activity but
they retain the ability to bind to platelets in the presence of botrocetin (Hilbert 1995). Nishikubo et al studied eight patients with the R611 mutation and a slight decrease in the HMW plasma multimers and they showed an increased abnormal proteolysis resulting in an additional 209 kDa fragment (Nishikubo 1995). The R611 mutation was also described in association with phenotypes characterised by a normal multimeric pattern (Lavergne 1997).

The R552 mutation was first described by Zhang et al (Zhang 1995). This mutation has been associated with a type 2A phenotype (Meyer 1997) and also with a type 2M VWD phenotype (Casana 1998). Meyer et al have shown that patients with R552 mutation demonstrated no binding to the GPIb in the presence of either ristocetin or botrocetin and their HMW multimers pattern was decreased or absent (Meyer 1997). The R in position 552 is flanked by residues in which naturally occurring mutations have been previously described (Ginsburg 1993). However, the R552 has not as yet been expressed in vitro.

In the RFHHC cohort, the kindred with R611C and R611L mutations exhibited a normal multimeric pattern consistent with type 2M phenotype. The kindred with the R552C mutation exhibited a hitherto unreported supranormal multimeric pattern that resembles the pattern seen in type 2M Vicenza (Mannucci 1988; Schneppenheim 2000). The two kindred with R611H mutations had a moderate reduction in the HMW multimers with a normal triplet structure and were associated with a severe decrease in function. These
kindred are identified as 'type 2 VWD unclassified'. The analysis of platelet VWF will be required to determine whether this mutation results in impaired synthesis of HMW multimers or increased proteolysis of VWF multimers.

The molecular visualisation of the eight mutations was based directly on the two VWF-A1 crystal structures instead of the homology models (Jenkins 1998), together with an improved understanding of VWF-A1 domain function (Perkins 1999). In agreement with the loss of activity that is correlated with these mutations, all eight mutations have structural consequences for the VWF-A1 structure on the upper side of the central β-sheet as viewed in Figure 6.2 that possesses the GPIb receptor binding site and is associated with type 2M mutations. Interestingly, several of the mutations lead to phenotypes that are variants of type 2M or type 2 VWD unclassified, and correspond to structurally distinct subsets of mutations. Most notably, R552 and R611 are located on the N-terminal side of the VWF-A1 domain on the opposite side to the active site cleft and participate in buried interactions. None of the eight mutations corresponded to type 2B mutations which are localised on the N-terminal face of the VWF-A1 domain in the vicinity of α-helix A2 and A3 on the lower face of the central β-sheet as seen in Figure 6.2a.

In summary, this study showed that type 1 VWD may be misclassified leading to the underdiagnosis of type 2 VWD, either type 2M, type 2A or as yet ‘type 2 unclassified’. This study shows that type 2M, and in general type 2 VWD, is more frequent than
previously thought and in addition to a simple quantitative deficiency, many patients with previously diagnosed type 1 VWD have qualitative abnormalities of VWF.

The correct classification of these patients is of clinical importance as it directly influences the choice of treatment required to achieve optimum haemostasis, especially under a haemostatic challenge such as the time of surgery. In type 1 VWD the majority of patients respond to DDAVP therapy, whereas the majority of patients with type 2 VWD require clotting factor concentrates (Mannucci 1998). However, consistently good responses to DDAVP were found in patients with R611 and R552 mutations (Lethagen 1998) and in a study by Nishikubo et al, a shortening of the BT and a partial correction of the multimeric pattern was found after DDAVP administration in patients with R611 mutations (Nishikubo 1995). Issues regarding the response to DDAVP in patients with type 2 VWD will be further addressed in Chapter 9.

There are many potential causes for the misclassification of type 1 VWD: the perceived rarity of qualitative variants; the poor sensitivity and limitations of currently available assays and the use of different functional assays for VWF, as described in detail in Chapter 5. Even though a discrepant VWF activity / antigen ratio is an important starting point for further evaluation of a qualitative type 2 VWF defect, this has been historically overlooked in the presence of the normal plasma multimers pattern.
A careful multimeric analysis plays an important role in the classification of VWD. Both type 1 and type 2M VWD are defined by normal multimers. However, subtly altered VWF multimeric structures have been described in type 2M VWD (Meyer 1997). Occasionally patients with type 2A VWD exhibit a full multimeric pattern on intermediate (1.4%) and high (1.8%) resolution gels, and only the low resolution gels (1.2%) and densitometry scanning can reveal the loss of the highest forms of the multimers. Moreover, as shown in this study, the interpretation of the multimeric structure cannot always provide an answer to a correct classification. Thus, the phenotypic analysis on its own cannot always establish the exact type of VWD in every affected individual. In these cases, the genotypic data can substantially aid in the diagnosis, especially in type 2 VWD, where mutations are localised within specific areas of the VWF gene. Identification of the potential mechanisms for the qualitative VWF defects related to the mutations that were found in this study in the VWF-A1 domain may help towards a better understanding of the VWF structure-function relationship.
CHAPTER 7

TYPE 2A VON WILLEBRAND DISEASE ASSOCIATED WITH CHRONIC THROMBOCYTOPAENIA – TWO CASE REPORTS ILLUSTRATING THE IMPORTANCE OF MOLECULAR BIOLOGY IN TYPE 2 VWD
7.1. INTRODUCTION

Type 2A VWD is characterised by the absence of the intermediate and large VWF multimers from plasma and a defective VWF–dependent platelet interaction. The inheritance of type 2A VWD is both dominant and recessive. The dominant type 2A VWD accounts for most of the type 2 VWD (Holmberg 1992) and the majority of these mutations are situated within a short segment of the A2 domain (between G742 to E875) and only a few mutations are found in the A1 domain. The most common mutation is R834W which accounts for a third of all type 2A VWD. Mutations in the propeptide region which interfere with the VWF processing and multimers assembly have also been recognised (Schneppenheim 1995, Gaucher 1996). Mutations in the VWF gene lead to type 2A VWD by two main mechanisms (Lyons 1992, Dent 1990): group 1 mutations cause a defective intracellular transport of VWF and impairs the assembly, storage and secretion of large VWF multimers in both plasma and platelets. Thus, individuals with group 1 mutations have absent intermediate and HMW multimers in platelets. Group 2 mutations render the multimers more sensitive to proteolysis in plasma. The protease cleavage site is at position Y842-M843 in mature VWF and the cleavage results form the action of a specific metalloproteinase which has been recently identified (Furlan 1996b). Individuals with group 2 mutations exhibit a full spectrum of platelet VWF multimers (Lyons 1992).

Type 2B VWD is characterised by an increased binding of VWF to the platelet GPIb/IX complex. This upregulated binding to platelets is reflected in vitro by an enhanced
ristocetin-induced platelet aggregation (RIPA) in the presence of low concentrations of ristocetin. The plasma HMW multimers are absent from the circulation but platelet VWF multimers are normal (Ruggeri 1980). Rare variants with a full multimeric pattern in the plasma are also described (Holmberg 1993). Associated thrombocytopenia in type 2B VWD is variable. It may depend on age, and it can be exacerbated by stress, bleeding, pregnancy or post DDAVP infusion. The thrombocytopenia is thought to be due to adsorption of the HMW multimers onto the platelets and subsequent rapid clearance from the circulation. However, recent studies have shown that in type 2B VWD thrombocytopenia is not due to platelet activation and consumption but to agglutination phenomena (Casonato 1999).

The majority of type 2B mutations are localised in a short segment of 38 amino acids (between 540-578 amino acids) within the A1 domain of VWF and in positions adjacent to the loop corresponding to the region involved in the GPIb binding.

In a patient with VWD, the presence of a low platelet count can pose a diagnostic challenge. In these circumstances two main possibilities should be considered: the presence of type 2B VWD and/or an associated second disorder, such as immune thrombocytopenic purpura (ITP). This chapter describes two patients with type 2A VWD in whom, because of an associated low platelet counts, a diagnosis of type 2B VWD was considered and investigated. These patients illustrate the importance of the molecular diagnosis and modelling in type 2 VWD and also the difficulties and the
therapeutic implications of a correct diagnosis. The methods used in the two studies will be described together and then the two Case reports, Results and Discussions will be presented separately.

7.2. MATERIALS AND METHODS

Phenotype analysis

Bleeding time (BT), FVIII:C, VWF:AC, VWF:Ag and RIPA were performed as described in Chapter 3. The PFA-100™ analysis was performed as described in Chapter 8. Plasma VWF multimer analysis and densitometric scanning were described in Chapter 6. Platelet VWF multimer analysis was performed at the Birmingham Children's Hospital centre as part of a collaborative project, following the methodology described by Lopez-Fernandez et al (Lopez-Fernandez 1986). The methodology for the densitometric scanning of platelet VWF multimers was similar to the techniques used for scanning the plasma VWF multimers.

Genetic analysis and molecular modeling

i. Screening for type 2A VWD mutations

The 3' end of exon 28 of VWF which encodes for the A2 domain of VWF was amplified by PCR using the following primers which yielded a fragment of 631 bp:
**Sense primer:** 5’ ATGGTTCTGGATGTGGCGT 3’ (primer 373)

**Antisense primer:** 5’ TGCTACATGCATCTGCCAASGA 3’ (primer 375a)

The PCR conditions were: denaturation 94°C for 30 sec, annealing 57°C for 30 sec and elongation 72°C for one min, for a total of 40 cycles. Despite altering the PCR conditions several times (by increasing the annealing temperature) two bands were consistently obtained when the PCR products were run on a 2% agarose gel. The band which corresponded to the 631 bp was then manually excised and purified according to the manufacturer’s instructions. Purified PCR products were then automated sequenced for both forward and reverse strands (for details see Chapter 3).

**ii. Screening for type 2B VWD mutations**

The 5’ end of exon 28 which encodes for the A1 domain of VWF gene was amplified by PCR 1 using the following primers (which yield a 936 bp PCR fragment):

**Sense primer:** 5’ TGGGAASTATGGAASGTGACATTG 3’ (226)

**Antisense primer:** 5’ CCGATCCCTCCAGGACGAASC 3’ (227a)

For the second PCR (PCR 2) the following primers were used (558 bp fragment):

**Sense primer:** 5’ GACATCTCG GAMINO ACIDS CCG CCGT TG3’ (28 F1)

**Antisense primer:** 5’ TGAGCAGTGTGGATGAGCTGG 3’ (28 R2)

For the third PCR (PCR 3) the following primers were used (fragment of 308 bp):
*Sense primer:* 5' GACATCTCG GAA CCG CCGT TG3' (28 F1)

*Antisense primer:* 5' AGGTCTTGAASATACACACTGTT3' (Ex 74)

The PCR conditions were: denaturation 94°C for 30 sec, annealing 59°C for 30 sec and elongation 72°C for 30 sec, for a total of 40 cycles. The PCR products were purified and automated sequenced for both forward and reverse strands as described in *Chapter 3*.

Restriction enzyme analysis was performed directly in the amplification mixture of the three PCR products.

In vitro expression work is currently being performed by site directed mutagenesis of full-length cDNA and transiently expression in COS-7 cells using the same methodology as described in detail by Enayat et al (Enayat 2000).

**Haplotype analysis**

The following polymorphic markers were analysed: the polymorphic regions of the tetranucleotide (TCTA) simple repeat in intron 40 of the VWF gene, indicated as VNTR I (Peake 1990) and VNTR II (Ploos van Amstel 1990) and the following restriction fragment length polymorphisms (RFLP) of the VWF gene were determined: an *Rsa I* polymorphism in exon 13 (+ indicates presence; - absence of the restriction site); an *Rsa I* polymorphism in exon 18, an *Hph I* polymorphism in exon 28; an *Aci I* polymorphism in exon 42 and an *Xmn I* polymorphism in exon 45 (Eikenboom 1998). The linkage studies were performed at Birmingham Children's Hospital as part of collaborative work.
iii. Molecular modeling

Molecular modeling of the mutations identified was performed by Pr. S.J. Perkins from the Department of Biochemistry RFH. Details of the methodology are described in Chapter 6.

Platelet serology

Platelet immunology tests were performed at the Blood Transfusion Centre Addenbrooke's Hospital Cambridge.

Platelet associated immunoglobulins (PAIg) were tested by direct platelet immunofluorescent test (PIFT) and by the indirect PIFT in the serum. Serum screening for antibodies directed against the major platelet glycoprotein GpIIb/IIIa, GPIb/IX and GpIa/IIa was performed by the monoclonal antibody immobilization of platelet antigen assay (MAIPA).

7.3. STUDY OF PATIENT RT WITH TYPE 2A VWD ASSOCIATED WITH CHRONIC THROMBOCYTOPAENIA

7.3.1. CASE REPORT PATIENT RT

Patient RT was a male who presented initially at his local hospital at the age of four with severe epistaxis and easy bruising and a platelet count of $16 \times 10^9/l$ (normal range 150-400 $\times 10^9/l$). He was one of ten siblings, with no family history of bleeding disorders or
consanguinity. A bone marrow biopsy showed increased megakaryocytes suggestive of an increased peripheral destruction of platelets and a diagnosis of ITP was made. Steroids (1mg/kg/day) were administered for several months with a variable and inconsistent increased in the platelet count (between 10 - 93×10⁹/l). At age 16 a splenectomy was performed for ITP which was complicated by severe bleeding which required multiple transfusions of blood and platelets and several months of intermittent hospitalisation. Post splenectomy there was no response in the platelet count and immunosuppressive therapy with vincristine was added without any benefit. He remained a regular attendant to his local hospital for frequent bleeding episodes mainly from his mouth. At age 36 he was referred to the RFHHC with an unclear diagnosis of refractory ITP.

7.3.2. INVESTIGATIONS AT THE RFHHC - PATIENT RT

Phenotype analysis

The phenotypic results are tabulated in Table 7.1. The RIPA showed a normal response to low doses of ristocetin (0% with 0.5 mg/ml; reference range 0-5%) and a decreased response with high dose ristocetin (38% with 1.25 mg/ml ristocetin; reference range > 50%). Plasma VWF multimers showed the complete absence of the HMW forms with an abnormal triplet pattern. In addition radiographic analysis suggested an increase in the protomeric bands but a densitometric study of the autoradiograph is not available. These findings suggested a diagnosis of VWD type 2A phenotype which is classically not
associated with a low platelet count, in contrast to type 2B VWD. The chronic thrombocytopaenia was hypothesised to be attributed to either an associated ITP, a form of congenital thrombocytopaenia or an unusual VWD phenotype, i.e. an atypical type 2B VWD with low platelet count but a non-enhanced RIPA.

Table 7.1. Laboratory data in the two kindreds with VWD and thrombocytopaenia.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Patient RT</th>
<th>Patient LC II-2</th>
<th>Mother to LC I-1</th>
<th>Daughter of LC III-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleeding time</td>
<td>15 min</td>
<td>&gt; 20 min</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>FVIII:C</td>
<td>47 IU/dl</td>
<td>46 IU/dl</td>
<td>58</td>
<td>36</td>
</tr>
<tr>
<td>VWF:Ag</td>
<td>35 IU/dl</td>
<td>47 IU/dl</td>
<td>52</td>
<td>48</td>
</tr>
<tr>
<td>VWF:AC</td>
<td>10 IU/dl</td>
<td>32 IU/dl</td>
<td>36</td>
<td>25</td>
</tr>
<tr>
<td>Plasma multimers</td>
<td>Absent HMW</td>
<td>Absent HMW</td>
<td>Absent HMW</td>
<td>Absent HMW</td>
</tr>
<tr>
<td>Platelet count</td>
<td>15-186 ×10^9/l</td>
<td>50-110 ×10^9/l</td>
<td>119×10^9/l</td>
<td>72×10^9/l</td>
</tr>
</tbody>
</table>

Genotype analysis

Screening for type 2A and 2B VWD mutations was performed by analysing the whole exon 28 of VWF by two separate PCR and direct automated sequencing (see Chapter 3). No mutations were identified in the VWF-A2 domain where most of the type 2A VWD mutations are localised. However, mutations associated with type 2A VWD have been found in other regions outside the A2 domain of the VWF gene, such as the D1 and D2 domains encoding for the VWF propeptide (Gaucher 1994, Gaucher 1995, Schneppenheim 1995).

Further analysis of the VWF gene with amplification and sequencing of exons 11, 12, 13, 14 and 15 failed to identify any mutations responsible for the VWD phenotype. This
work was performed in Lille as part of a collaborative project with the French INSERM network.

Screening of the VWF-A1 domain for type 2B mutations did not show any mutations in the 5’ region of exon 28.

Platelet immunology

PAIg were positive for IgM by direct PIFT and the serum screen for platelet antibodies by an indirect PIFT was positive for IgM. Serum screening for antibodies directed against the major platelet glycoprotein GpIIb/IIIa, GpIb/IX and GpIa/IIa by MAIPA was negative. Although these results are inconclusive, a diagnosis of ITP can not be excluded.

7.3.1 DISCUSSION - PATIENT RT

Patient RT was diagnosed with type 2A VWD associated with chronic thrombocytopenia probably due to ITP. Since treatment with clotting factor concentrates rich in VWF has been started, this has significantly reduced his bleeding symptoms and substantially improved his quality of life.

This case illustrates how both VWD and ITP can be present in the same patient and can cause diagnostic confusion. Patient RT was initially diagnosed with refractory ITP which had major therapeutic implications, as he underwent a splenectomy followed by severe bleeding complications compounded by an hitherto unrecognised VWD. A diagnosis of
VWD was made with a delay of over 30 years after presentation when the patient has been referred to a specialised centre. The absence of a family history of VWD in this case may have further contributed to the delay in the diagnosis.

A pattern of mucosal bleeding can be caused by a defect in primary haemostasis and investigations for VWD and platelet function defects should be considered. In the context of VWD with unexplained chronic thrombocytopaenia but no increase response to ristocetin, type 2B VWD should still be considered, as atypical type 2B phenotypes (i.e. RIPA not enhanced) have been reported (Ribba 1994; Gaucher 1995). In patient RT a possible atypical type 2B VWD was ruled out as no mutation associated with type 2B was found. Type 2A VWD remains the most likely diagnosis in this patient and the causative mutation probably lies outside the A2 domain of VWF. As there is no family history of VWD it is possible that patient RT may have a recessive form of type 2A VWD, previously named IIC, which is characterised by a marked increase of the protomeric forms of VWF, recessive inheritance and the gene defect lying in the propeptide region of VWF (D1 and D2 domains) which has been shown to be required for multimerisation. However, in this case no blood samples from the family members were available for study.

In patient RT a diagnosis of chronic ITP was supported by the bone marrow findings, the platelet serology and the partial response to treatment with steroids. Platelet immunology has a controversial role in establishing an immune cause for
thrombocytopenia. Screening for PAIg using a platelet immunofluorescent test although highly sensitive has a relatively low specificity. If PAIg are present this is best confirmed with an antigen capture assay, such as the MAIPA assays, which have a sensitivity and specificity of around 80% (Kiefel 1987) and are considered better than the PAIg assays in discriminating immune from non-immune thrombocytopenia (Brighton 1996). Platelet serology has a limited role in the investigation of ITP, in particular before treatment options are considered and especially as a negative predictor. However, their interpretation should be made in the clinical context with value as an adjunctive test.

In conclusion, this case highlights the importance of screening for VWD which should be considered in patients who present with mucocutaneous bleeding pattern of unknown cause. VWD has been previously misdiagnosed and treated as ITP (Rick 1987) but the association of both conditions, VWD and ITP, is not often recognised. The 2B subtype of VWD should be carefully ruled out in the differential diagnosis of chronic thrombocytopenia, especially if a significant bleeding history is present. Molecular analysis remains an essential tool to investigate type 2 VWD especially when the phenotype is unclear.
7.4. STUDY OF PATIENT LC WITH TYPE 2A VWD ASSOCIATED WITH CHRONIC THROMBOCYTOPAENIA

7.4.1. CASE REPORT - PATIENT LC

Patient LC was a female who presented at another hospital at the age of 18 with severe menorrhagia and a decreased platelet count varying between 50 - 110 x 10⁹/l. At age 26 she had a bone marrow biopsy which was compatible with a diagnosis of ITP. She was then commenced on steroids (1mg/kg/day) which were ineffective in raising the platelet count and were associated with unacceptable side effects, such as cushingoid features. Further investigations at the local hospital yielded a diagnosis of VWD type 2A. The chronic intermittent thrombocytopaenia was attributed to an associated ITP.

Despite regular treatment with cryoprecipitate and oral contraceptives, at the age of 30 she required a hysterectomy for severe uncontrollable menorrhagia. The post-operative evolution was complicated by two thromboembolic events: a deep leg vein thrombosis and an episode of pulmonary embolism. In 1992 she was found PCR positive for hepatitis C virus (HCV), infection which she had probably acquired following a blood transfusion in 1988. She underwent a trial of treatment with interferon for HCV infection but this had to be discontinued as the platelet count dropped below 50 x 10⁹/l. As the patient moved into the RFH catchment area, in April 2000 she was referred to the RFHHC for continuation of care.
7.4.2. INVESTIGATIONS AT THE RFHHC - PATIENT LC

Family history

A family history of VWD was elicited in several family members from three generations (Figure 7.1). The index case, patient LC is designated as II-3. The index' two brothers II-1 and II-2 were known to be registered with VWD elsewhere but no further information was available. The index’ mother (I-1) and her affected daughter (III-1) were both registered with ‘type 2B VWD’ and their phenotypic data is presented in Table 7.1 (page 149) however no data on the RIPA was available. All of the affected family members reported a significant muco-cutaneous bleeding history.
Figure 7.1. Family tree and haplotype analysis of kindred I.C.
**Phenotype analysis**

The laboratory phenotypic data in patient II-3 are presented in Table 7.1 (page 149). The plasma VWF multimers were performed on the available samples in two affected family members (II-3 and III-1) showing the absence of the HMW multimers (Figure 7.2) and in two unaffected family members (II-4 and III-2) showing a normal pattern (Figure 7.2). The densitometric scanning traces confirmed the loss of HMW in the affected patients (Figure 7.2) and when expressed as a percentage of the total, the proportion of the HMW multimers (> 9 mers) was reduced in the affected patients as compared to the normal individuals. The percentages of HMW forms of the total were as follows: individual II-4: 25% of total; patient II-3: 7% of total; patient III-1: 9% of total and individual III-2: 26% of total.

The platelet VWF multimers were analysed in the index case II-3 and showed the absence of the HMW forms with the preservation of some of the intermediate molecular weight forms (Figure 7.3). The densitometric scanning traces confirmed the loss of HMW and when expressed as a percentage of total the proportion of the HMW multimers (> 9 mers) was 3% in the index case compared to 27% in the normal platelet control (Figure 7.3).
Figure 7.2. Upper part: autoradiograph of the plasma VWF multimers in four family members of index II-3. Lower part: densitometry scanning of the above autoradiograph showing the absence of HMW in patients II-3 and III-1.
Figure 7.3.
*Upper part:* autoradiograph of platelet VWF multimers from normal platelets. *Lower part:* densitometry scanning of the above autoradiograph showing decrease in the HMW multimers in patient II-3.
Genotype analysis and molecular modeling

i. Screening for type 2A VWD mutations

To screen for type 2A mutations the 3' end of exon 28 which encodes for the A2 domain was amplified as described in Methods. A single base pair change (a nucleotide substitution C to T at position 4789 of the mature VWF polypeptide) was found in both mother (II-3) and the affected daughter (III-1) corresponding to R834W substitution which has been previously described in association with type 2A VWD (http://mmg2.im.med.umich.edu/vWF).

ii. Screening for type 2B VWD mutations

To screen for type 2B VWD mutations the 5' end of exon 28 was analysed initially using the PCR 1 primers (see Methods). In the index case (II-3) at position 4122 in the nucleotide sequence a single base change C for T was identified in a heterozygous form (Figure 7.4). This nucleotide change encoded for the amino acid substitution A528V in the mature VWF polypeptide. A search of the database for VWF mutations and polymorphisms did not find any previous reports on this substitution (http://mmg2.im.med.umich.edu/vWF). In order to verify that this amino acids change is a mutation rather than a polymorphism, a DNA panel from 50 normal unrelated individuals was checked by amplification of 5' end of exon 28 followed by digestion with restriction enzyme DdeI. The A528V substitution was not present in 100 normal alleles.
Figure 7.4. Sequence analysis of 5′ part of exon 28 (PCR 1)

**Upper part:** in the affected daughter (III-1) at position 4122 the substitution T to C appears homozygous (represented by a single peak as arrowed).

**Lower part:** in the mother LC (II-2) at position 4122 the substitution T to C appears heterozygous (represented by two peaks as arrowed).

This substitution results in the A528V mutation.
DNA was also available from the index affected daughter (III-1) and amplification of 5’ end of exon 28 using PCR 1 showed the presence of the same substitution encoding for A528V but in a homozygous form (Figure 7.4). The homozygosity found in the affected daughter seemed unlikely, as it would imply that either she had inherited the same mutation from her father (who has no clinical or phenotypic characteristics of VWD) or that the daughter has another identical de novo mutation on the second allele. As both these hypothesis were highly unlikely, further analysis was undertaken which showed that the seeming homozygosity of this mutation is due to the presence of a polymorphism within the sequence of the sense primer (primer 226) that was used for PCR 1 (Eikenboom 1994). Testing for failure of amplification of one allele was checked by performing several PCRs with a combination of different primers. By using two different sets of primers (PCR 2 and PCR 3) which yielded fragments of 588 bp and 308 bp length respectively, the A528V substitution was detected in a heterozygous form in both mother and daughter (Figure 7.5 and Figure 7.6).
Figure 7.5.

Sequence analysis of 5' part of exon 28 using PCR 2 in patient LC (II-3) (upper part) and in the affected daughter III-1 (lower part): the heterozygous substitution T to C at position 4122 is represented as two peaks (arrowed). This substitution results in the A528V mutation.
Figure 7.6.

Sequence analysis of 5' of exon 28 (PCR 2) in patient LC (II-3) (upper part) and in the affected daughter III-1 (lower part): the heterozygous substitution T to C at position 4122 is represented as two peaks (arrowed). This substitution results in the A528V mutation.
**Restriction enzyme digestion**

To further confirm the presence of the novel A528V mutation and the heterozygous state, restriction enzyme digestion using the *DdeI* restriction enzyme was performed for the affected daughter (III-1). In the presence of the A528V mutation a restriction site for *DdeI* is abolished. Restriction digestion was performed using the PCR products from the three PCR (PCR 1, PCR 2 and PCR 3). The digestion patterns showed that by using PCR 1 products the A528V mutation appears in a homozygous form (Figure 7.7) in contrast to using the other two sets of primers (PCR 2 and PCR 3) which showed that the mutation is in a heterozygous state (Figure 7.8 and 7.9).

*In vitro* expression studies of the function of the recombinant VWF containing the A528V mutation are currently in progress as part of a collaborative project with the Birmingham Children's Hospital.
Figure 7.7. Digestion of the 936 bp PCR 1 fragment with DdeI restriction enzyme

i. Normal sequence - PCR 1 fragment of 936 bp

ii. Homozygous digestion pattern PCR 1 containing the A528V mutation - the mutation abolishes a restriction site at position 303

iii. Heterozygous digestion pattern PCR 1 - contains both a normal and a mutated allele
**Figure 7.8.** Digestion of the 558 bp PCR 2 fragment with $DdeI$ restriction enzyme

i. Normal sequence - uncut PCR 2 fragment of 308 bp

ii. Homozygous digestion pattern PCR 2 containing the A528V mutation - the mutation creates a restriction site at position 83

iii. Heterozygous digestion pattern PCR 2 - contains both anormal and mutated allele
Figure 7.9. Digestion of the 308 bp PCR 3 fragment with DdeI restriction enzyme.

Uncut = uncut PCR 3 fragment; M = mother II-2 (heterozygous); D = daughter III-1 (heterozygous); Mk = marker (100 bp ladder)

i. Normal sequence - uncut PCR 3 fragment of 558 bp

```
<table>
<thead>
<tr>
<th>20 bp</th>
<th>20 bp</th>
<th>426 bp</th>
<th>92 bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>40</td>
<td>446</td>
<td>558</td>
</tr>
</tbody>
</table>
```

ii. Homozygous digestion pattern PCR 3 containing the A528V mutation - the mutation abolishes a restriction site at position 446

```
| 20 bp | 20 bp | 518 bp |
```

iii. Heterozygous digestion pattern PCR 3 - contains both a normal and a mutated allele

```
<table>
<thead>
<tr>
<th>20 bp</th>
<th>20 bp</th>
<th>426 bp</th>
<th>92 bp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>518 bp</td>
<td></td>
</tr>
</tbody>
</table>
```
**Haplotype analysis**

Haplotype analysis was performed on the available DNA samples from family members II-3, II-4, III-1, and III-2. Linkage analysis studies demonstrated linkage between the VWD phenotype and a shared haplotype in the mother and affected daughter (Figure 7.1). This haplotype was not present in the unaffected daughter or the father. Thus, the affected daughter (III-1) inherited the allele which probably carries the two mutations identified (the novel A528V and the previously described R834W mutation) from the mother (II-3) in a dominant fashion. The father is clinically asymptomatic and his phenotype is normal. However, to rule out that neither of the two mutations are present in the paternal VWF genes, the father's DNA is being checked by PCR analysis and sequencing.

**iii. Molecular modeling of the R834W and V528A mutations**

The molecular modeling of the R834W mutation showed that this was at the rear of the VWF-A2 domain where R834 may be crucial for its folding (Figure 7.10). The A528V mutation was located adjacent to the spatial region in the VWF-A1 domain which contains other known type 2B mutations (Figure 7.11).

**Platelet immunology**

PAIg and platelet glycoprotein specific antibodies were not detected. These results are consistent with a non-immune cause of thrombocytopenia.
Location of the R834 mutation site (type 2A mutation) (arrowed) in a homology model of the VWF-A2 domain (side view). The VWF-A2 domain is shown in two orthogonal perspectives rotated by 90° along the vertical axis. The central beta-sheet is seen edge-on. The mutation residues are identified by spheres at their alpha-carbon atom.
Figure 7.11.

Location of the A528V mutation site in the proximity of the other previously described type 2B VWD mutations (in green) in a homology model of the VWF-A1 domain (side view). The VWF-A1 domain is shown in a perspective in which the central beta-sheet is seen edge-on. The previously known type 2M mutations are shown in blue and the new type 2M mutation identified in Chapter 6 are shown by yellow spheres. The mutations residues are identified by spheres at their alpha-carbon atom.
**Trial of desmopressin in patient LC (II-3)**

To study the response to desmopressin and its potential therapeutic benefits a trial dose of DDAVP (0.3 µg/kg intravenously) was administered in the index case (II-3) under close supervision. Blood samples were collected pre and at 15 min, 30 min, 1 hr, 2 hr and 4 hr post DDAVP infusion and the following parameters were analysed in all blood samples: VWF:Ag, VWF:AC, FVIII:C, platelet count and the closure time (CT) by PFA-100. In addition, the RIPA test and plasma VWF multimer analysis were performed pre and at one and 4 hr post DDAVP. Following DDAVP normalisation of the VWF:Ag, VWF:AC and FVIII:C levels (> 50 IU/dl) were recorded as soon as at 15 min post DDAVP and lasted until 4 hr post infusion (Figure 7.12).

![Figure 7.12](image)

**Figure 7.12.** Levels of VWF:Ag, VWF:AC and FVIII:C levels pre and post DDAVP.
A normal reduced response to low doses of ristocetin was measured with the RIPA test was observed pre DDAVP and the RIPA remained reduced at one and 4 hrs post DDAVP (Table 7.2).

<table>
<thead>
<tr>
<th>RIPA</th>
<th>Pre DDAVP</th>
<th>1 hr</th>
<th>4 hr</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mg/ml</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.25 mg/ml</td>
<td>5%</td>
<td>8%</td>
<td>0</td>
<td>90%</td>
</tr>
<tr>
<td>1.5 mg/ml</td>
<td>75%</td>
<td>95%</td>
<td>80%</td>
<td>93%</td>
</tr>
</tbody>
</table>

Table 7.2. RIPA results pre and post DDAVP in index II-3..

The plasma VWF multimeric pattern showed absent HMW forms pre DDAVP. At 1 hr post DDAVP there was a slight increase in the HMW forms and at 4 hr post infusion the multimeric pattern returned to baseline with the loss of the HMW forms (Figure 7.13). The visual appraisal of the multimeric patterns was confirmed by the densitometric traces which showed the absence of the HMW in the pre and 4 hr samples and some increase in the HMW at 1 hr post DDAVP but still reduced as compared to normal plasma (Figure 7.13). When expressed as a percentage of the total, the HMW (> 9 mers) forms in the baseline sample represented 3%, in the 1 hr post DDAVP sample 10% and at 4 hr post DDAVP 3% as compared to the normal plasma which showed a percentage of the HMW of 37% of the total.
Abnormal triplet post DDAVP

Normal plasma Pre DDAVP 1 hr post DDAVP 4 hr post DDAVP

Figure 7.13.
Upper part: autoradiograph of the plasma VWF multimers pre and post DDAVP in patient II-3. Lower part: densitometry scanning of the above autoradiograph showing the absence of HMW multimers pre and at 4 hr post DDAVP in patient II-3.
At the time of DDAVP infusion the baseline platelet count was within normal range (147 × 10⁹/l) as in this patient the thrombocytopenia occurs intermittently. Post DDAVP administration there was a minimal decrease in the platelet count at 1 hr post infusion (Figure 7.14) however the change in the platelet count is probably not significant.

Figure 7.14. Platelet count pre and post DDAVP in index II-3.
The closure times (CT) with two cartridges (CT-ADP and CT-EPI) (as described in Chapter 8) were minimally shortened after DDAVP and they remained abnormally prolonged throughout the test (Figure 7.15).

![Graph](image)

**Figure 7.15.** Closure times (CT-ADP and CT-EPI) pre and post DDAVP in index II-3.
Patient LC represents a kindred with type 2A VWD phenotype and unexplained chronic intermittent thrombocytopenia in whom a potential mixed type 2A/2B genotype was identified. Due to a consistently decreased RIPA, type 2B VWD was initially not suspected in the index case. However, the other affected family members registered elsewhere were labelled as 'type 2B VWD' based on the low platelet count (despite no data on the RIPA testing). In the framework of this thesis, studies of the phenotype, genotype, molecular modeling and a desmopressin trial were performed in order to clarify the diagnosis in this kindred.

The R834W mutation which is the commonest type 2A VWD mutation (Lyons 1992, Ginsburg 1989, Sugiura 1992, Inbal 1992, Hagiwara 1996) was identified. However, a second mutation A528V was identified and molecular modelling suggests that this is a novel candidate mutation for type 2B VWD. The functional characteristics of this novel mutation are awaited from the expression studies. The presence of a 2B subtype could explain the chronic intermittent thrombocytopenia for which no other cause has been identified as present. However, interestingly, despite the presence of a potential type 2B mutation, RIPA was not enhanced in this kindred. Although a non-enhanced RIPA argues against a type 2B phenotype (which is defined by an increased platelet agglutination with low dose of ristocetin and thrombocytopenia), there are two reports in the literature of kindreds with a non-enhanced RIPA (i.e. 2A phenotype) in whom type 2B mutations
were found (Ribba 1994, Gaucher 1995). These patients with type 2A phenotype and a discrepant type 2B genotype had a non-enhanced RIPA, a decreased plasma VWF HMW multimers and associated thrombocytopenia. Peculiar characteristics of the platelets or plasma VWF have been proposed to explain the discrepancy between the phenotype and genotype, but the exact underlying pathophysiological mechanisms are not clear. Furthermore, analysis of the platelet VWF can be useful to identify a type 2B, as platelet VWF has a normal multimeric pattern and may show an increased response to low concentrations of ristocetin in contrast to plasma VWF (Gaucher 1995). Thus, in the context of VWD in the presence of a non-enhanced RIPA and unexplained low platelet counts, a type 2B VWD diagnosis should be considered in the differential diagnosis of chronic thrombocytopenia.

Investigations for ITP showed that the platelet serology was negative hence the original diagnosis of ITP was unlikely. Chronic thrombocytopenia is a common clinical problem. In isolation and without an obvious cause usually leads to a diagnosis of ITP, which is a diagnosis of exclusion (George 1996). In the index case a presumed diagnosis of ITP was made several years ago and resulted in treatment with steroids.

Screening for type 2B mutations in this kindred identified a novel heterozygous candidate mutation A528V which may explain the particular phenotype with associated thrombocytopenia. The molecular analysis in this kindred has underlined the importance of choosing the correct primers for PCR analysis. As previously reported, the gene-
However, several patients with type 2B have been successfully treated with DDAVP with a good response and no fall in the platelet count (Castaman 1996). If there is a drop in the platelet count post DDAVP this is usually immediate, at 15 min post DDAVP and tends to correct at 3 hr post infusion (Casonato 1999). Mckeon et al (Mckeon 1996) have shown that the thrombocytopenia seen after administration of DDAVP in patients with type 2B VWD is primarily an \textit{in vitro} effect influenced by the anticoagulant used and the type of blood collected (whole blood \textit{versus} fingerstick).

In patient LC a minimal reduction in the platelet count without clinical significance was recorded at one hour post DDAVP. Serial plasma multimeric analysis showed that at one hr post DDAVP although some HMW multimers were released, they were still reduced and by 4 hr post DDAVP the HMW multimers were completely removed from the circulation by the excessive proteolysis. Although there was some release of HMW at 1 hr post DDAVP, there was no correction of the HMW forms which would be expected in a patient with type 2A VWD and an underlying mutation belonging to group 2. RIPA remained non-enhanced pre and at one and 4 hrs post desmopressin suggesting that DDAVP did not release any abnormal VWF protein with enhanced affinity for platelets.

The PFA-100™ analysis was also used to assess the DDAVP response but, although in general the CT is useful in monitoring the response to DDAVP (as described in Chapter 8) in this patient the CT post DDAVP did not parallel the increments obtained in the FVIII and VWF levels. The abnormally prolonged CT observed post DDAVP is probably
due to the release of abnormal dysfunctional VWF secondary to DDAVP infusion and to the slight decrease in the platelet count post DDAVP.

In conclusion, molecular analysis and modelling suggest that the unusual type 2A phenotype and low platelet count present in this kindred could be explained by a potential compound \textit{allelic} 2A/2B genotype. Results are awaited from the \textit{in vitro} expression studies which may bring further clarifications in this complex case. The importance of the molecular characterisation for the correct diagnosis and classification of type 2 VWD is re-emphasized.
CHAPTER 8

ASSESSMENT OF PLATELET FUNCTION ANALYSER (PFA-100™) IN PATIENTS WITH VWD: ROLE IN THE DIAGNOSIS, ITS EFFICACY IN TREATMENT MONITORING AND ANALYSIS OF THE DETERMINANTS OF PFA-100™
8.1 INTRODUCTION

Historically, the bleeding time (BT) has been considered an essential screening test for abnormalities of primary haemostasis and it has been extensively employed in the screening and diagnosis of VWD. However, more recently, the platelet function analyser (PFA-100™) has emerged as an alternative to the BT for assessing primary haemostasis. The usefulness of the PFA-100™ in screening for VWD, with the exception of detecting type 2N and some mild forms of type 1 VWD is well established (Fressinaud 1998). However, limited data are available regarding the power of PFA-100™ to discriminate between various subtypes of VWD. Similarly, there is limited data on the utility of PFA-100™ to monitor the treatment in patients with VWD (Fressinaud 1999).

Several factors which can affect the closure times (CT) as measured by the PFA-100™ have been identified, such as VWF (Fressinaud 1998), haematocrit (Harrison 1999), platelet count (Harrison 1999), platelet functions (Cattaneo 1999), platelet receptors GPIb, GPIIb-IIIa and platelet receptor polymorphisms (Di Paolo 1999) but the magnitude of these influences on the CT is unclear. Of these, the von Willebrand factor (VWF) appears to have a strong influence on the CT (Fressinaud 1998). However, the plasma VWF levels are subject to large variations and one of the main determinants is the ABO blood group, as lower levels of VWF are found in individuals with blood group O (Gill 1987). This suggests that blood group could also have an influence on the CT and similarly, blood group O individuals could have higher CT, which could potentially have important implications in establishing the normal ranges according to the blood group.
As the PFA-100™ is a relatively new and under evaluation test, this chapter addresses some of the unresolved issues regarding this test. Thus, the chapter will concentrate on three main aims: to evaluate the role of the PFA-100™ in discriminating different subtypes of VWD; to assess the influence of the blood group and the type and severity of VWD on the variations in the CT, along with the effects of other determinants of the CT, such as haematocrit and platelet count (Harrison 1999); and lastly, the use of PFA-100™ to monitor treatment with desmopressin and clotting factor concentrates was investigated in eight patients with VWD.

8.2. MATERIALS AND METHODS

Patients and controls

Twenty healthy donors who were not on aspirin, aspirin containing medication or non-steroidal anti-inflammatory drugs were investigated to establish the in-house normal range for the PFA-100™. The normal ranges were calculated as the mean ± SD.

A group of 53 patients with VWD (28 female, 25 male) who regularly attend the RFHHC were included in the study with their informed consent. These patients have a well established diagnosis of the type of VWD and the majority have been described and analysed in Chapter 6. The subtypes of VWD in this group as defined by the revised criteria [Sadler 1994] were as follows: 32/53 (60%) were type 1 VWD, 12/53 (23%) were type 2M, 7/53 (13%) were type 2A, and one patient each with type 2B and type 3 VWD.
For both controls and patients whole venous blood was collected in vacutainer tubes containing 0.105m sodium citrate (Becton Dickinson Vacutainer Systems Europe) and into EDTA and all samples were analysed within four hours after venepuncture.

**Bleeding time**

BT was assessed by the Simplate II method using disposable devices according to the manufacturer instructions (Organon Teknika Corp, Durham, NC).

**PFA-100™ device (Dade Behring)**

The PFA-100™ is a simple device where blood is aspirated via a capillary through an aperture which contains a membrane coated with collagen and an additional platelet agonist (ADP or epinephrine). As platelets pass through the capillary and the aperture they are activated and subsequently form a platelet plug which occludes the aperture. The time required to obtain the occlusion of the aperture is defined as the (CT). All patients and control samples were tested with two cartridges originating from the same batch of cartridges: collagen/epinephrine (CT-EPI) and collagen/ADP (CT-ADP).

VWF:AC, VWF:Ag and FVIII:C were measured as described in Chapter 3. Haematocrits and platelet counts were performed using an automated cell counter (Adiva, Bayer Diagnostics).

Desmopressin (DDAVP) was administered in a standard dose of 0.3μg/kg, i.v. over 20 minutes. Haemate P (Centeon, Germany) was administered in three patients who had
been previously treated with clotting factor concentrates in a dose of 60 IU/kg intravenously.

Statistical analysis

Statistically, the differences between the laboratory characteristics in different types of VWD were assessed by ANOVA followed by the Fisher’s least significant multiple comparison test. The determinants of the CT and the BT were identified by stepwise regression analysis (Statview SE Software) using as independent variables the platelet count, haematocrit, the blood group (categorised as O and non-O), the type of VWD (categorised as type 1, 2 and 3) and the severity of VWF as reflected by the VWF:AC and VWF:Ag levels. Three different regression models were used: model 1, where the VWD was categorised according to quantitative (type 1 and 3) and qualitative (type 2) defects; model 2, where the VWF:AC and VWF:Ag were tested as independent variables; and model 3 where the VWF:AC/VWF:Ag ratio was tested (Table 2). The data was log-transformed before the application of parametric tests. CT values were analysed as a continuous variable by assigning to every infinite CT (> 300s) a fixed value of 300s.

8.3. RESULTS

The laboratory characteristics of the patients with VWD analysed in this study are presented in Table 8.1.

*PFA-100™ testing*
The normal ranges for CT-EPI were 110 ± 36s and for the CT-ADP 82 ± 21s. In patients, CT were prolonged with both cartridges in 50/53 patients (sensitivity 94%). CTs with both cartridges were within the normal ranges in three patients with mild type 1 VWD. In general, patients with normal or nearly normal CTs had mild VWD, with minimal bleeding symptoms, a slight decrease in the VWF levels and normal BT (with the exception of one patient with type 1 VWD with normal CTs and slightly prolonged BT at 10 min). Thus, the CTs had a good correlation with the severity of VWD, as abnormal CTs were associated with the more severe forms of the disease.

Table 8.1. Laboratory data of patients with VWD tested with PFA-100™

<table>
<thead>
<tr>
<th>Types and Subtypes</th>
<th>1</th>
<th>2A</th>
<th>2B</th>
<th>2M</th>
<th>3</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of patients</td>
<td>32</td>
<td>7</td>
<td>1</td>
<td>12</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>CT-ADP (sec)</td>
<td>198 ± 76</td>
<td>284 ± 27*</td>
<td>300</td>
<td>250 ± 49*</td>
<td>300</td>
<td>0.01</td>
</tr>
<tr>
<td>CT-EPI (sec)</td>
<td>246 ± 64</td>
<td>287 ± 23</td>
<td>300</td>
<td>265 ± 51</td>
<td>300</td>
<td>0.38</td>
</tr>
<tr>
<td>BT (min)</td>
<td>11 ± 7</td>
<td>19 ± 2</td>
<td>20</td>
<td>16 ± 7</td>
<td>20</td>
<td>0.06</td>
</tr>
<tr>
<td>VWF:Ag (IU/dl)</td>
<td>31 ± 26</td>
<td>23 ± 10</td>
<td>41</td>
<td>33 ± 17</td>
<td>4</td>
<td>0.06</td>
</tr>
<tr>
<td>VWF:AC (IU/dl)</td>
<td>21 ± 14**</td>
<td>11 ± 5**</td>
<td>22**</td>
<td>16 ± 7.5**</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>FVIII:C (IU/dl)</td>
<td>51 ± 31**</td>
<td>38 ± 9.5**</td>
<td>35**</td>
<td>48 ± 11**</td>
<td>3</td>
<td>0.0001</td>
</tr>
<tr>
<td>VWF AC/AG ratio</td>
<td>0.71 ± 0.24</td>
<td>0.58 ± 0.32</td>
<td>0.53</td>
<td>0.5 ± 0.13</td>
<td>0.25</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Values are given as arithmetic means ± SD.

*Differences between VWD types and subtypes were assessed by ANOVA followed by the Fisher’s least significant multiple comparison test.

* significantly different from type 1 (p<0.05).

** significantly different from type 3 (p<0.05).
**Bleeding time**

The BT was available for 41/53 (77%) patients and it was prolonged (> 8 minutes) in 24/41 patients (sensitivity 58%). The remaining 17/41 (41%) patients exhibited a normal BT (15 patients type 1 and two patients with type 2M VWD).

**Haematocrit and platelet count**

In 6/53 (11%) patients the haematocrits were below the reference ranges (less than 35% for female and less than 38% for male). Low platelets counts were recorded in 4/53 (8%) patients (normal range 140-400 ×10^9/l). Of these three patients with thrombocytopenia, one patient was classified with type 2B VWD and two patients with type 2A and type 2M VWD had a coexistent idiopathic thrombocytopenic purpura.

**Variables influencing the CT and BT**

Among the different variables tested by stepwise regression analysis (VWF:AC, VWF:Ag, type of VWF defect, blood group, platelet count and haematocrit) using different regression models (Table 8.2), the VWF:AC emerged consistently as the main determinant of the prolongation of the three haemostatic tests (CT-ADP, CT-EPI and BT) studied. Interestingly, the reduction of the VWF:AC had a more pronounced effect on the prolongation of the BT compared to CT-ADP and it had less influence on the CT-EPI. The CT-ADP and the BT (mean ± SD) were more prolonged in qualitative versus quantitative defects (264 ± 44 sec versus 201 ± 76 sec, p = 0.001 and 17.6 ± 5.min versus
11.5 ±7.5 min, p = 0.09). The CT-ADP did not however differ significantly between type 2 VWD subtypes (Table 8.2).

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Regression Model</th>
<th>Independent Variable</th>
<th>Partial $r^2$</th>
<th>Slope</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT-ADP</td>
<td>Model 1</td>
<td>Type of defect</td>
<td>0.14</td>
<td>0.14</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Model 2</td>
<td>VWF:AC</td>
<td>0.30</td>
<td>-0.29</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>Model 3</td>
<td>Platelet Count</td>
<td>0.06</td>
<td>-0.33</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VWF:AC/AG ratio</td>
<td>0.16</td>
<td>-0.30</td>
<td>0.01</td>
</tr>
<tr>
<td>CT-EPI</td>
<td>Model 1</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Model 2</td>
<td>VWF:AC</td>
<td>0.16</td>
<td>-0.16</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Model 3</td>
<td>VWF:AC/AG ratio</td>
<td>0.01</td>
<td>-0.16</td>
<td>0.05</td>
</tr>
<tr>
<td>BT</td>
<td>Model 1</td>
<td>Type of defect</td>
<td>0.14</td>
<td>0.27</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Model 2</td>
<td>VWF:AC</td>
<td>0.36</td>
<td>-0.46</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Model 3</td>
<td>VWF:AC/AG ratio</td>
<td>0.13</td>
<td>-0.46</td>
<td>0.03</td>
</tr>
</tbody>
</table>

The determinants of CT and the BT changes were identified by stepwise regression analysis using as independent variables the platelet count, the haematocrit, the blood group and the type or severity of VWD in three different regression models. In model 1, VWD was categorised according to the quantitative (type 1 and 3) or qualitative defect (type 2). In model 2, the VWF:AC and VWF:Ag ratio were tested as independent variables whereas the VWF AC/AG ratio was tested in model 3.

**PFA-100™ use in monitoring treatment with desmopressin**

Four patients had a DDAVP infusion and blood samples were collected pre and two hours post infusion. The results are presented in Table 8.3.
### Table 8.3.

Laboratory data on the BT, the CT-ADP, the CT-EPI, the VWF activity (VWF:AC), the VWF antigen (VWF:Ag) and the factor VIII (FVIII:C) levels pre and at two hours post DDAVP in patients with VWD.

<table>
<thead>
<tr>
<th></th>
<th>DDAVP</th>
<th>BT</th>
<th>CT-ADP</th>
<th>CT-EPI</th>
<th>VWF:AC</th>
<th>VWF:Ag</th>
<th>FVIII:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>min</td>
<td>sec</td>
<td>sec</td>
<td>IU/dl</td>
<td>IU/dl</td>
<td>IU/dl</td>
</tr>
<tr>
<td>Type 1 VWD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>20</td>
<td>-</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>9</td>
<td>7</td>
<td>40</td>
</tr>
<tr>
<td>Post</td>
<td>9</td>
<td>-</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>26</td>
<td>34</td>
<td>79</td>
</tr>
<tr>
<td>Type 1 VWD</td>
<td></td>
<td>5</td>
<td>188</td>
<td>270</td>
<td>4</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>Pre</td>
<td>5</td>
<td>-</td>
<td>148</td>
<td>187</td>
<td>29</td>
<td>40</td>
<td>52</td>
</tr>
<tr>
<td>Post</td>
<td>-</td>
<td>-</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>13</td>
<td>23</td>
<td>31</td>
</tr>
<tr>
<td>Type 2M VWD</td>
<td></td>
<td>-</td>
<td>250</td>
<td>281</td>
<td>38</td>
<td>92</td>
<td>100</td>
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<tr>
<td>Pre</td>
<td>-</td>
<td>-</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>22</td>
<td>34</td>
<td>41</td>
</tr>
<tr>
<td>Post</td>
<td>-</td>
<td>-</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>83</td>
<td>137</td>
<td>145</td>
</tr>
</tbody>
</table>

**PFA-100™ use in monitoring treatment with Haemate P**

Three patients (type 2A, type 2B and type 3 VWD) previously treated with clotting factor concentrates and known to be unresponsive to DDAVP received an infusion of Haemate P (Centeon, Germany) in a dose of 60 IU/kg. In all three patients after Haemate P a shortening of the BT was observed but the CT remained unchanged (>250 sec) at 6 hours post infusion (Figure 8.1).
specific primer 226 failed to amplify the allele which contains the A/G polymorphism at position 131, which introduces a mismatch between the primer and the complementary VWF gene sequence, leading to failure of amplification of the respective allele and a false homozygous pattern (Eikenboom 1994). On the crystal structure of the A1 domain the A528V mutation was shown to be located in the proximity of other type 2B VWD mutations. To further verify that the A528V mutation leads to a type 2B phenotype \textit{in vitro} expression studies are currently ongoing. If the recombinant product shows properties consistent with a 'gain of function' then this novel mutation can be classified as type 2B VWD.

In patient LC the platelet VWF multimers demonstrated an absence of the HMW multimers forms. In type 2B VWD the platelet VWF multimers are normal. R834W belongs to type 2A group 2 mutations, where there is an increased proteolysis but a normal synthesis of the VWF multimers and platelet VWF multimers are normal (Lyons 1992). Thus, the platelet multimeric pattern remains unexplained as it cannot be attributed by either of the two mutations alone. One can speculate that the absent platelet VWF multimers could be the result of the combined effect of the two mutations. This discrepancy may be answered by the results of expression studies.

The DDAVP trial performed in the index case provided several additional informations. In type 2B VWD desmopressin is thought to induce platelet aggregation and aggravate the thrombocytopenia (Holmberg 1983) which renders it contraindicated in this subtype.
Figure 8.1. The response of the BT and the CT pre and post Haemate P in three patients with type 2A, type 2B and type 3 VWD.
8.4. DISCUSSION

The CT as measured by the PFA-100™ were studied in a group of 53 patients with different types of VWD. These results confirmed that the PFA-100™ has a higher sensitivity (94%) than the classical BT (58%) in detecting VWD. Thus, by performing the BT alone the diagnosis of VWD would have been missed in nearly half of these patients (42%). In contrast, by assessment of the CTs only 6% of patients with VWD would have been missed.

Simple algorithms for analysis of the PFA-100™ results have been proposed [Favoloro 1999; Ortel 2000] stating that finding a normal CT can exclude a defect in the primary haemostasis and only an abnormal result should always be confirmed by further tests. However, in this study three patients had normal PFA-100™ testing but they were diagnosed with type 1 VWD based on other criteria as described in Chapter 4. These data add to the difficulties in diagnosing type 1 VWD, especially the mild forms as discussed in detail in Chapter 4, and it also demonstrates that finding of a normal PFA-100™ cannot on its own exclude a diagnosis of VWD. If there is a strong clinical suspicion of VWD then the diagnosis should be further investigated with other tests irrespective of the PFA-100™ results. Thus, although the CT have a very high predictive value of VWD it has very little negative predictive value and the decision that a patient is not affected by VWD should not rely on PFA-100™ testing alone.
The available literature also suggests that if only the CT-EPI is prolonged and the CT-ADP is normal, an aspirin effect should be suspected. However, the finding of a CT-ADP prolongation irrespective of the CT-EPI (normal or prolonged) indicates a qualitative platelet defect or VWD and platelet aggregation and VWF studies are necessary [Favoloro 1999; Ortel 2000].

In this study both cartridges were found to be equally sensitive to VWD in contrast to other reports where CT-ADP was found to be superior to CT-EPI, with CT-ADP sensitivities varying between 87% (Schlammadinger 2000) to 100% (Fressinaud 1998). However, when the analysis of the CT was performed on the subtypes of VWD, only the CT-ADP was significantly different in the various groups, whereas the CT-EPI and the BT were not.

The VWF:AC did not differ between the subtypes of VWD as the severity of the disease was very variable in each group. Similarly, the VWF:AC/VWF:Ag ratio was not significantly different between the subtypes, reflecting the small number of patients analysed in each group and also the use of the VWF:AC assay instead of the classical VWF:RiCo assay. However, interestingly, the CT-ADP was significantly longer in patients with type 2A and type 2M as compared to type 1 VWD, reflecting not only the severity of the disease but also its value as a global test for primary haemostasis in the presence of qualitative defects in VWF.
Among the variables which can influence the CT analysed in this study, the VWF:AC emerged as the main predictor of the CT with both cartridges and of the BT, accounting for about 30% of their variation. As a direct consequence, the VWF:AC/VWF:Ag ratio and the type of VWD also emerged as important variables. The dependency of the BT on the VWF levels is in contrast to some of the published studies (Rodeghiero 1992). 14% of the variation in the CT-ADP and the BT could be accounted for by the type of VWD (as quantitative or qualitative) which indirectly emphasizes the importance of other variables which define the type of VWD, such as the multimeric structure of VWF and the platelet VWF (Fressinaud 1999, Cattaneo 1999).

In contrast to CT-ADP, the CT-EPI was not influenced by the type of VWD and the VWF:AC had only a small effect on its variation, suggesting that this cartridge is of less use in the assessment of the diagnosis and classification of VWD.

The platelet count had a borderline significant influence on the CT-ADP cartridge and no effect on the CT-EPI. The haematocrit had no influence on neither the CT or the BT. The lack of significance of the platelet count and haematocrit variables is probably due to the small number of patients who had thrombocytopenia or low haematocrits and were included in this study.

With regard to the blood group, these data demonstrated that in patients with VWD the CT was not affected by the blood group. This is in contrast with a recent report which
found higher CT in normal individuals with blood group O (Lasne, 2000). However, although the plasma VWF are lower in individuals with blood group O, the platelet VWF, which is independent of the blood group (Rodeghiero 1992), is crucial for establishing and maintaining primary hemostasis as reflected by the BT. Furthermore, this study found that the BT was also independent from the blood group in patients with VWD which confirms (Rodeghiero 1992) but also contradicts some of the available literature (Caekbeke 1989). As the CT is an *in vitro* alternative for the BT and the BT is independent of the blood group, it would be expected that the CT is also independent of the blood group.

In the second part of this study the use of PFA-100™ to monitor treatment was studied. Two patients with type 1 VWD were analysed and they were unresponsive to DDAVP as the VWF levels did not correct at two hours post infusion. Similarly, at two hours post DDAVP infusion, the CTs remained prolonged (>250 sec in one patient and a slight shortening of the CT without normalisation was observed in the second one) showing that the lack of correction of the CTs reflected the poor response to DDAVP.

Fressinaud *at al.* reported a correction of the CT at 30 minutes post DDAVP infusion in all their patients who responded to DDAVP. However, at three hours post DDAVP infusion the CT were mildly prolonged or had returned to baseline levels [Fressinaud 1999]. These workers defined as a good response to DDAVP if a full correction of the CT was achieved at 30 min post infusion [Fressinaud 1999]. However, the correction of
the CT was a short lasting effect and if the response to DDAVP was defined as the correction of the CT at three hours post infusion, the majority of their patients would have been considered unresponsive. Testing the CT at longer intervals post DDAVP infusion is probably more appropriate especially when the CT is used to monitor treatment in patients with VWD receiving DDAVP.

The response to DDAVP was assessed in one patient with type 2M VWD with the R611H mutation (previously described in Chapter 6) who was unresponsive to DDAVP as defined by low VWF levels (< 50 IU/dl) and very prolonged CT at two hours post DDAVP. It has been suggested that among patients with type 2M the response to DDAVP may vary according to the genetic defect [Fressinaud 1999]. In contrast to the findings in this patient, other investigators have reported a good response to DDAVP with normalisation of the CT in patients with type 2M VWD and the R611H mutation [Fressinaud 1999].

The fourth patient who received DDAVP had type 2A VWD with the R834C mutation, which belongs to group 2 mutations where there is increased proteolysis of VWF (Lyons 1992). This patient (patient LC who is described in detail in Chapter 7) was found to be responsive to DDAVP as the VWF levels did correct at two hours post DDAVP but the CT remained unchanged post DDAVP.
The response to DDAVP by monitoring the CT was studied in four patients. A good correlation was found between the response to DDAVP and the correction of the CT in two patients with type 1 and one patient with type 2M VWD and no correlation was found in the patient with type 2A VWD. As the number of patients analysed here is small, no conclusion can be drawn on the value of the CT in monitoring treatment with DDAVP. However, it appears that at least in type 2 VWD the response to DDAVP may depend on the underlying genetic mutation and this hypothesis merits further investigations.

The CT was also used to monitor the response to treatment with clotting factor concentrates (CFCs) in three patients with VWD. It was observed that the CT did not correct after the administration of Haemate P despite the normalisation of plasma FVIII/VWF levels. In contrast to the CT, the BT shortened without normalisation in two patients at two hours post Haemate P. In explanation to the inability of the CT to reflect the response to CFCs, two main factors should be considered. Firstly, although the plasma VWF levels were corrected, the infusion of concentrate did not correct the platelet VWF, which is essential for the formation of the platelet plug [Mannucci 1995] and an important determinant of the CT [Fressinaud 1999]. The three patients who received an infusion of Haemate P may have underlying quantitative and/or qualitative abnormalities in the platelet VWF (with the additional contribution of thrombocytopenia in the type 2B patient). The multimeric content of these concentrates may be another contributing factor to the lack of correction of the CT after administration of CFCs. The PFA-100™ is sensitive to the high molecular weight forms of the multimers [Fressinaud 1999], which
are haemostatically most effective [Furlan 1996] and which are not entirely normal in the Haemate P (HMW represent 84.1% of the corresponding bands in normal plasma) [Dobrkovska 1998].

In summary, this study has confirmed the superiority of the PFA-100™ to the traditional BT in screening for VWD. An important finding was that both the CT and the BT are independent from the blood group, which should not be taken into consideration when the normal ranges are established. It was also shown that only a third of the variation of the CT and the BT is due to the plasma VWF levels, and other determinants remain important. The most useful cartridge in assessing VWD was the CT-ADP with a higher prolongation reflecting the severity of the reduction of VWF levels and it may also point towards a qualitative defect in VWF. However, the degree of prolongation of the CT-ADP is unable to discriminate between the subtypes of VWD and it remains of value only as a screening tool. The PFA-100™ may be useful to monitor therapy with DDAVP where a continued prolongation of the CT suggest a poor response to DDAVP, however it has no role in monitoring treatment with clotting factor concentrates.
CHAPTER 9

ISSUES RELATED TO THE TREATMENT OF VON WILLEBRAND DISEASE
Chapter 9 addresses issues related to the treatment in VWD and is divided into three sections. Section 9.1 presents an audit of the treatment in patients with VWD during 1997 and a review of the treatment patterns between 1980-1997 at the RFHHC. Section 9.2 assesses the treatment with desmopressin and section 9.3 is dedicated to issues related to prophylaxis of surgery in patients with VWD.


An important way of understanding the treatment of VWD is to review the past experience of treatment at a single institution. This section of the thesis is an audit focusing on the clinical management of VWD at the RFHHC during 1997 which is then extended to an 18 years review in order to analyse the changing patterns in the treatment of VWD.

9.1.1 INTRODUCTION

The aim of treatment in VWD is to correct the dual haemostatic defect in primary haemostasis and coagulation by correcting the prolonged bleeding time and the deficiency of plasma factor VIII coagulant activity (FVIII:C). There are two main forms of treatment of VWD: desmopressin and transfusion with blood products.
Over the last two decades the treatment of VWD has undergone major changes secondary to changes in the available choice of product and with the introduction of prophylaxis treatment in late 1970s.

The usefulness of cryoprecipitate in treating individuals with bleeding disorders was recognised in the mid 1960s (Perkins 1967; Bennett 1967). Cryoprecipitate was formerly advocated as the principal replacement therapy for patients with symptomatic VWD as it contained normal multimeric distribution and moderate concentrations of VWF. Early studies showed that cryoprecipitate transiently normalised FVIII:C levels, shortened or normalised the bleeding time and it was generally effective in controlling or preventing bleedings (Perkins 1967; Bennett 1967). However, from 1985 there was a move towards the more purified and virally inactivated plasma concentrates, as virucidal methods cannot be applied to cryoprecipitate and it carries a small risk of transmitting blood borne infections.

Desmopressin (1-deamino-8-D-arginie vasopressin, abbreviated DDAVP) was first introduced in 1977 (Mannucci 1977) and since then it has been the mainstay of treatment in VWD as discussed in the next section. By mid 1980s, virally inactivated clotting factor concentrates (CFC) rich in VWF became available and they revolutionised the management of VWD. At the RFHHC two main intermediate purity CFC (with FVIII specific activity of less than 10 IU/mg active protein) have been used: BPL 8Y (Elstree, UK) and Haemate P (Centeon, Germany). In vitro and clinical studies have clearly
shown that CFC such as Haemate P (Berntrop 1989) and NHS 8Y (Pasi 1990; Cumming 1990) are highly efficacious alternatives to cryoprecipitate for the treatment of VWD. Both concentrates are manufactured from human plasma by cryoprecipitation / adsorption, using as virucidal methods dry heating (80°C for 72 hours) for 8Y and pasteurization (60°C for 10 hours) for Haemate P. However, they lack the largest, most haemostatically active multimeric forms of VWF. Haemate P contains about 2.5 IU ristocetin cofactor activity for each IU of FVIII:C and the average content of high molecular weight VWF multimers has been determined to be 84.1% of the corresponding bands in normal human plasma (Dobrovska 1998; Metzner 1998). These plasma derived clotting factor concentrates have a well established record of viral safety, although there remains the risk of transmission of non lipid enveloped viruses such as hepatitis A and parvovirus B19, that are resistant to current virucidal methods (Mannucci 1992; Yee 1996).

In 1997, the United Kingdom Haemophilia Centre Directors Organisation (UKHCDO) published the guidelines for diagnosis, management and choice of therapeutic products to treat VWD (UKHCDO Guidelines 1997a, UKHCDO Guidelines 1997b). The aims of this study were to audit the clinical practice at the RFHHC in treating VWD during 1997 in the light of the published guidelines and to assess how these recommendations have been implemented into practice. In the second part of the audit, the patterns and the evolution of treatment of VWD during 18 years, between 1980-1997 were reviewed.
9.1.2 MATERIALS AND METHODS

i. Design of the study

Each patient registered with VWD who received treatment with CFC or DDAVP during 1997 was analysed in the audit. Data were collected retrospectively from patients records and details of type and dose of the product used, the reasons for treatment administration and mode of administration were recorded. By using the internal computerised data base established at the RFHHC since 1980, data of all patients diagnosed with VWD who received any form of treatment to prevent or treat a bleeding event between 1980 and 1997 were collected. The type and amount of treatment, together with the date, place and reason for treatment were recorded.

ii. Types of product

Three main forms of treatment were used at the RFHHC between 1980 and 1997: cryoprecipitate, DDAVP and intermediate purity clotting factor concentrates (CFC).

Cryoprecipitate had been extensively used in the closed plastic blood bag system up until 1988. Each bag contained approximately 10–15 ml of factor VIII concentrate (80-225 units factor VIII per bag) (Bennett 1967).

DDAVP was administered in a dose of 0.3 μg/kg, diluted in 250 ml of normal saline, by intravenous route, over 30 minutes.
Clotting factor concentrates rich in VWF that have been in use at RFHHC were BPL 8Y (Elstree, UK) and Haemate P (Centeon, Germany), with the choice of concentrate and dose depending on the nature of bleeding.

iii. Statistics

Linear regression analysis was performed to determine the statistical significance for the change in product use over time.

9.1.3. RESULTS

i. Audit of treatment of von Willebrand disease during 1997

During 1997 there were 442 patients with VWD registered at the RFHHC, of whom a total of 30 patients were treated with CFC and another 10 patients received DDAVP.

a) Clotting factor concentrate usage during 1997

A total of 1.2 million IU of CFC were used during 1997. The indications for treatment were spontaneous or traumatic bleeds and prophylactic treatment prior to surgery. The classification of the 30 patients who received CFC according to the VWD subtype is shown in Figure 9.1: 12/30 - type 1 VWD, 2/30 - type 2A VWD, 3/30 - type 2B VWD, 3/30 - type 2M VWD and 10/30 were type 3 VWD.
The two patients with type 2A VWD had both low levels of VWF activity (< 15 IU/dl, normal range 50 –150 IU/dl) and a history of severe bleeding in the past and were treated with CFC for an upper gastro-intestinal bleeding and multiple dental extractions, respectively. Three individuals type 2M VWD were treated with CFC: one patient underwent a lower caesarian section under CFC cover, as she had persistently low levels of VWF pre delivery (VWF:Ag 32 IU/dl, VWF:AC 21 IU/dl); one patient was known to have ischaemic heart disease and another patient with a VWF:AC of 11 IU/dl necessitated multiple dental extractions and DDAVP was considered inadequate for haemostatic cover.

The clinical and laboratory details of patients with type 1 VWD who received CFC are summarised in Table 9.1.
Table 9.1.

Clinical and laboratory details of patients with type 1 VWD who were treated with clotting factor concentrates during 1997.

**b) Desmopressin usage during 1997**

During 1997 a total of 10 patients (9 patients with type 1 VWD and one patient with type 2A VWD) received 11 infusions of DDAVP, in a dose of 0.3 μg/kg, intravenously. One patient with mild type 1 VWD was treated with two doses of DDAVP on two consecutive days for a head injury.

iii. *Analysis of treatment of von Willebrand disease between 1980 and 1997*
a) Patients registered with VWD

The number of individuals diagnosed with VWD and registered at RFHHC has increased over the years. In 1980 there were 85 patients registered with VWD rising to 442 in 1997, representing a 520% increase (Figure 9.2).

Figure 9.2. The increase in the number of patients with VWD registered at the RFHHC between 1980-1997.

b) Treatment with cryoprecipitate

In 1980, a total of 98 infusions of cryoprecipitate were administered to 13 patients with VWD. Cryoprecipitate was the main form of treatment for patients with VWD until 1988; the last treatment with cryoprecipitate was administered at the RFHHC in early 1990.
c) Treatment with DDAVP

Individuals with VWD registered at RFHHC first received treatment with DDAVP in 1981. Since then, there has been a significant increase in the total number of patients who have been treated with DDAVP and in the total number of DDAVP infusions administered per year (p = 0.0004, Figure 9.3).

![Figure 9.3. Treatment with DDAVP at the RFHHC between 1980-1997.](image)

**Figure 9.3.** Treatment with DDAVP at the RFHHC between 1980-1997.

d) Treatment with clotting factor concentrates

The total amount of CFC administered in patients with VWD at the RFHHC had significantly increased between the period 1980 to 1997 (p = 0.0001) from 0.08 million IU in 1980 to 1.2 million IU in 1997, respectively (Figure 9.4). The median clotting
factor usage (IU/year) has also followed a significant increase during the study period (p = 0.0001) (Figure 9.5).

**Figure 9.4.** Total amount of CFC used at the RFHHC between 1980-1997.

![Graph showing total amount of CFC used at RFHHC between 1980-1997.](image)

**Figure 9.5.** The lower, median and upper ranges of CFC usage between 1980-1997.

![Graph showing lower, median, and upper ranges of CFC usage between 1980-1997.](image)
In 1994, over 2.9 million IU CFC were used and this high peak can be explained by the heavy usage of CFC by four patients with VWD:

The first patient had type 2A VWD and hereditary telangiectasia and presented with severe recurrent upper gastro-intestinal bleeding. He required intensive daily treatment and intermittent prophylaxis with Haemate P, three times every fortnight, cumulating for 1994 a total of 552,500 IU CFC.

The second patient with type 3 VWD underwent a tonsillectomy during 1994, for which he required five weeks of daily treatment with Haemate P, using a total of 670,530 IU CFC [Alusi 1995].

The third patient with severe type 2M VWD was on prophylactic treatment with Haemate P (23 IU/ kg) three times per week to control severe recurrent epistaxis and during 1994 he used a total of 320,180 IU CFC.

Finally, the fourth patient with type 3 VWD required a total of 670,090 IU CFC for prophylaxis and / or daily treatment for recurrent gastro-intestinal bleeding occurring during 1994.

e) Usage of DDAVP versus clotting factor concentrate

A direct comparison between the usage of DDAVP and CFC during the study period was difficult to perform as there is no common quantifiable measure, i.e. number of infusions of DDAVP versus number of units CFC. Therefore, a comparison between the number of patients treated with either product as a percentage of treated patients in respect with the number of registered patients was performed (Figure 9.6). There was an
increase in the percentage of individuals who received treatment with CFC between 1980-1997 ($p = 0.07$), which was paralleled by a significant increase in the usage of DDAVP ($p = 0.0001$).

**Figure 9.6.** Percentage of patients with VWD treated with DDAVP or clotting factor concentrate / year, between 1980 –1997 at the RFHHC.
9.1.4. DISCUSSION

This retrospective analysis analysed the treatment modalities in patients with VWD in 1997 in order to verify the appropriateness and the standards of treatment of VWD at the RFHHC.

The study showed that ten patients with VWD received DDAVP and thirty patients were treated with CFC during 1997. Several patients had contraindications to DDAVP, such as type 3 and type 2B VWD and received CFC as the treatment of choice. However, the audit showed that several patients with type 1, type 2A and type 2M VWD received inappropriate treatment with CFC on the basis of age or reduced VWF levels — where it was felt that DDAVP would not raise the VWF to sufficiently high levels to provide an adequate haemostasis. However, in these cases the preference for CFC was made without a preliminary test dose of DDAVP. For example, patient No.12 (see Table 9.1), aged 60, with type 1 VWD and borderline VWF levels was treated with CFC for a post traumatic haematoma, when arguably DDAVP could have been tried first.

Two pregnant patients with type 1 and type 2M VWD underwent a lower caesarian section under CFC cover. It is known that in pregnancy the VWF levels do not always normalise and there is an increased risk of post partum haemorrhage in patients with low levels of VWF. These two patients had pre-delivery VWF levels below 40 U/dl and they received CFC to cover the surgical delivery. The use of DDAVP in pregnancy is generally avoided as a possible oxytocic effect on uterus and hyponatraemia in the
newborn have been reported [Chediak 1986]. However, DDAVP administration has been advocated immediately after delivery to reduce the extent of post partum blood loss [UKHCDO Guidelines 1997b].

In the second part of this study the products and amount of treatment used to treat patients with VWD in the course of 18 years, between 1980 and 1997 was analyzed. As expected, there has been an increase in the number of patients attending the RFHHC and therefore, a similar increase in the number of patients needing treatment.

Although effective, treatment with cryoprecipitate was discontinued in the early 1990 because of the continuing risk of transmission of blood-borne viruses (Guidelines 1992). Moreover, it has been shown that the bleeding time is not always corrected after cryoprecipitate infusion (Rodeghiero 1992).

Intermediate purity FVIII-VWF concentrates have been increasingly used either on demand or as prophylaxis, proving especially useful to treat soft tissue and post operative bleeding (Rodeghiero 1992). The main products used at RFHHC over the last 17 years have been Haemate P (Centeon, Germany) and 8Y (BPL, Elstree, UK). The number of units of CFC used for VWD has been substantially increasing over the years and in 1994 it reached a peak of nearly three million units CFC, making VWD patients amongst the highest user of CFC registered at the RFHHC (Miners 1998).
Desmopressin was first administered to patients with VWD at the RFHHC in 1981 and the usage of DDAVP has generally increased over the years, with more patients being successfully treated with DDAVP. DDAVP has numerous advantages over CFC, with significant reductions in costs (an average dose of intermediate purity CFC costs £1300 versus an average dose of 21μg DDAVP which costs £27). Furthermore, desmopressin has the added advantage of safety, with avoidance of transfusion transmitted infections diseases and minimal side-effects (Mannucci 1997).

At the RFHHC there was an awareness that increasingly more patients are receiving treatment with CFC in preference to DDAVP, on the grounds of the proven efficacy and easy availability of the CFC. One aim of this audit was to verify the above. The analysis however, did not show a tendency to switch patients from treatment with DDAVP to CFC. During the years, the increase in the usage of CFC was mirrored by the increase in the usage of DDAVP and the indications for treatment with a specific product were appropriate in most cases.

In summary, auditing the practice at the RFHHC during 1997 regarding the management of VWD showed reasonable adherence to the guidelines on the VWD treatment. The use of clotting factor concentrates was appropriate in most cases. However, patients with relative contraindications to DDAVP did not always have a preliminary test dose to assess responsiveness to DDAVP. This highlighted the need for patients with type 1 and type 2 VWD, especially those with low levels of VWF, to have a trial of DDAVP before
using CFC. DDAVP testing in patients with severe VWD (type 3 VWD or severe type 1 and 2 as defined by very low levels of VWF) will be discussed in the following section where the DDAVP trial is performed prospectively in 17 patients.

The analysis of the 18 years, between 1980-1997, showed that the usage of CFC and DDAVP is on the increase, making VWD an expensive condition to manage. As more patients are now on prophylactic treatment this adds extra costs, e.g. switching to prophylaxis costs an additional £547 per averted bleed (Miners 1998). Possible cost savings could be achieved, where appropriate, with a more extensive usage of DDAVP treatment reserving the usage of CFC for carefully selected cases. Overall, this review showed that regular auditing the VWD treatment and its changing patterns is a useful and necessary exercise, in order to maintain the optimal standards of treatment.

The specific treatment of patients with VWD in the context of surgery will be analysed in section 9.3
9.2. DESMOPRESSIN – LITERATURE REVIEW AND STUDY OF ITS USEFULNESS IN THE TREATMENT OF PATIENTS WITH SEVERE FORMS OF TYPE 1 AND TYPE 2 VON WILLEBRAND DISEASE

9.2.1 DESMOPRESSIN – GENERAL BACKGROUND

Desmopressin (1-deamino-8-D-arginine vasopressin, abbreviated DDAVP) is a synthetic analogue of the naturally occurring antidiuretic hormone L-arginine vasopressin, first synthesized in 1967 (Zaoral 1967). Compared to vasopressin, DDAVP has a greater potency and a more protracted antidiuretic effect, but with a marked reduction in the pressor activity. Thus, DDAVP has a very limited vasoconstrictor and oxytocic effect on the uterus or gastrointestinal tract. The haemostatic properties of DDAVP were discovered in mid 1970s, when DDAVP administered to normal volunteers led to an increase in plasma concentration of FVIII, VWF and tissue plasminogen activator (tPA) (Mannucci 1977). DDAVP causes a release of endogenous VWF from stores in endothelial cells probably via a second messenger (possibly by a monocyte derived platelet activation factor, Hashemi 1993) or in the presence of another cofactor (Tuddenham 1982). The increase in FVIII can be accounted for by two main mechanisms: one mechanism relies on the primary action of DDAVP on the release of VWF with consecutive stabilization of plasma FVIII (Mannucci and Cattaneo 1992) and secondly an direct release of FVIII from its site of synthesis and storage in the sinusoid liver endothelial cells (Nachman 1975) which is independent of the release of VWF as a carrier protein (Mazurier 1994). DDAVP has also an important effect on platelets.
inducing platelet adhesiveness which is dependent on the platelet VWF content and the platelet receptor GPIIb/IIa (Lethagen 1992).

The release of tPA is short lived and most of the generated plasmin is complexed to $\alpha_2$-antiplasmin without an increase in fibrinolysis (Levi 1992).

The mechanisms of action of DDAVP are still not fully understood. The haemostatic effect of DDAVP seems to be mediated via the V2 receptors that regulate water reabsorption in the kidney with no effect on the V1 receptor present in the smooth muscle cells. However, the site for the V2 receptor is probably situated extrarenal as anephric patients still have a normal response to DDAVP (Mannucci 1975). Proposed sites for the V2 receptors include endothelial cells, megakaryocytes, blood monocytes and mast cells. The plasma half life of DDAVP is about 4-5 hours irrespective of the mode of administration. The half life of FVIII is between 5-8 hours and for VWF between 8-10 hours (Mannucci 1997).

Intravenous DDAVP is usually given in a dose of 0.3 $\mu$g/kg diluted in saline and infused over 20-30 minutes. This dose produces a maximal effect on FVIII and VWF which increase to 3-5 times above the baseline and reach a peak at about 30-60 minutes post infusion (Mannucci 1981). Alternatively the same dose can be given subcutaneously but the peak concentration of FVIII is reached later, at 1-2 hours post administration (Kohler 1986). The third option of DDAVP administration is intranasally as drops or spray, with the added benefit of self and treatment at home. The usual dose for the intranasal route is 300 $\mu$g in each nostril in adults (150 $\mu$g in a child) (Lethagen 1987). The increase in
factor levels is slightly lower (2-3 times above baseline) and the peak is reached at 90-120 minutes post intranasal spray. Further doses of DDAVP can be administered at 12 hour intervals though the response should be monitored as there may be a decrease in the response (tachyphylaxis) but this is rarely clinically relevant (Mannucci 1992).

The major clinical indication of the use of DDAVP as a haemostatic agent are VWD and mild haemophilia A. In addition, DDAVP is used extensively in other conditions such as platelet function defects, uraemia, drug induced bleeding disorders and as a blood saving agent during surgery.

In VWD, DDAVP is considered the treatment of choice for patients with type 1 and some type 2 VWD. In patients with type 2B VWD the use of DDAVP is controversial. Some considered that DDAVP is contraindicated as it results in worsening of the thrombocytopenia which is probably caused by agglutination and not aggregation of the circulating platelets (Casanato 1999). However, in some patients with type 2B VWD who had only a mild decrease in the platelet counts, DDAVP proved clinically efficacious (Casonato 1994, Mckeown 1996).

Type 3 VWD patients do not respond to DDAVP presumably because of complete lack of VWF synthesis (Mannucci 1997). However, a subgroup has been recently identified in which FVIII becomes normal after DDAVP, even though the BT remains markedly prolonged (Castaman 1994).
Monitoring the response to DDAVP usually relies on measuring the FVIII and VWF levels and the BT. Recently, the collagen binding assay has been shown to provide potential improvement to laboratory monitoring of desmopressin therapy (Favaloro 1994). Another emerging tool is the platelet function analyser (PFA-100™) which is believed to be adequate to monitor the response to DDAVP and may replace the bleeding time (Cattaneo 1999). In this thesis the PFA-100™ response to DDAVP infusion was studied in four patients and the results are presented in Chapter 8.

DDAVP has relatively few side-effects. The most common ones are facial flushing, transient headache, a slight drop in blood pressure and a small increase in pulse rate. The risk of water intoxication, hyponatraemia and convulsions are due to its antidiuretic effect and this is important in young children (Smith 1989). Thus, in children less that 2 years of age DDAVP is relatively contraindicated. Caution should be exerted in patients with advanced atherosclerosis and cardio-vascular disease and in these situations DDAVP is best avoided. During pregnancy, DDAVP seems safe for both mother and fetus (Ray 1998), but there are few published data.

DDAVP is a very valuable drug and amongst its advantages are the avoidance of plasma derived factor concentrates, thereby minimizing the danger of immunological or infectious complications, the avoidance of needles if applied as a nasal spray and considerable reductions in cost in comparison with factor concentrates.
9.2.2 INFUSION TRIAL WITH DESMOPRESSIN IN PATIENTS WITH SEVERE HEREDITARY TYPE 1 AND TYPE 2 VON WILLEBRAND DISEASE

9.2.2.1 INTRODUCTION

Desmopressin has an established role in the management of patients with type 1 VWD due to its ability to increase the concentration of VWF and FVIII in plasma and shorten the BT. Desmopressin has reduced the need for blood products in patients with type 1, but it is ineffective in type 3 and in some cases of severe type 1 and type 2 VWD. While DDAVP is effective in about 70-75% of all VWD (Mannucci 1985) and in about 90% of patients with type 1 VWD (Nolan 2000), there is limited published data assessing the efficacy of this treatment strategy in the severe forms of type 1 and in the type 2 VWD.

The aim of this study was firstly, to evaluate the clinical and laboratory response to DDAVP in patients with severe forms of type 1 and type 2 VWD. For type 2 VWD the response to DDAVP may depend on the molecular mechanism which might predict the response to DDAVP but this hypothesis has not yet been tested in large clinical trials. Thus, the second aim of this study was to analyse the possible correlations between the response to DDAVP and the underlying mutations found in patients with type 2 VWD.

9.2.2.2 PATIENTS AND METHODS

This study was performed on patients registered at the RFHHC and recruited from patients' database (Chapter 3). This study was part of a multicentre clinical trial involving five European Haemophilia Centres (EU Grant BMH4-CT97-2256).
Patients were eligible for enrollment if they had severe hereditary VWD type 1 or type 2 (except type 2B). Severe VWD was defined by a significant bleeding history (more than one episode of severe bleeding) and one of the following historic data: a prolonged BT (>15 min by Simplate II method), VWF:AC < 15 IU/dl or FVIII:C < 20 IU/dl. The exclusion criteria were type 3 VWD, type 2B VWD, acquired VWD, age ≤ 12 or ≥ 65 years, cardiovascular disease, epilepsy and previous reactions to DDAVP. Approval of the local ethical committees and informed consent from the patients was obtained.

17 patients (six female, 11 male) with a median age of 42 (range 19 to 59 years) who were registered with different types of severe VWD were enrolled (Figure 9.7).

![The type of VWD in the 17 patients who received DDAVP](image)

**Figure 9.7.** Classification of patients with VWD who received a trial dose of DDAVP.

DDAVP (Minirin, Ferring, Malmo, Sweden) was administered in a dose of 0.3 μg/kg intravenously over 20 minutes diluted in 50 ml of 0.9% normal saline. At the time of the infusion no patient was bleeding and no therapy has been administered in the previous two weeks. Citrated whole blood was collected by venesection before the infusion and
then at 30 minutes, 1 hour, 2 hours and 4 hours post DDAVP. In those patients who did not have a baseline BT recorded in the notes, the BT was performed before the DDAVP infusion. The BT was performed at 2 hours post DDAVP in all patients except in one patient with type 2M VWD who refused the procedure.

The FVIII:C, VWF:Ag and VWF:AC levels were performed on all the blood samples (as described in Chapter 3). In two patients (with type 1 and type 2M VWD, respectively) plasma multimeric analysis was also performed pre and post DDAVP infusion.

A patient was defined as responsive to DDAVP if at 2 hours post DDAVP there was a three fold increase in both FVIII:C and VWF:AC levels as compared to the baseline levels and the 2 hours post infusion FVIII:C and VWF:AC levels were both greater than 30 IU/dl; in addition the BT at 2 hours post DDAVP should be 12 minutes or less. A partial response to DDAVP was defined if at 4 hours post infusion both the FVIII:C and VWF:AC levels were normalised (≥ 50 IU/dl).

9.2.2.3 RESULTS

Five out of the 17 patients (29%) were responsive to DDAVP. Three patients experienced mild facial flushing but no other side effects to DDAVP were observed.

Peak plasma FVIII:C levels occurred at 30 minutes post DDAVP infusion in 13/16 (81%) patients and at 60 minutes post infusion in three patients. For VWF levels the peak was reached at 30 minutes post DDAVP infusion in 7/16 (44%) and at 60 min post infusion in the remaining in 9/16 (56%) patients.
Table 9.2 shows the ranges obtained for FVIII:C, VWF:Ag, VWF:AC and BT at various time points for all the patients with different types of VWD who had the DDAVP trial:

**Table 9.2.**

**DATA ON VWF:AG, VWF:AC, FVIII:C AND BT PRE AND POST DDAVP**

<table>
<thead>
<tr>
<th>ALL TYPES OF VWD</th>
<th>BASAL</th>
<th>30 MIN</th>
<th>1 HOUR</th>
<th>2 HOURS</th>
<th>4 HOURS</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT (min)</td>
<td>5 -&gt;20</td>
<td>-</td>
<td>-</td>
<td>3.5-&gt;20</td>
<td>-</td>
</tr>
<tr>
<td>FVIII:C (IU/dl)</td>
<td>9-72</td>
<td>82-190</td>
<td>72-265</td>
<td>42-250</td>
<td>19-160</td>
</tr>
<tr>
<td>VWF:Ag (IU/dl)</td>
<td>6-45</td>
<td>12-180</td>
<td>21-170</td>
<td>22-126</td>
<td>12-114</td>
</tr>
<tr>
<td>VWF:AC (IU/dl)</td>
<td>3-44</td>
<td>12-131</td>
<td>16-126</td>
<td>14-130</td>
<td>8-108</td>
</tr>
</tbody>
</table>

The response to DDAVP was analysed according to the type of VWD.

1. **Type 1 VWD**

3/10 (30%) patients with severe type 1 VWD were responsive to DDAVP. FVIII:C levels normalised at 2 hours post infusion and in 9/10 (90%) patients the levels remained normal until 4 hours. However, the VWF:AC levels corrected in 6/10 (60%) patients at 2 hours post DDAVP and remained within normal ranges in 4/10 (40%) patients at 4 hours post infusion. Thus, a partial response to DDAVP was obtained in 4/10 (40%) patients with severe type 1 VWD. A graphic representation of the levels of FVIII:C, VWF:Ag and VWF:AC pre and at several time points post DDAVP is showed in Figure 9.8.
Figure 9.8. FVIII and VWF levels pre and post DDAVP in patients with type 1 VWD.
Type 2M VWD

Two unrelated patients with type 2M VWD were included in this study and their detailed analyses were presented in Chapter 6. The R611H mutation was present in both patients in a heterozygous form.

Both patients responded to DDAVP in terms of VWF and FVIII levels but the BT was not available in patient 2 post DDAVP infusion. The laboratory data of these patients is presented in Table 9.3. In addition, in patient 1 plasma multimeric analysis was performed on all blood samples collected pre and post DDAVP. On visual inspection there was a minimal reduction in the HMW multimers in the baseline sample. At 30 minutes post DDAVP there was a correction of the multimers with an increase in the HMW forms which persisted until 4 hours post infusion. These findings were confirmed by densitometric scanning: at baseline the percentage of HMW (>9 mers) was 12% of the total as compared to 23% HMW in a normal pooled plasma control. At 30 min post DDAVP the percentage of HMW increased to 20% of the total multimers and the increase persisted at 4 hours post DDAVP.

Table 9.3.
Data on two patients with type 2M VWD pre and post DDAVP

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patient 1</th>
<th>Patient 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>FVIII</td>
<td>VWF:Ag</td>
</tr>
<tr>
<td>30 min</td>
<td>44</td>
<td>25</td>
</tr>
<tr>
<td>1 hour</td>
<td>152</td>
<td>98</td>
</tr>
<tr>
<td>2 hours</td>
<td>132</td>
<td>75</td>
</tr>
<tr>
<td>4 hours</td>
<td>135</td>
<td>53</td>
</tr>
</tbody>
</table>
In patient 2 the platelet count pre DDAVP was $237 \times 10^9/l$ and at 2 hours post DDAVP it showed a very modest decrease to $210 \times 10^9/l$ (normal range 140-450 $\times 10^9/l$).

iii. Type 2A VWD

Three patients with type 2A VWD were studied and all three were unresponsive to DDAVP. A partial response was obtained in one patient who had normalised the FVIII:C and VWF:AC levels at 4 hours post infusion. Two patients were heterozygous for C509G and I865T (previously described in type 2A VWD mutation database) and in the third patient the causative mutation has not as yet been identified.

The C509G mutation has been described in association with group 1 mutations in which there is an impaired synthesis of the multimers (Lyons 1992). Administration of DDAVP in the patient with C509G mutation resulted in a minimal increase in the VWF levels (VWF:AC increase from 5 to 16 IU/dl at 2 hours) and no change in the BT (30 min pre and at 2 hours post DDAVP).

The I865T mutation has been associated with group 2 mutations where there is an increase in proteolysis of the normally secreted multimers (Lyons 1994). In the patient with I865T mutation, there was a three fold increase in FVIII levels and a 2.2 fold increase in the VWF:AC levels at 2 hours post DDAVP infusion and the BT remained unchanged (at 20 min). However, this patient achieved a partial response to DDAVP.

In the third patient with type 2A VWD (patient RT described in detail in *Chapter 7*) there was an 2.4 fold increase in FVIII:C and a 2.5 fold increase in VWF:AC levels at 2 hours.
post DDAVP infusion and BT shortened from 15 min to 8 min. In addition to VWD this patient has a low platelet count secondary to an associated chronic idiopathic thrombocytopenia. As detailed in Chapter 7, type 2B VWD was excluded in this patient however DDAVP was administered under close supervision as at the time of DDAVP infusion the platelet count was less than 50 × 10^9/l.

iv. Type 2N compound heterozygous VWD

Two patients with type 2N VWD were studied and the laboratory data is presented in Table 9.4.

<table>
<thead>
<tr>
<th>Table 9.4</th>
<th>DATA IN TWO PATIENTS WITH TYPE 2N VWD PRE AND POST DDAVP</th>
</tr>
</thead>
<tbody>
<tr>
<td>TYPE 2N</td>
<td>PATIENT 1</td>
</tr>
<tr>
<td>Parameters</td>
<td>FVIII</td>
</tr>
<tr>
<td>Basal</td>
<td>13</td>
</tr>
<tr>
<td>30 min</td>
<td>168</td>
</tr>
<tr>
<td>1 hour</td>
<td>155</td>
</tr>
<tr>
<td>2 hours</td>
<td>115</td>
</tr>
<tr>
<td>4 hours</td>
<td>75</td>
</tr>
</tbody>
</table>

Patient 1 type 2N VWD

This patient has reduced FVIII:C levels and also reduced VWF levels with a discrepant ratio of FVIII:C/VWF:Ag of 0.42. As discussed in Chapter 3, a discrepant ratio of FVIII:C/VWF:Ag of less than one (0.4-0.7) is suggestive of a compound heterozygosity for type 2N VWD (Eikenboom 1993, Zhang 1993). Genetic studies in this patient have demonstrated that he is a compound heterozygous for type 1/2N VWD with one allele
carrying the R91Q mutation and a second null allele (which expresses undetectable levels of mRNA). This underlying genotype explains the presence of reduced levels of VWF in addition to reduced FVIII levels.

Patient 1 had an excellent response to DDAVP with an 8.8 fold increase in FVIII levels and a 3.7 fold increase in the VWF:AC levels at 2 hours post DDAVP. In addition, the BT shortened from 11 min to 7.5 min at 2 hours post DDAVP.

**Patient 2 type 2N VWD**

This patient was classified as type 2N VWD based on an autosomal recessive family history of VWD, bleeding symptoms and reduced levels of FVIII:C (9 IU/dl). In addition baseline VWF were also reduced (VWF:Ag 17 IU/dl, VWF:AC 15 IU/dl), with a discrepant ratio FVIII:C/VWF:Ag of 0.52. FVIII binding assay was performed as described in Chapter 3 and it was consistent with a homozygous binding pattern with a FVIII binding activity of less than 1 IU/dl. In classical type 2N VWD the plasma multimeric pattern is normal. Interestingly, in this patient the plasma multimers analysis showed a decrease in the HMW and intermediate MW multimers and a normal RIPA, suggestive of a type 2A phenotype. The molecular analysis is not yet available, however the complex phenotype found in this patient is suggestive of the presence of two genetic defects on the VWF gene accounting for a compound heterozygous 2A/2N mutation.

Patient 2 was unresponsive to DDAVP as FVIII increased 4.5 fold and VWF:AC increased 1.9 fold at 2 hours post DDAVP with levels of less than 30 IU/dl.
9.2.2.4 DISCUSSION

The response to DDAVP infusion was assessed in a group of 17 patients with severe hereditary VWD. Only a third of them were responsive to DDAVP according to the strict criteria used. Other studies have suggested less stringent criteria for defining the response to DDAVP. In a recently published study looking at 91 patients with type 1 VWD the response to DDAVP was defined by the normalisation (≥ 50 IU/dl) of FVIII:C, VWF:Ag and VWF:AC levels at 30 minutes post DDAVP (Nolan 2000). However, FVIII:C levels alone represent an unreliable and an insufficient guide of assessing the response to DDAVP in patients with VWD (Nolan 2000). Previous studies have found that the majority of patients (80% - 90%) with type 1 VWD are responsive to DDAVP (Mannucci 1998, Nolan 2000). However, in the group of patients with type 1 VWD analysed here, 70% of them were DDAVP unresponsive. In type 1 VWD the administration of DDAVP will lead to a release of normal VWF from the stores. The correction of the BT can be explained by DDAVP-mediated release of VWF which contains an increase amount of HMW multimers which are important for platelet adhesion to subendothelium. The low rate of response to DDAVP obtained in this study in patients with type 1 VWD may reflect the selection of patients with severe forms of VWD, the criteria used to define the DDAVP response and the levels of platelet VWF.

This cohort of patients had severe forms of the disease with low baseline levels of FVIII and VWF and markedly prolonged BT. Previous studies have shown that a prerequisite
for a good response to DDAVP is a minimum resting level of FVIII and VWF of 10 IU/dl (Mannucci 1997).

Another explanation as to why the majority of patients in this study were unresponsive to DDAVP is because strict criteria to define the response were used. However, a partial response to DDAVP was obtained in 40% of patients with type 1 VWD and normalisation of FVIII at 4 hours post infusion was obtained in 90% of patients with type 1 VWD. Achieving a partial response is clinically relevant as it identifies patients in whom DDAVP is likely to provide effective haemostasis at the time of minor surgical procedures (UKHCDO Guidelines 1997b).

It has also been suggested that a quantitative or qualitative defect in the platelet VWF is predictive of the response to DDAVP, so patients with type 1 ‘platelet low’ or ‘discordant’ have a poor response to DDAVP (Mannucci 1985). In contrast, DDAVP appears effective in patients with type 1 VWD and normal platelet VWF levels which may reflect the pool of VWF contained in the endothelial cells (Rodeghiero 1988).

In this study platelet VWF was not assessed, but it may have influenced the response rate to DDAVP. In addition to platelet VWF, it has been suggested that the determination of VWF:Ag II (the VWF propeptide) which indicates the release capacity of VWF from endothelial cells might predict the efficiency of DDAVP in type 1 VWD patients (de Romeuf 1998)
In patients with qualitative defects of VWF (i.e. type 2 VWD) DDAVP administration will lead to release of abnormal VWF. The three patients with type 2A VWD analysed here were unresponsive to DDAVP irrespective of the underlying molecular defect, whether type 1 or type 2 group.

In the patient with type 2A VWD with an unknown mutation but likely to be in the VWF propeptide affecting the multimerisation of VWF, behaving like a group 1 mutation, as described in Chapter 7 a shortening of the BT was observed. However according to the criteria of this study he was unresponsive to DDAVP. The additional thrombocytopenia present in this patient is an important contributing factor as the response to DDAVP depends also on the platelet number and content. DDAVP is ineffective in patients with severe thrombocytopenia and a critical number of platelets (of ~ 50×10^9/l) is a prerequisite for a good response (Mannucci 1988). Another determinant of the response to DDAVP is the presence of the platelet membrane receptor (GPIIb/IIIa) and the content of the platelets (the presence of VWF and dense granules) (Lethagen and Nilsson, 1992). In our small cohort of patients with type 2A VWD there was no difference in the response to DDAVP irrespective of the underlying mutation and the responsible mechanism for type 2A VWD. Due to small numbers it is difficult to draw conclusions regarding the correlation between the type of mutation and the response to DDAVP. Further studies to assess this interplay between the genotype and phenotype and their prediction of the DDAVP response in type 2A VWD are needed.
The two patients with type 2M VWD who were heterozygous for R611H mutation had a good response to DDAVP. The mutation R611H was initially described as a type 2B-like mutation as it was found in patients with DDAVP induced thrombocytopaenia (Castaman 1995) however this finding has not been confirmed by subsequent studies which assigned the R611H mutation to type 2M or 2A classification (Hilbert 1995, Nishikubo 1997). Nishikubo et al. found no significant variation in the platelet count after DDAVP in a patient with R611H mutation, similar to our findings present in patient 2 with type 2M VWD. These workers also observed a correction of the HMW multimers in plasma following the DDAVP infusion.

More recently, Lethagen et al. (Lethagen 1998) debated whether patients with R611 mutations should actually be classified as type 1 VWD based on similar laboratory data to type 1 and a good clinical response to DDAVP. It was suggested that perhaps type 1 VWD should include patients with circulating VWF containing mutant subunits provided that the multimeric and the function of the VWF are nearly normal (Lethagen 1998).

In the two patients with type 2N compound heterozygous there was some increase of VWF and a variable and short lived increase in FVIII levels. In one patient with type 2N VWD a good response to DDAVP was observed. The VWF capacity to bind FVIII was not tested post DDAVP but the literature suggests that there is usually no improvement post DDAVP (Mazurier 1994). A study of eight patients with type 2N VWD had shown that FVIII levels did increase after DDAVP in all patients irrespective of the basal level of FVIII or the genetic mutation (Mazurier 1994). These workers showed the extent of
the FVIII response was variable whereas the increment of VWF was consistent from one patient to another. Moreover, the FVIII response was more transient than the VWF response and FVIII half disappearance time was approximately three hours. In contrast, another study found that there was no increase in the FVIII levels following DDAVP infusion in a patient with type 2N VWD (Lopez-Fernandez 1992).

In summary, only 30% of the severe forms of type 1 and type 2 patients with VWD were responsive to DDAVP. At present there is no accurate test to predict the response to DDAVP and therefore individual testing is the only way to assess the response. The study showed that it was not possible to predict the response to DDAVP based on the baseline levels of FVIII:C and VWF profile, therefore a DDAVP trial should be performed irrespective of the baseline levels of FVIII:C or VWF or the underlying genetic defect. The hypothesis that responsiveness to DDAVP in type 2 VWD may be related to specific mutations in the VWF gene merits further investigations. For patients with type 1 VWD, investigation of the platelet VWF and VWF:AgII levels may be a useful predictor of the response to DDAVP. The wider application of these tests in clinical practice is needed in order to confirm their potential to accurately predict the response to DDAVP without a need for individual testing. In conclusion, a DDAVP trial is currently recommended in every patient diagnosed with VWD (except type 2B and type 3), preferably at the time of diagnosis and especially before deciding for treatment with FVIII/VWF concentrates.
9.3. REVIEW OF THE MANAGEMENT OF ELECTIVE SURGERY WITH DESMOPRESSIN AND CLOTTING FACTOR CONCENTRATES IN PATIENTS WITH VON WILLEBRAND DISEASE

9.3.1. INTRODUCTION

Despite the common occurrence of VWD, the optimal intensity and duration of therapy required to achieve haemostasis in affected patients in both surgical and non-surgical settings are not yet established. Moreover, the correlation between in vitro laboratory testing and clinical efficacy is poorly understood.

Patients with VWD are difficult to manage particularly in the setting of surgery because the haemostatic response in this context cannot be accurately predicted, especially for patients who have not received their first haemostatic challenge. Furthermore, patients with severe VWD who need extensive surgery or surgery involving sites with an increased fibrinolysis, such as oropharyngeal mucosa, digestive tract or uterus, have undoubtedly an increased bleeding risk. There are very few large prospective or retrospective studies about the appropriate prophylactic regimens during surgery and in general the management of these patients is guided by the type of surgery, the type and severity of VWD and the choice of treatment. However, there are no clear guidelines on the optimal dosage, the duration of treatment and the adequate monitoring of these patients and in general there is lack of agreement among the clinicians.
As described in section 9.2, the treatment of choice in VWD is desmopressin (DDAVP). For patients unresponsive to DDAVP or if DDAVP is contraindicated, clotting factor concentrates (CFCs) are successfully used, despite their limited and inconsistent effects on the BT (Rodeghiero 1992). The CFCs usually used to treat VWD are plasma derived intermediate purity FVIII concentrates rich in VWF. Their therapeutic success in preventing or treating postoperative bleeding is due to their high FVIII content, which is the main determinant of postoperative bleeding. However, there is no accepted standardisation for VWF-containing concentrates making dosage recommendations difficult. The content of VWF multimers present in a concentrate is an important characteristic of these products as the high molecular weight forms of the multimers are haemostatically most effective (Furlan 1996).

Other CFCs used for the treatment of VWD are high purity VWF products which have a low FVIII content and an almost normal multimeric pattern, and recently recombinant human VWF has been developed and is currently under study.

Despite the routine use of the plasma-derived concentrates only limited clinical data are available on which to base dosage and frequency of infusions. Moreover, at present, no published data are available from systematic clinical trials from which to establish a firm relationship between the dosage of a specific product or target plasma level of VWF activity and clinical efficacy (Chang 1998).
The laboratory monitoring of patients undergoing surgery is controversial. The majority of the treating centres monitor only the FVIII:C levels, as the assay is relatively easy and widely available (Foster 1995). However, for the muco-cutaneous type of surgery it appears that the correction of the BT and the contribution of platelet VWF are important factors (Mannucci 1998).

To address some of these issues, a retrospective survey spanning 10 years of experience in the management of planned surgery in patients with VWD registered at the RFHHC is presented in this section. Thus, in the context of elective surgery, treatment options, protocols and the outcome of haemostasis were analysed. The RFHHC experience was then compared to the limited data available in literature.

### 9.3.2 MATERIALS AND METHODS

#### i. Patients selection

The records of all patients with VWD who underwent a planned surgical event, invasive procedure or obstetric delivery by Caesarian section between 1988 and 1997 were reviewed. A total of 65 patients with VWD regularly followed up at RFHHC who underwent surgery during the study period were identified. These patients underwent 103 surgical procedures which required prophylactic treatment with either desmopressin or CFCs. If a patient underwent more than one similar surgical event, which was treated and responded in a similar fashion (in most cases this was a dental procedure) only one entry was made for the respective patient.
CFCs were the treatment of choice in patients with type 2B and type 3 VWD where DDAVP is contraindicated or ineffective. In type 1 and certain type 2 VWD cases, several patients underwent a trial of DDAVP administered intravenously in a dose of 0.3 μg/kg before the planned surgery. The patient was considered DDAVP responsive if within an hour of DDAVP administration the FVIII/VWF levels were normalised (≥ 50 IU/dl).

One patient with type 3 VWD who underwent a tonsillectomy in 1994 was excluded from this analysis, as his management and response to therapy were unusual. He required five weeks of daily treatment and several months of intermittent treatment with Haemate P for severe postoperatively bleeding (Alusi 1995).

**ii. Type of surgery**

All surgical procedures were performed at the Royal Free Hospital and were divided into five main groups: major surgery (abdominal, intra-cranial and orthopaedic surgery), minor surgery (invasive procedures such as arthroscopy, GI endoscopy, cystoscopy, urethral dilatation or minor surgery such as uterine surgery, skin excision or varicose vein strip), dentistry (dental extraction, invasive dental surgery or dental cleaning), ENT surgery (tonsillectomy, adenoidectomy, nasal package/cauterisation under regional anaesthesia, rhinoplasty) and obstetric delivery by Caesarian section.

**iii. Treatment protocols**
Treatment with desmopressin

Desmopressin was administered in a dose of 0.3 μg/kg intravenously over 30 minutes.

The number of DDAVP doses and the interval of administration were recorded.

Treatment with clotting factor concentrates

Data collection included details of the treatment with intermediate purity FVIII/VWF concentrates, either BPL 8Y (British Plasma Laboratory, Elstree, UK) or Haemate P (Centeon, Germany). The type of product, the preoperative dose, the post operative dose within the first 24 h and the dose, duration and frequency of treatment received after the first 24h were recorded. One patient received treatment with a high purity VWF concentrate by continuous infusion on a clinical trial basis and he was excluded from this analysis.

Antifibrinolytics

The antifibrinolytic of choice was Tranexamic acid which was usually administered in connection with muco-cutaneous surgery, including dental surgery. The route of administration was either orally in a dose 15-20 mg/kg, four times a day, for 7 to 10 days or more often as a 5% mouthwash for dental procedures (10 ml, four times a day).

Laboratory monitoring

FVIII:C and VWF:Ag were measured as described in Chapter 3. VWF activity (VWF:AC) was measured by either ristocetin cofactor activity using fresh platelet method or by the in-house ELISA assay as described in Chapter 3.
Postoperative complications
The complications considered in the postoperative period were bleeding which was either higher than expected perioperative oozing or immediately postoperative, haematoma formation or severe bleeding which required surgical re-intervention.

Effectiveness of haemostasis
The effectiveness of postoperative haemostasis was considered excellent if there were no bleeding or other complications, moderate if there was some bleeding, but no further action was needed and poor if there was significant bleeding which required further treatment.

9.3.3 RESULTS
During a ten year period, elective surgery was performed in 65 patients, of whom 27 (42%) had prophylactic treatment with DDAVP and 38 (58%) received treatment with CFCs.

Treatment with desmopressin for elective surgery in patients with VWD
Twenty seven patients with VWD had 35 planned surgical procedures under DDAVP cover. The age at the time of surgery was between 14-57 years, 18 patients were female and 9 male. 25/27 (93%) patients were type 1 VWD and two (7%) patients were type 2M VWD. The severity of VWD in type 1 patients varied between severe (VWF:AC 6
IU/dl) to borderline/normal (VWF:AC 50 IU/dl). The two patients with type 2M VWD had baseline phenotypic characteristics of FVIII:C 40 IU/dl, VWF:AC 7 IU/dl, VWF:Ag 19 IU/dl and FVIII:C 100 IU/dl, VWF:AC 9 IU/dl, VWF:Ag 47 IU/dl. Both patients with type 2M VWD underwent dental treatment.

Details of the surgical events and treatment with DDAVP are shown in Table 9.5.

<table>
<thead>
<tr>
<th>Type of surgery</th>
<th>No of surgical events (%)</th>
<th>No of DDAVP median (range)</th>
<th>No of days median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major</td>
<td>3 (8)</td>
<td>5 (2-5)</td>
<td>5 (2-5)</td>
</tr>
<tr>
<td>Minor</td>
<td>10 (27)</td>
<td>2 (1-6)</td>
<td>1.5 (1-6)</td>
</tr>
<tr>
<td>Dentistry</td>
<td>19 (51)</td>
<td>1 (1-2)</td>
<td>1 (1-2)</td>
</tr>
<tr>
<td>ENT</td>
<td>3 (8)</td>
<td>2 (2-4)</td>
<td>2 (1-4)</td>
</tr>
<tr>
<td>Total</td>
<td>35 (100%)</td>
<td>2 (1-6)</td>
<td>2 (1-6)</td>
</tr>
</tbody>
</table>

Table 9.5. Details of treatment with DDAVP.

The interval between DDAVP infusions varied between 12 to 48 hours. Tranexamic acid was added in 30/35 (86%) muco-cutaneous surgical procedures, including all 19 dentistry events. FVIII:C levels were monitored routinely on a daily basis. Within an hour post DDAVP, a 2-3 fold increase in the FVIII:C levels was seen, with normalisation of the FVIII:C post infusion level (in only one patient with type 1 VWD who underwent dentistry the FVIII:C raised from 16 IU/dl to 38 IU/dl post DDAVP infusion).
The effectiveness of DDAVP treatment was rated as excellent for 32 surgical events (91%). In two patients with type 1 VWD the haemostasis was moderate. One patient had oozing following a dental extraction and required a second dose of DDAVP and the other patient, who underwent a hysterectomy, developed a small haematoma and required six daily doses of DDAVP. In only one patient with mild VWD was the effectiveness of treatment with DDAVP considered poor. This patient underwent a rhinoplasty and had two doses of DDAVP 12 hours apart. Two days later, the patient had to be readmitted to hospital with extensive bruising and secondary infection at the surgical site, and a third dose of DDAVP was given.

_Treatment with intermediate purity FVIII/VWF concentrates for elective surgery in patients with VWD_

Between 1988 and 1997 a total of 38 patients with VWD underwent 68 surgical procedures under cover with CFCs. The median number of surgical events/year was 5 (range 0-12). The median age of the patients at the time of surgery was 42 years (range 3-77), 22 (58%) were female and 16 (42%) were male. The classification of the patients according to the VWD type showed that 26 (68%) had type 1 VWD, 3 (8%) had type 2A VWD, 3 (8%) had type 2B VWD and 3 (8%) had type 3 VWD. The type of CFCs used to treat the surgical events was BPL 8Y for 52 events (76%) and Haemate P for 16 events (24%). Tranexamic acid was added in 26 (38%) surgical events, including all dental procedures.

Details of the surgery are outlined in Table 9.6.
In the postoperative period, FVIII:C levels were monitored at least once a day and the levels were kept above 50 IU/dl in all cases.

The effectiveness of haemostasis was rated as excellent in 56 events (82%) and moderate in six events (9%) where there was some postoperative bleeding, usually oozing or small haematomas, but no further action was needed. However, for another six events (9%) the haemostasis was considered poor, as there was significant bleeding postoperatively which required further treatment as detailed in Table 9.7. The dosage of CFCs used to treat the bleeding complications varied between 16 – 41 IU/kg (median 27 IU/kg) and the median number of days of treatment was one (range 1-4).

<table>
<thead>
<tr>
<th>Type of surgery</th>
<th>No of surgical events (%)</th>
<th>Dose prior to surgery IU/kg</th>
<th>Dose first 24 hrs postop IU/kg</th>
<th>Postop dose IU/kg/day</th>
<th>No of days of Rx.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major</td>
<td>10 (15)</td>
<td>54 (41-77)</td>
<td>47 (24-62)</td>
<td>43 (25-78)</td>
<td>10 (4-14)</td>
</tr>
<tr>
<td>Minor</td>
<td>26 (38)</td>
<td>48 (14-70)</td>
<td>26 (24-37)</td>
<td>37 (13-58)</td>
<td>4 (1-16)</td>
</tr>
<tr>
<td>Dentistry</td>
<td>18 (27)</td>
<td>34 (20-67)</td>
<td>-</td>
<td>23 (16-30)</td>
<td>1 (1-3)</td>
</tr>
<tr>
<td>ENT</td>
<td>9 (13)</td>
<td>48 (42-61)</td>
<td>32 (28-37)</td>
<td>32 (18-49)</td>
<td>6 (1-11)</td>
</tr>
<tr>
<td>Delivery</td>
<td>5 (7)</td>
<td>52 (24-62)</td>
<td>31 (30-31)</td>
<td>37 (32-43)</td>
<td>7 (1-13)</td>
</tr>
<tr>
<td>Total</td>
<td>68 (100%)</td>
<td>48 (14-77)</td>
<td>31 (24-62)</td>
<td>37 (13-78)</td>
<td>4 (1-16)</td>
</tr>
</tbody>
</table>

Table 9.6. Details of treatment with clotting factor concentrate.
<table>
<thead>
<tr>
<th>VWD type</th>
<th>Type of surgery</th>
<th>Type CFC</th>
<th>FVIII:C baseline IU/dl</th>
<th>FVIII:C post infusion IU/dl</th>
<th>Dose preop IU/kg</th>
<th>Dose postop IU/kg/day</th>
<th>Bleeding complication and additional Rx</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tonsillectomy</td>
<td>HP</td>
<td>40</td>
<td>115</td>
<td>44</td>
<td>22</td>
<td>Rebleeding at one wk, further HP</td>
</tr>
<tr>
<td>2b</td>
<td>TOP</td>
<td>HP</td>
<td>68</td>
<td>136</td>
<td>49</td>
<td>49</td>
<td>Rebleeding at 2 wk, further HP</td>
</tr>
<tr>
<td>1</td>
<td>Caesarian section</td>
<td>HP</td>
<td>56</td>
<td>-</td>
<td>24</td>
<td>-</td>
<td>Secondary PPH day 5, Blood Tx</td>
</tr>
<tr>
<td>3</td>
<td>Dental extraction</td>
<td>8Y</td>
<td>5</td>
<td>66</td>
<td>54</td>
<td>31</td>
<td>Rebleeding at day 5, further 8Y</td>
</tr>
<tr>
<td>1</td>
<td>Tonsillectomy</td>
<td>HP</td>
<td>40</td>
<td>115</td>
<td>44</td>
<td>22</td>
<td>Rebleeding at day 7, further HP</td>
</tr>
<tr>
<td>1</td>
<td>Cerebral angiography</td>
<td>8Y</td>
<td>40</td>
<td>90</td>
<td>34</td>
<td>24</td>
<td>Day 3 changed to HP as small haematoma present, continue until day 8</td>
</tr>
</tbody>
</table>

Table 9.7. Details of the six patients with bleeding complications who required additional treatment and in whom haemostasis was considered poor.

9.3.4. DISCUSSION

This is one of the largest retrospective study that analysed over 100 surgical procedures in patients with different types of VWD who required prophylactic treatment before surgery. The surgical events varied from oral surgery to major surgery and the haemostasis was provided by either DDAVP or CFCs. In 1995 the results of an international prospective study on the use of FVIII concentrates in surgery and severe bleeds had analysed 76 surgical events which were covered with CFCs and emphasized the large variations in the modalities of treatment in patients with VWD at the time of surgery (Foster 1995).
In this study DDAVP was used in 34% of all types of surgical procedures and it was efficacious in the majority of surgical events (91%). In one patient who did not have a DDAVP test prior surgery and underwent a rhinoplasty under DDAVP cover the efficacy of treatment was poor. These data re-emphasise the importance of assessing the response to DDAVP in every patient for whom treatment with DDAVP is contemplated as the response is variable and unpredictable as detailed in section 9.2. Two patients with type 2M VWD, where DDAVP is generally considered unsuitable, were successfully managed with DDAVP cover for dental procedures. However, the role of DDAVP is not clear in the context of major surgery, but in the population analysed here DDAVP was used with optimal results to manage three major surgical events (two appendicetomies and one hysterectomy).

Tranexamic acid is particularly indicated in muco-cutaneous bleeding events. From this study it appeared that tranexamic acid was used more often in association with DDAVP then with CFCs. An explanation for this discrepancy is that more muco-cutaneous surgical procedures were performed under DDAVP cover than under CFCs treatment.

In the majority of surgical events, CFCs were used for patients with various types of VWD where DDAVP was ineffective or contraindicated. Two types of CFCs were used: BPL 8Y and Haemate P, both of which have been shown to be highly efficacious (Pasi 1990, Berntrop 1989). The majority of surgical procedures analysed in the present study
were covered with BPL 8Y (77%) as the data was collected until 1997. However, from 1997 onwards, as more evidence suggested that Haemate P is probably the 'golden standard' concentrate, the practice at the RFHHC has changed and the concentrate of choice became Haemate P, which is one of the recent advances in the availabilities of therapies for VWD.

A recent survey published from the Nordic countries has looked at their experience in using Haemate P for patients with VWD undergoing surgery and analysed 35 surgical events covered with Haemate P or AHF-Kabi (Fraction I-O) (Lethagen 1999). In their experience doses of 30-40 IU/kg of Haemate-P preoperatively and between 20-30 IU/kg postoperatively once daily were found to provide satisfactory haemostatic control. In comparison, from this study it appears that higher preoperative (median 48 IU/kg, range 34-54 IU/kg) and postoperative (median 37 IU/kg, range 23-43 IU/kg) doses of CFCs have been used at the RFHHC. This may reflect the variation in the type and quality of CFCs that were used at the RFHHC where two different types of concentrates with different characteristics (BPL 8Y and Haemate P) have been used. Moreover, the dosage of CFCs used at the RFHHC was guided by the FVIII:C levels, which were consistently normalised. In contrast, in the Nordic survey neither the FVIII:C levels nor the BT were routinely monitored in connection with surgery (Lethagen 1999). There are other groups who similarly found that at least during oral surgery the monitoring of FVIII/VWF levels was probably not necessary (Federici 2000).
Factor VIII:C levels are available in ‘real-time’ and therefore enable the clinician to make rapid decisions regarding dosage. In the RFHCC cohort, the FVIII:C was the only parameter constantly monitored throughout all the surgical events covered with either DDAVP or CFCs and it proved sufficient to ensure an adequate haemostasis. Although monitoring of the BT has been recommended in muco-cutaneous surgery (Mannucci 1998), the BT was not monitored during surgery at the RFHHC.

In the Nordic survey (Lethagen 1999) once daily dosing interval was satisfactory, despite the manufacturer's recommendation that in the first three days postoperatively dosing should be a 12 hours interval. In the RFHHC the doses were given between 12 to 24 hours in the immediate postoperative period and they were individualised depending on the FVIII:C levels and the clinical response, which were closely monitored.

The retrospective analysis of the RFHHC cohort showed that the peri-operative treatment with CFCs guided by the FVIII:C levels was efficacious in the majority of surgical procedures and was associated with a low complication rate. In only five surgical events (7%) was the haemostasis unsatisfactory and these patients required additional CFCs to treat postoperative bleeding complications, which occurred despite the normalisation of FVIII:C levels throughout the perioperative period. This is comparable with the Nordic survey where they found bleeding problems in 6% of their cohort (Lethagen 1999). Whether the monitoring of other parameters, such as the ristocetin cofactor activity levels, the collagen binding assay or the BT measurements would have been useful in
these patients with bleeding complications is debatable. In addition, administration of platelets concentrates could also have a beneficial role by establishing and maintaining the primary haemostasis due to their content of VWF.

In the surgical context in patients with VWD pure VWF concentrates characterised by a high specific activity of VWF and a low FVIII concentration have also been used. However, a higher dose of these concentrates is required and due to the delayed elevation of FVIII, either the VWF concentrate needs to be started 12 hours before surgery or a FVIII concentrate infusion is given in addition. If a pure VWF concentrate is used, the monitoring of treatment relies on the FVIII:C levels and /or ristocetin cofactor activity levels (Goudemand 1999).

In summary, during the ten year period reviewed the prophylactic treatment of surgical procedures used at the RFHHC followed the recommendations published in 1997 (UKHCDO Guidelines 1997b), but with individualisation of treatment regimens as clinically indicated. A large proportion of all types of surgical procedures were managed with DDAVP cover, which is a safer and cheaper option than CFCs. In conclusion, this study demonstrates that with appropriate haemostatic cover the surgical risk of bleeding is associated with a low risk of complications and provides useful clinical evidence on the management of surgery in patients with VWD.
CHAPTER 10

CONCLUSIONS
10.1. GENERAL CONCLUSIONS

This thesis has addressed several unresolved issues related to the diagnosis, classification and treatment of von Willebrand disease. All studies presented in this thesis have focused on a group of patients registered with VWD at the Royal Free Hospital in whom prospective and retrospective studies were performed.

Establishing a correct diagnosis is the starting point for any analysis or treatment approach. The majority of patients with VWD are diagnosed as type 1 VWD, as they present with a quantitative reduction of the amount of VWF, which is structurally and functionally normal. However, although the diagnosis of VWD is based on clinical and laboratory criteria, there is continuous debate on which of these criteria are essential for a correct diagnosis. In a retrospective study presented in Chapter 4, it was shown that the currently proposed criteria for a definite diagnosis of type 1 VWD (i.e. a reduction in VWF levels, a significant bleeding history and a family history for VWD/or genetic abnormality) were difficult to fulfil in the majority of patients. Of the three parameters involved in the diagnosis, the bleeding history was shown to be of prime importance in the clinical decision to diagnose and treat type 1 VWD. It has also been shown that individuals with similar low levels of VWF have similar bleeding histories irrespective of the blood group, and hence the distinction between different blood groups and separate normal ranges for VWF should not influence the diagnosis in symptomatic individuals. As long as clear and validated criteria for VWD are lacking, it seems reasonable to treat
those individuals with symptoms and low VWF levels as having VWD, irrespective of the inheritance patterns or blood group.

The correct recognition of the type of VWD relies on accurate and reproducible phenotypic testing. The functional analysis of VWF has been extensively explored throughout this thesis in an attempt to identify the best functional assay currently available by performing several comparative studies. In Chapter 5 it was shown that the ELISA VWF activity method is in poor agreement with the standard VWF:RiCo and insensitive to qualitative variants, as it failed to detect the disproportionate reduction in VWF activity in patients with type 2 VWD. The use of the ELISA based assays led to the misclassification of qualitative variants of VWD. The VWF:RiCo assay, despite technical difficulties and labour intensity, remains of primary value in assessing the ability of VWF to bind to GpIb. However, the VWF:RiCo assay reflects only one function of VWF, which is a large and multifunctional protein. One of the other important functions of VWF is the binding to collagen, which is mediated via the HMW multimers and which can be assessed in vitro by the VWF:CBA. The VWF:CBA has recently received a revived interest and it has been proposed as an alternative to the plasma multimeric analysis. However, as demonstrated in Chapter 5.2, the VWF:CBA was unable to detect type 2M VWD because the assay is not sensitive in the presence of the HMW multimers. However, the use of VWF:CBA in combination with the VWF:RiCo assay may allow a more powerful and complete approach to the diagnosis of qualitative variants. Furthermore, both assays are of value because one might envisage the possibility of
VWD variants with normal plasma multimers and a decreased collagen binding activity, which although not yet described, may be found through future research.

Another important issue in the correct classification of VWD was illustrated by the patients described in Chapter 6, who were initially classified as type 1 VWD because of the association of reduced levels of VWF and apparently normal plasma multimers. However, repeated laboratory testing indicated that qualitative defects were present in all the 17 kindreds studied and these were overlooked at the time of the initial diagnosis. Only when the ratios between the VWF:Activity/VWF:Ag were specifically sought it became apparent that these ratios were disproportionate and hence suggestive of the possibility of type 2 VWD. The qualitative defects suspected in these patients were decreased binding to platelets in the presence of normal multimers, which would reclassify these patients as type 2M VWD. However, prior to this study the presence of VWF qualitative defects in patients with normal multimers was poorly described and thought to be very rare. Based on the reappraisal of the phenotypes, the genotypes and molecular modelling at least 9/17 kindreds were reclassified as having qualitative VWF abnormalities (type 2M, type 2A or type 2 unclassified VWD). The results of the study emphasised the need to calculate the VWF:Ac/VWF:Ag ratio at the time of diagnosis in every patient with suspected VWD irrespective of the presumed subtype, as a rapid and simple initial step before further investigations. An unexpected finding of this study was the presence of abnormal multimeric patterns in 4/9 kindreds investigated (in whom underlying mutations were identified). These plasma multimeric abnormalities were not
previously recognised and they became apparent on repeated analysis and scanning of the autoradiographs.

Targeted DNA analysis to amplify the A1 domain of VWF encoded by 5' part of exon 28 was performed in the group of patients studied in Chapter 6 as they were suspected of being misclassified as type 2M VWD. The finding of eight mutations (of which four novel candidate mutations for type 2 VWD) in 9/17 kindreds studied led to an examination of the structure/function relationship of the A1 domains of VWF and \textit{in vitro} expression studies in two kindreds.

The determination of a mutation \textit{per se} is of little use in the absence of a rational explanation of the effect of the mutation on the protein and an association with the phenotype. To study the effect of missense mutations resulting in amino acid substitutions, the standard approach is to perform \textit{in vitro} expression studies. The value of such an approach was demonstrated in the study of kindred 1 from \textit{Chapter 7} who harbours the V516F mutation. Expression studies showed that the effect of this mutation was to cause a reduced ability of VWF to bind to platelets and not a quantitative deficiency. Although the plasma VWF multimers were normal and in the VWF-A1 domain molecular views the V516 mutation was located in the cleft near the region responsible for ristocetin binding, the recombinant product showed a decrease in the HMW multimers and a decreased binding to botrocetin. Thus, the classification of this mutation, whether 2A or 2M, remains unresolved.
Several of the patients studied in this thesis in whom mutations responsible for qualitative defects in VWF were identified remain at present diagnosed as 'type 2 unclassifiable'. This implies that the current classification of the VWD, which is based on a phenotypic approach, does not have enough flexibility to accommodate all VWD variants. However, it is essential that the framework of the classification remains simple to allow the clinician to make a rapid diagnosis focusing on two main questions: is this VWD? And if so, is it a quantitative or a qualitative variant? In order to make a clear demarcation between quantitative and qualitative VWF defects, firstly, good and reproducible phenotypic assays are needed and secondly, the ratio between the VWF activity and antigen should always be calculated to facilitate a rapid initial diagnosis. Once the qualitative nature of the disease has been suspected, the importance of molecular strategies cannot be underestimated. The use of genetic analysis and molecular modelling in type 2 VWD will also lead to molecular insights into the effect of particular mutations and understanding of the structure-function relationship. However, as exemplified in several kindreds studied in this thesis at the present time there are certain limitations in trying to neatly fit each form of VWD into a certain subtype of the current classification.

Another approach to study the structure/function relationship was the use of the tri-dimensional crystal structure of the VWF-A1 domain which allowed the determination of the mutations site. The location of type 2M VWD mutations were found either on the
surface of the GpIb binding region or near the active site cleft of the A1 domain. In contrast, the novel type 2B mutation identified in patient LC in Chapter 7 was located in the N-terminal region of the A1 domain where all previously described type 2B mutations are clustered. The structural analysis of the A1 domain and mutation sites has provided insights into important regulatory and structural elements within the domain demonstrating the utility of a molecular modelling approach.

Two interesting cases of type 2A VWD and associated thrombocytopenia are reported in Chapter 7. These patients illustrate the difficulties of a correct diagnosis and the need for a high index of suspicion of an atypical form of type 2B VWD. Moreover, the importance of molecular biology in the diagnosis of type 2 VWD is re-emphasized with particular attention to the use of correct primers for the DNA amplification.

A chapter of this thesis is dedicated to the PFA-100™ which is a relatively new test currently under evaluation for the diagnosis and treatment monitoring in VWD. In Chapter 8 it was shown that the PFA-100™ is of value only as a screening test for VWD and not as a discriminatory tool for the subtype of VWD. At the outset of the study no data on the influence of the blood group on the PFA-100™ was available. An analysis of the main determinants of the PFA-100™ showed that the closure times were independent of the blood group and one of the main determinants of the PFA-100™ were the VWF activity levels which account for a third of the variations of this test.
Regarding treatment of VWD, although this is well established, the usefulness of desmopressin in severe forms of type 1 and type 2 VWD is not fully known. Seventeen patients with severe forms of type 1 and 2 VWD were administrated DDAVP and a good response to DDAVP was obtained in a third of them. Thus, the study showed that a selected group of patients with severe forms of VWD can benefit from DDAVP treatment but that individual testing of the response to DDAVP still remains mandatory.

Very little data are available on the optimal management of surgery in patients with VWD and at present there are no guidelines on this topic. In Chapter 9 a large retrospective body of data is analysed concerning the management of surgery in VWD, underlining the optimal doses of clotting factor concentrates which were found adequate for a good haemostasis and the monitoring of such cases.

Stemming from this thesis several projects are still ongoing relating to the in vitro expression work of novel mutations and further genotyping of patients with type 2 VWD. Throughout the thesis the complex nature of VWD and VWF was demonstrated and the need for further research into this field became obvious. Several suggested research venues as originating from the studies described in the thesis, are detailed below:
10.2. SUGGESTED FUTURE RESEARCH

1. The diagnostic criteria for type 1 VWD were shown to be very stringent when applied retrospectively in a population with VWD. However, this approach may be biased as full data on the patients were not always available or they were not sought at the time of diagnosis. Therefore, these diagnosis criteria should be checked prospectively in selected patients with type 1 VWD. In patients with possible type 1 VWD especially if they lack a family history of VWD, an underlying genetic mutation should be sought, as finding a mutation at the VWF gene locus is a defining criteria of VWD according to the present classification. So far searching for mutations in type 1 VWD has been very limited because it is a very laborious and difficult task which implies the amplification of all 52 exons of the VWF gene. However, with the prospect of automated and multiplex PCR technologies this should become more feasible in the future. In the frame of prospective studies, the relationship between the VWF levels and blood groups and the effect of the blood group on the diagnosis should be further reassessed.

2. A variety of functional tests are required to demonstrate the definite presence of a quantitative or qualitative VWF abnormality. In the presence of reduced plasma VWF levels, the platelet VWF levels should also be studied especially in type 2 VWD forms where the mutational analysis can be more readily available. The presence of normal or reduced platelet VWF may provide insight into the mechanism of the mutations causing the disease and can help with a better classification.
3. Better phenotypic tests are needed which should ideally be reproducible and easy to perform in order to allow a confident diagnosis. A proposed assay which is expected to be of value is the recently developed VWF:RiCo using the recombinant GpIb fragment which should be assessed in a large population with various types of VWD.

4. The VWF:CBA can be of use in cases where the type of VWD whether 2A or type 2M, is unclear as it may provide useful information in addition to the multimeric pattern. However, larger prospective studies on the role of VWF:CBA in various type 2 (including type 2M VWD) are still needed.

5. The relationship between the response to DDAVP and the underlying mutation in type 2 VWD merits further studies in a larger group of patients. If the underlying mutation for type 2 VWD can predict the response to DDAVP, this may alleviate the need for individual testing, which is currently the only way to assess the response to DDAVP therapy.

6. The effects of candidate mutations should be demonstrated where possible by in vitro expression studies and the molecular effects should be investigated on the protein structure.

Ever since the first description of von Willebrand disease in 1926 the understanding of VWD and VWF has increased enormously. However, as more questions are being answered further ones arise, feeding a perpetual interest in VWD in order to allow a global understanding of this complex and fascinating disease.
References List


Favaloro E., Henniker A., Facey., Hertzberg M,(2000b) Discrimination of von Willebrands Disease (VWD) Subtypes: Direct Comparison of von Willebrand Factor:


http://mmg2.im.med.umich.edu


