Investigation of determinants of clearance of von Willebrand factor in individuals with type 1 VWD

by

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Dedication

For Ralph

Always bear in mind that your own
resolution to succeed is more important
than any other thing

Abraham Lincoln

Declaration

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This thesis is the result of my own investigations, except where otherwise stated. Other sources are acknowledged giving explicit references. A bibliography is appended.

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Abstract

The release and clearance of von Willebrand Factor (VWF) in a group of patients with the quantitative deficiency type 1 von Willebrand Disease (VWD) was investigated. This was done by analysis of circulating VWF and VWF released from the endothelial pool on infusion of a vasopressin analogue. A variety of parameters were investigated pre-and post infusion in order to identify *VWF* gene linked and non-linked variables that could affect VWF clearance. Increased clearance of plasma VWF in a significant proportion of type 1 VWD patients was shown but this was not consistently associated with steady-state levels of VWF, indicating that circulating plasma VWF levels are not a consistent reflection of the VWF life-cycle in this patient group. An association between galactose exposure and reduced levels of VWF was demonstrated by the increased binding of the lectins *Ricinus communis* and *Erythina crystagalli*, this was unrelated to clearance. In addition, no significant ABO blood group effect on VWF clearance was demonstrated. The absolute level of ADAMTS-13, and the susceptibility of VWF to cleavage by ADAMTS-13 were not associated with the clearance rate of VWF in patients with type 1 VWD. Three novel candidate mutations were identified in association with significantly accelerated VWF clearance. Notably, candidate mutations were generally identified in patients with steady-state VWF levels reduced to <20 $IUdL^{-1}$ and family analysis suggests absolute linkage with the *VWF* gene.

Despite demonstrating an increased rate of clearance in the majority of patients with type 1 VWD, no single underlying common characteristic or variable was predominant within this study group.

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Abstracts

Millar CM, Riddell AF, van Mourik JA, Starke R, Mackie I, Bowen DJ, Brown SA. VWF clearance and type 1 VWD. European Workshop on von Willebrand factor and von Willebrand disease, 2007. *Oral presentation*

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Millar CM, Riddell AF, Griffeon A, Brown SA, Brinkman H-J, van Mourik JA. Blood: 2005: 106: 505-506a. Von Willebrand Factor Propeptide: Response to 1-Deamino-8-D-Arginine Vasopressin and Investigation of Relationship with Plasma Clearance of Von Willebrand Factor Antigen in Type 1 Von Willebrand Disease. Blood: 2005: 106: 505-506a.

Millar CM, Starke R, Riddell AF, Griffioen A, Mackie I, Brown SA. Investigation of relationship of von Willebrand factor antigen and collagen-binding activity in patients with type 1 von Willebrand disease. Journal of Thrombosis and Haemostasis; suppl July 2005.

Millar CM, Starke R, Riddell AF, Griffioen A, Mackie I, Brown SA. Investigation of relationship of von Willebrand factor antigen clearance with ADAMTS-13 activity and Tyr1584Cys polymorphism in type 1 von Willebrand disease. Journal of Thrombosis and Haemostasis suppl July 2005.

Millar CM, Starke R, Riddell AF, Griffioen A, Mackie I, Brown SA. Investigation of relationship of von Willebrand factor antigen clearance with ADAMTS-13 activity and Tyr1584Cys polymorphism in type 1 von Willebrand disease. British Journal of Haematology, 2005;129 (suppl 1):46. *Oral Presentation*

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Millar CM, RA, Bell RJ, Harrington CM, Pascoe GM, Lee CA, Brown SA. Study of VWF antigen clearance in patients with type 1 von Willebrand disease and reporting of a familial tendency. British Journal of Haematology, 2003;121 (suppl 1):21. *Prize awarded*: *Haemostasis and Thrombosis session*

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Abbreviations and Nomenclature

Nomenclature

The type 1 VWD patients and kindred studied are numbered. Haemophilia A controls are prefixed 'Control' and patients with a previous diagnosis of type 2M VWD are prefixed 'M'.

The nucleotide sequence numbering used throughout this thesis follows the recommendations by the Subcommittee on von Willebrand factor of the Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis. (Sadler, 1994). The numbering starts from the transcription site (+1). Amino acid numbering is form the first amino acid of the signal peptide (the initiator methionine), thus 764 is the first amino acid of mature VWF (Goodeve and Peake, 2001). The cDNA sequence and amino acid residues used in this thesis are given in Appendix 1 and reported mutations are listed in the ISTH-VWF mutation database (www.vwf.group.shef.ac.uk/).

Commonly used Abbreviations

ASGPR	asialoglycoprotein receptor
ADAMTS-13	a disintegrin and metalloprotease with thrombospodin motifs
AHF	Antihaemophilic factor
cAMP	cyclic adenosine monophosphate
APTT	activated partial thromboplastin time
bp	base pairs
BSA	bovine serum albumin
BT	bleeding time
CI	confidence interval
CK	cysteine knot
CLB	cell lysis buffer
Con A	Conconavalin A
CV	coefficient of variation
Da	dalton
DDAVP	1-deamino-8-d-arginine vasopressin
cDNA	complementary DNA
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
ECA	*Erythina crystagalli* agglutinin
ELISA	enzyme-linked immunosorbant assay
FFP	fresh Frozen Plasma

FVIII	Factor VIII
Gal	galactose
Galgt2	N-acetylgalactosaminyltrasnferase
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
GP	glycoprotein
h	hour
HMW	high molecular weight
HRP	horse radish peroxidise
HUVEC	human umbilical vein endothelial cells
ISTH	International Society on Thrombosis and Haemostasis
KCl	potassium chloride
kDa	kilodalton
kg	kilogram
L	litre
µL	microlitre
mL	millilitre
LMW	low molecular weight
M	molar
mg	milligram
$MgCl_2$	magnesium chloride
min	minute
mL	millilitre
mol	moles
mRNA	messenger ribonucleic acid
n	nano
NLB	nuclear lysis buffer
OBS	Owren's buffered saline
OD	optical density
OPD	O-Phenylenediamine
PACE	paired amino acid cleaving enzyme
PAS	Periodic Acid Schiff
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFA-100™	platelet function analyser
PK	pharmacokinetic
RCA-I	*Ricinus communis* agglutinin I

RIPA	ristocetin induced platelet aggregation
SSC	scientific and standardisation committee
SDS	Sodium Dodecyl Sulphate
sec	second
SNP	single nucleotide polymorphism
T_0	pre-DDAVP
$T_1 - T_6$	hourly time intervals 1 h to 6 h post DDAVP
$t_{1/2}$	half-life
TE	Tris EDTA
TBS	tris buffered saline
Tris	Trishydroxymethylaminomethane (2-amino-2-hydroxymethyl-1,3-propanediol
TSP-1	Thrombospondin-I
TTBS	Tween tris buffered saline
TTP	thrombotic thrombocytopenic purpura
Tw	Tween 20
UKHCDO	United Kingdom Haemophilia Centre Doctors' Organisation
UL	unusually/ultra-large
VWD	von Willebrand disease
VWF	von Willebrand factor
VWF	VWF gene
VWF:Ag	VWF antigen
VWF:CB	VWF collagen binding activity
VWF:RCo	VWF ristocetin cofactor activity
VWFpp	VWF propeptide
V2R	vasopressin 2 receptor
WPB	Weibel-Palade body

Chapter 1

INTRODUCTION

1n 1926, Erik von Willebrand, a physician based in Helskinki, first reported a novel inherited bleeding disorder in a large family who presented with a variety of bleeding symptoms ranging from mild to life threatening. (Von Willebrand 1926) The underlying cause of the bleeding diatheses could not be determined at the time. It is now known that the bleeding disorder was associated with abnormalities of a glycoprotein now known as von Willebrand factor (VWF). VWF plays an important role in platelet adhesion, thrombus formation and coagulation. The functional deficiency of VWF, known as von Willebrand disease (VWD) is the most common inherited bleeding disorder in humans. While many details regarding the biosynthesis, secretion and function of VWF are well established, aspects of the mechanisms of clearance of VWF remain unclear.

1.1 HISTORICAL PERSPECTIVE

Von Willebrand described the bleeding symptoms of a large family who lived on Föglö, an island of the Åland archipelago in the Gulf of Bothnia between Sweden and Finland. (Von Willebrand 1926) The propositus, a five year old girl, had suffered from severe spontaneous bleeding since birth. Numerous family members including the parents exhibited severe and fatal bleeding patterns. Four of the proband's five sisters had died in infancy from intestinal, nasal and wound bleeding. The propositus herself died during her fourth menstrual period at the age of 13 years. Von Willebrand studied a further 58 family members and found 22 to have similar symptoms to the propositus, reporting consistent prolongation of the bleeding time and a predominantly mucocutaneous bleeding pattern in affected members. He distinguished this bleeding disorder from haemophilia, calling it hereditary pseudohaemophilia and concluded that the inheritance was of an X-linked dominant pattern. Von Willebrand proposed that the bleeding diathesis resulted from the combined effect of a platelet function disorder and systemic vessel wall lesion. Collaboration with a German physician, Dr Rudolf Jürgens also led to the suggestion that there was an abnormality in platelet function and for some time the condition was termed von Willebrand- Jürgens thrombopathy.(von Willebrand 1933)

The distinction between 'pseudohaemophilia' and haemophilias A and B remained purely theoretical until the early 1950s by which time improved methods for measuring factor VIII (FVIII) had been devised. In 1953 two cases of a combined haemostatic defect were described, namely prolonged bleeding time and decreased FVIII activity.(Alexander 1953) Although the bleeding symptoms were similar to those reported by von Willebrand, in these cases platelet function appeared to be

normal. The idea that haemophilia and VWD could result from deficiencies of distinct proteins arose following observations by a Swedish group studying transfusional therapy. This group reported a number of patients with a severe bleeding disorder characterised by factor VIII deficiency and prolonged bleeding time.(Blomback, *et al* 1956, Nilsson, *et al* 1957b) At around the same time, FVIII activity was successfully recovered during the purification of fibrinogen from Cohn's fraction I; this was designated fraction I-0. (Nilsson, *et al* 1957b) Treatment with fraction I-0, but not fibrinogen was shown to swiftly control bleeding symptoms as well as correct the bleeding time in the aforementioned patients.(Nilsson, *et al* 1957a) Following treatment with fraction I-0, the half-life of FVIII (then known as antihaemophilic factor, AHF) was shown to differ between these patients and those with haemophilia A. In addition, infusion of Fraction I-0 was shown to result in a secondary rise in FVIII activity in these patients. Finally, the prolonged bleeding time was found to be corrected following infusion of fraction I-0 prepared from haemophilia A plasma.(Nilsson, *et al* 1957b) Together, these findings suggested that the prolonged bleeding time in this group of patients was due to a lack of a plasma factor (VWF) and not to a primary platelet dysfunction or capillary wall defect. The subsequent finding of decreased platelet adhesiveness in these patients suggested this as a pathogenic mechanism for the deficiency of VWF,(Salzman 1963) and provided an explanation for the improvement in both primary haemostasis and bleeding time observed following infusion of fraction I-0.

The next milestone in the history of VWD was the isolation and characterisation of VWF in the early 1970s. A cross-reactive protein demonstrated in haemophilia A patients was shown to be reduced or absent in patients with VWD.(Zimmerman, *et al* 1971) Meanwhile, an antibody been raised that, as well as inhibiting AHF activity in normal plasma, was shown to reduce the platelet retention of normal blood in glass bead columns and block the capacity of purified AHF to correct the decreased platelet retention of VWD.(Bouma, *et al* 1972) It was concluded that either normal plasma contained a bifunctional molecule with both FVIII and VWD correcting activity, or that the two activities were located on different molecules with antigenic determinants that could not be differentiated in the available serum. Similar studies were subsequently published and although definitive evidence that the correcting factor of VWD was identical to VWF was yet to come, it had become customary to refer to the correcting factor as VWF. The antigen detected by the precipitating antibodies against FVIII had become known as 'FVIII-related antigen'. Concurrently, Howard and Firkin demonstrated that while the antibiotic ristocetin caused aggregation of platelets derived from both normal individuals and haemophilia

patients resulted from, this was not found in platelets from patients with VWD.(Howard and Firkin 1971) These studies led to a clearer understanding of the functions of VWF and the beginning of a classification of VWD. Assays for the quantification of FVIII-related antigen were developed with most laboratories at this time employing the rocket immunoelectrophoresis technique of Laurell.(Laurell 1966)

Further laboratory investigation of the family originally described by von Willebrand showed concordant reductions in VWF antigen levels (VWF:Ag) and ristocetin cofactor activity (VWF:RCo), consistent with a partial quantitative deficiency of VWF. Following cloning of the *VWF* gene in 1985, genotypic analysis identified heterozgosity for a nucleotide deletion in exon 18 in five surviving siblings of the original propositus.(Zhang, *et al* 1992a) The five girls, including the propositus, who died from uncontrolled bleeding, are likely to have been homozygous for the deletion.

1.2 VON WILLEBRAND FACTOR GENE AND PROTEIN

Von Willebrand factor (VWF) is a plasma glycoprotein(Legaz, *et al* 1973, Shapiro, *et al* 1973) that plays a central role in the critical initial phases of haemostasis. VWF has two main haemostatic functions: firstly, it binds to platelet membrane receptors (glycoprotein Iba and the integrin $a_{IIb}ß_3$) and to the subendothelial matrix. In doing so, VWF mediates both the adhesion and aggregation of platelets. Secondly, VWF is a specific carrier of FVIII in plasma, protecting FVIII from proteolytic degradation and thus prolonging its half-life in the circulation,(Morfini, *et al* 1993) and localising FVIII at sites of vascular injury. The complex structure of VWF facilitates these varied interactions, as well as its binding to many ligands.

1.2.1 VWF gene

The gene that encodes VWF (*VWF*) is located on chromosome 12p13.3 and was cloned and characterised by four independent groups in 1985.(Ginsburg, *et al* 1985, Lynch, *et al* 1985, Sadler, *et al* 1985, Verweij, *et al* 1986) *VWF* spans 178 kb, is composed of 52 exons and is transcribed into an 8.7-kb *m*RNA. The pseudogene is located on chromosome 22q11.2 and corresponds to exons 23 to 34 of *VWF*, demonstrating 97% homology (Mancuso, *et al* 1991) Exon 1 of *VWF* encodes the 5' untranslated sequence and the initiation codon is located in exon 2. Exons 2 to 18 encode the signal peptide and propeptide and the remainder of the exons encode the mature VWF peptide.

1.2.2 Structure of the VWF monomer

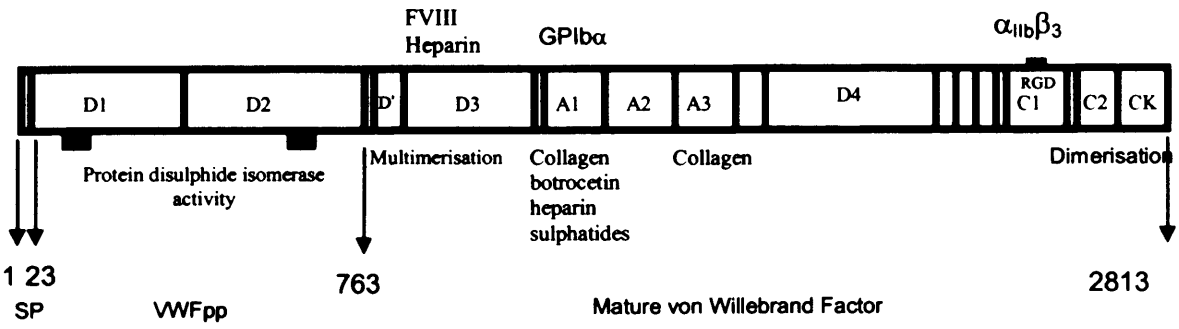
VWF is synthesised in endothelial cells and megakaryocytes as a precursor molecule of 2813 amino acids, known as pre-pro-VWF.(Bonthron, *et al* 1986) Pre-pro-VWF is composed of a 22-residue signal peptide, a 741 propeptide and a 2050-residue mature VWF subunit.(Sadler 1998) The molecular weight of the pre-pro VWF is approximately 270 kDa. (Shapiro, *et al* 1973) The molecular weight of the mature sub-unit is 220 kDa and circulates as multimers of up to 20 MDa.(Counts, *et al* 1978, Hoyer and Shainoff 1980). The complete amino acid sequence of VWF was elucidated from the cDNA sequence for pre-pro-VWF,(Appendix 1) and the recommended nomenclature for its numbering is to start from the first amino acid of the signal peptide (the initiator methionine), thus 764 is the first amino acid of mature VWF (Fig.1.1).(Goodeve and Peake 2001) The domain structure of pre-pro VWF is D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK: the four repetitive domains are shown in Fig.1.1 and the extents of the domains are given in Table 1.1. VWF is heavily glycosylated with the carbohydrate component constituting around 20% of its molecular weight. The carbohydrate component is distributed amongst 22 oligosaccharide chains, 12 of which are asparagine- (N-) linked and 10 serine or threonine- (O-) linked glycosylation sites.(Matsui, *et al* 1992, Titani, *et al* 1986) Approximately 12% of the N-linked oligosaccharides bear ABH antigens.(Sodetz, *et al* 1979) The structure and function of the oligosaccharide structures of VWF is discussed in Section 1.4.

Table 1.1 Domain structure of pre-pro VWF.

Residues are numbered from the initiator methionine (+1) of pre-pro-VWF

VWF Domain	Amino acid residues	Exon(s)
D1	34 – 386	3 – 10
D2	387 – 745	11 – 17
D'	769 – 866	18 - 20
D3	867 – 1241	20 – 28
A1	1242 – 1479	28
A2	1480 – 1672	28
A3	1673 – 1875	28 – 32
D4	1947 – 2295	35 – 39
B1-B3	2296 – 2399	40 – 42
C1	2400 – 2516	44
C2	2544 – 2663	45 – 48
CK	2724 - 2813	52

Figure 1.1 Schematic structure of pre-pro-VWF. Showing internal homologies (A-D) and functional regions. The locations of binding sites for several ligands, and the regions involved in dimerisation and multimerisation are shown. The locations of the protein disulphide isomerise activity of the propeptide are shown. Amino acid residues are indicated for the beginning and end of the signal peptide (SP), propeptide (VWFpp) and mature VWF. Amino acid residues are numbered from the initiator methionine (+1).



1.2.3 VWF biosynthesis and multimeric structure

VWF is synthesised in endothelial cells and megakaryocytes giving rise to distinct pools of platelet, plasma and subendothelial VWF.(Jaffe, *et al* 1973, Nachman, *et al* 1977) Prior to its secretion VWF undergoes a number of intracellular modifications. (Fig. 1.2) Firstly, the VWF signal peptide is cleaved in the rough endoplasmic reticulum and 12 N-linked oligosaccharide chains are added to each VWF monomer.(Wagner and Marder 1984) Following this initial glycosylation, dimers of pro-VWF are formed through disulphide bonding of the carboxyl terminal 151 residues of the VWF subunit, which incorporates the CK domain.(Katsumi, *et al* 2000) The dimers of pro-VWF then translocate to the Golgi bodies and trans-Golgi network where they undergo multimerisation, sulphation and further glycosylation, including modification of the N-linked oligosaccharide chains and the addition of 10 O-linked oligosaccharide chains. The propeptide is cleaved and remains noncovalently associated with VWF while in the cell. The mechanisms that determine whether newly synthesised VWF is secreted into the circulation or stored within endothelial cell-specific storage organelles, the Weibel-Palade bodies(Weibel 1964) or a granules of platelets remain unclear. Multimerisation may continue while VWF is stored and the extent of multimerisation may differ between VWF molecules, resulting in a range in the size of circulating VWF multimers. A detailed discussion of the structures and possible functions of the oligosaccharide side chains is presented in Section 1.4.

1.2.4 VWF propeptide

The VWF propeptide (VWFpp) was identified as a 741 amino acid residue containing the D1 and D2 domains during the characterisation of full-length VWF cDNA.(Ginsburg, *et al* 1985) This was shown to be identical to a previously known VWF-associated protein (VWF antigen II).(Fay, *et al* 1986, Montgomery and Zimmerman 1978) Propeptides are frequently referred to as 'intramolecular chaperones' in view of their functional roles in the processing and folding of their mature proteins and.(Shinde, *et al* 1999) The main functions of VWFpp appear to be its facilitation of VWF multimerisation and its trafficking into storage organelles.(Haberichter, *et al* 2003, van Mourik and Romani de Wit 2001, Wise, *et al* 1988) and several mutations within VWFpp have been shown to interfere with VWF multimerisation.(Allen, *et al* 2000, Gaucher, *et al* 1998, Schneppenheim, *et al* 1995) VWFpp forms a disulphide-linked intermediate with the D'D3 domain of VWF in the endoplasmic reticulum that rearranges to form multimers in the Golgi.(Purvis and Sadler 2004, Voorberg, *et al* 1990, Wagner, *et al* 1986) Following assembly of the VWF multimers in the trans-Golgi network, VWFpp is cleaved from the amino-

terminal region of VWF by a paired amino acid cleaving enzyme (PACE), also known as furin.(Wagner, *et al* 1987) Like mature VWF, VWFpp is either secreted into the circulation or stored in the platelet α granules or Weibel-Palade bodies. Upon stimulation, both the cleaved VWFpp and mature VWF have been shown to be released in equimolar amounts.(Sadler 1998, Wagner, *et al* 1987) Although some plasma VWFpp may derive from platelets, this is not thought to be of major significance except in situations of massive platelet activation.(Vischer, *et al* 1997) The function of VWFpp in the circulation remains unclear.

Figure 1.2 Biosynthesis of VWF: (Ruggeri 1999)The signal peptide is cleaved and initial glycosylation takes place as the polypeptide is transported into the rough endoplasmic reticulum (RER). Once in the RER, dimers of pro-VWF are formed which are transported to the Golgi apparatus where they undergo post-translational glycosylation and sulphation. Finally, the propeptide is cleaved from the dimers in the trans-golgi network (TGN) and fully functional VWF multimers are stored in Weibel-Palade bodies (WPB).
C-carboxy terminal; N-amino terminal

1.2.5 Storage and secretion of VWF

Plasma VWF

Plasma VWF is derived almost entirely from endothelial cells,(Nichols, *et al* 1995a) and two distinct pathways of secretion have been identified.(Ruggeri and Zimmerman 1987) (Fig. 1.3) Approximately 95% of endothelial-derived VWF is secreted via the constitutive pathway in which VWF (composed of dimers and small multimers) is continuously released from the endothelial cell. The remainder is stored within Weibel-Palade bodies and secreted via the regulated pathway on stimulation by secretagogues (Table 1.2): this VWF is composed of the largest multimeric species.(Sporn, *et al* 1986, Sporn, *et al* 1989) Many of the secretagogues, including thrombin, histamine, complement and fibrin induce intracellular influx of calcium ions. Other secretagogues (notably adrenaline, adenosine triphosphate and adenosine diphosphate) induce release of VWF by an increase in intracellular levels of cyclic adenosine monophosphate (cAMP). Both adrenaline and adenosine potentiate the effect of thrombin induced VWF release. Possible mechanisms of release of 1-deamino-8-d-arginine vasopressin (desmopressin or DDAVP) are discussed in Section 1.5.

Platelet VWF

Platelet VWF is stored within α granules and its secretion occurs only by a regulated pathway following platelet activation at a site of injury. While platelet-derived VWF has not been shown to contribute significantly towards the circulating pool of plasma VWF, up to 20% of the total VWF present in blood is found in platelets.

Figure 1.3 Pathways of VWF secretion. 95% of endothelial cell-derived VWF is secreted via the constitutive pathway whereby VWF is continuously released from the endothelial cell. The remaining 5% is stored within Weibel-Palade (WP) bodies and secreted via the regulated pathway on stimulation by secretagogues.
ER endoplasmic reticulum

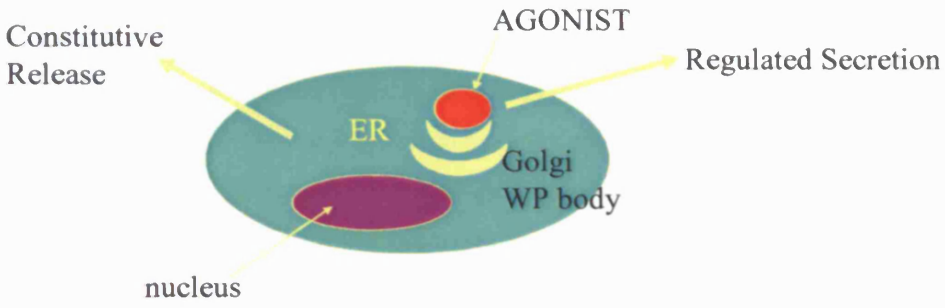


Table 1.2 Agonists that induce VWF release from endothelial cells
Intracellular response to agonist is given as mechanism of exocytosis: $[Ca^{++}]_i$ for intracellular calcium flux; cAMP for increase in intracellular levels of cyclic adenosine monophosphate. PMA - phorbol myristate acetate. DDAVP - desmopressin. PAF - platelet activating factor. VWGF - vascular endothelial growth factor. ATP – adenosine triphosphate. ADP – adenosine diphosphate.

Agonist	Mechanism	Reference
Thrombin	$[Ca^{++}]_i$	(Levine, *et al* 1982)
Histamine	$[Ca^{++}]_i$	(Hamilton and Sims 1987)
Fibrin	$[Ca^{++}]_i$	(Ribes, *et al* 1987)
Calcium ionophore	$[Ca^{++}]_i$	(Sporn, *et al* 1986)
PAF	$[Ca^{++}]_i$	(Hashemi, *et al* 1993)
VEGF	$[Ca^{++}]_i$	(Brock, *et al* 1991)
Leukotrienes	$[Ca^{++}]_i$	(Datta, *et al* 1995)
Endothelin	$[Ca^{++}]_i$	(Pruis and Emeis 1990)
Complement C5b-9	$[Ca^{++}]_i$	(Hattori, *et al* 1989)
Trypsin	$[Ca^{++}]_i$	(Collins, *et al* 1993)
Bradykinin	$[Ca^{++}]_i$	(Tranquille and Emeis 1991)
Endotoxin	?	(Schorer, *et al* 1987)
PMA	Protein kinase C	(Giddings and Shall 1987)
ATP & ADP	cAMP & $[Ca^{++}]_i$	(Vischer and Wollheim 1998)
Vasopressin/DDAVP	cAMP	(Kaufmann, *et al* 2000)
Adrenaline	cAMP	(Vischer and Wollheim 1997)
Prostacyclin	cAMP	(Hegeman, *et al* 1998)

1.2.6 Relationship between structure and function of VWF

Endothelial damage and exposure of the subendothelium promotes a highly regulated and integrated series of events that result in the formation of a platelet plug, stabilisation by fibrin deposition and clot retraction. VWF supports thrombus formation by maintaining platelet adhesion to sites of injury and promoting platelet-platelet aggregation. Platelets respond rapidly to alterations of endothelial cells by firmly attaching to the site of lesion where exposure of subendothelial components may have occurred. The first layer of platelets is in contact with the thrombogenic surface (adhesion), whereas subsequent growth of the haemostatic plug depends on platelet-platelet interactions (aggregation). Both aspects of platelet function are influenced by the interaction of VWF with specific platelet membrane glycoprotein (GP) receptors. The biological function of VWF is dependent on the distinct domains in the constitutive sub-unit, each one containing the structural elements responsible for the specificity and affinity of different interactions. The association between VWF region and function is shown in Fig. 1.1.

VWF interaction with platelets

VWF interacts with platelets via two platelet receptor complexes: GPIba and the integrin $a_{IIb}ß_3$, composed of the glycoproteins IIb and IIIa. The primary binding domain for the platelet receptor GPIba has been mapped to the VWF-A1 domain, although the exact residues are not known.(Celikel, *et al* 1998, Emsley, *et al* 1998) This binding, which is reversible, is modulated by high shear conditions *in vivo* (around 1000 s^{-1}) and can be simulated *in vitro* using the negatively charged antibiotic ristocetin.(Scott, *et al* 1991) On this first layer of adherent platelets, further VWF binds and uncoils, thus presenting the next attachment layer for the attraction of more platelets. Activation of $a_{IIb}ß_3$ mainly occurs via signalling initiated by membrane receptors that bind collagen or by stimulation by agonists released (e.g. ADP) or locally generated (e.g. thrombin) Adhesive ligands, mainly fibrinogen and VWF, bind via activated $a_{IIb}ß_3$ on the membrane of the adherent platelets and become the substrate for the additional recruitment and attachment of incoming platelets. The interaction of VWF with $a_{IIb}ß_3$ occurs via the peptide sequence RGDS, residues 1744 – 1747, located in the carboxy-region of the VWF-C1 domain.(Beacham, *et al* 1992, Plow, *et al* 1985) Recent studies have shown that under conditions of extreme shear stress, the interaction between GPIba and VWF can result in activation-independent platelet aggregation: at shear rates above 10 000 s^{-1}, exposed active VWF-A1 domains are able to bind GPIba, promoting additional platelet recruitment that results in an exponential rise in shear rate. (Ruggeri, *et al* 2006) Up to shear rates of 20,000 s^{-1}, the aggregates have been

shown to be reversible; above this threshold the adhered platelets appear to be capable of persisting as the core of aggregates for several minutes.(Ruggeri, *et al* 2006)

VWF interaction with collagen

VWF constitutively present in the subendothelial matrix is sufficient to stimulate initial platelet adhesion, the exact mechanism of which is unclear. VWF can bind to the fibrillar collagens types I and III and the non-fibrillar collagen type VI. Collagen type VI, found in most connective tissues, is abundant in the subendothelial matrix. VWF binding to type VI collagen is primarily via the A1 domain,(Hoylaerts, *et al* 1997) and to the fibrillar collagens, types I and III via the A3 domain.(Cruz, *et al* 1995, Lankhof, *et al* 1996)

VWF interaction with FVIII

Each VWF monomer has one binding domain, located in the amino terminus in the D' domain (amino acid residues 764 to 1035) that is able to bind one FVIII molecule *in vivo*. However, only 1-2% of available VWF monomers are occupied by FVIII, resulting in the plasma molar ratio of FVIII monomer to VWF monomer of 1:50.(Vlot, *et al* 1998)

VWF interaction with heparin

The interaction of VWF with the subendothelial matrix may also involve the heparin and sulphatide binding sites of VWF via an interaction with sulphated sugars on proteoglycans. There are two heparin binding sites: two clusters of amino acids within the VWF-A1 domain,(Rastegar-Lari, *et al* 2002) and a lower affinity heparin binding site within the D' and D3 domains.

1.2.7 VWF in the circulation

VWF circulates in plasma as a tight, non-covalently linked complex with FVIII. Several mechanisms may explain the necessity for this complex formation:

- VWF stabilises the heterodimeric structure of VWF.
- VWF protects FVIII from proteolytic degradation by phospholipid-binding proteases such as activated protein C (APC) and activated factor X (FXa).
- VWF interferes with the binding of FVIII to negatively-charged phospholipid surfaces.
- VWF inhibits the binding of FVIII to activated factor IX (FIXa).
- VWF prevents the cellular uptake of FVIII.

VWF is released into the circulation uncleaved, and the physiological reduction in multimer size occurs mainly through a process of controlled cleavage thatresults in rapid proteolysis of the highly thrombotic unusually/ultra large (UL) multimers to smaller, less adhesive forms. This cleavage is the function of a plasma metalloprotease that was first identified in 1996,(Furlan, *et al* 1996, Tsai 1996) although the suggestion of a VWF 'splitting enzyme' were first made by Bloom and colleagues as long ago as 1973.(Bloom 1973) The metalloprotease has subsequently been purified and cloned and found to be a member of the ADAMTS family (a disintegrin and metalloprotease with thrombospondin motifs), designated ADAMTS-13, a multidomain protease that is highly specific for VWF.(Fujikawa, *et al* 2001, Gerritsen, *et al* 2001, Zheng, *et al* 2001) ADAMTS-13-mediated proteolysis of VWF occurs at a single peptide bond Tyr ^{1605} – Met ^{1606} in the A2 domain,(Dent, *et al* 1990) generating two fragments of molecular mass 176 kDa (residues 843 to 2050) and 140 kDa (residues 1 to 842) that are responsible for the 'satellite bands' observed flanking the major bands on VWF multimer gels.(Dent, *et al* 1991, Furlan, *et al* 1993) It has recently been shown that ADAMTS-13 is synthesised in human endothelial cells, and released constitutively.(Turner, *et al* 2006) Although both the A1 and A3 domains of VWF may contain binding sites for ADAMTS-13, the A3 domain appears to be the preferred binding site for the metalloprotease under conditions of flow.(Dong, *et al* 2003) It has been suggested that the fragments of VWF released into the circulation following ADAMTS-13 proteolysis may no longer be subject to shear stress and therefore do not bind to platelets and rather it is the VWF-platelet string remaining at the cell surface that participates in the subsequent stages of clot formation.(Dong 2005) Although platelet VWF was previously believed to be segregated from ADAMTS-13 activity, a recent study has demonstrated platelets to contain functionally active ADAMTS-13.(Liu, *et al* 2005) In addition to modifying the release of UL-VWF from platelets, this second pool of ADAMTS-13 may also encounter the UL-VWF released from endothelial cells.

The glycoprotein thrombospondin-1 (TSP-1) has also been shown to regulate the size of VWF multimers. TSP-1 is stored in platelet a granules and is released following platelet activation and like ADAMTS-13, binds to the VWF A3 domain.TSP-1 cleaves disulphide bonds between VWF multimers,(Xie, *et al* 2001) and in wild type mice the multimer size of platelet- but not endothelial-derived VWF has been shown to reduce more rapidly than TSP-1 null mice, suggesting TSP-1 may be more relevant for the control of platelet VWF multimers.(Pimanda, *et al* 2004)

1.2.8 Modifiers of VWF level

VWF levels vary considerably within the general population with 95% of values occurring between 50 and 200% of the mean. This variation is due to several genetic and environmental determinants.(Levy and Ginsburg 2001) Twin studies have shown that around 60% of the variation in VWF levels is genetically determined, of which approximately one third is due to the influence of ABO blood group.(Gill, et al 1987, Orstavik, et al 1985) Linkage analysis has confirmed a direct functional effect of the ABO locus on plasma VWF levels,(Souto, et al 2000) and a detailed discussion of possible mechanisms for the ABO effect is presented in Section 1.4. The remainder of the genetic variability is yet to be determined although ethnic influences on VWF levels have been suggested.(Miller, et al 2001) Other factors shown to influence VWF levels include age, oestrogen, exercise, stress, pregnancy, acute phase reaction, diurnal variation, thyroid status and menstrual cycle.(Bennett and Ratnoff 1972, Gill, *et al* 1987, Hansen, *et al* 1990, Jern, *et al* 1989, Kadir, *et al* 1999)

1.2.9 VWF clearance

While the biosynthesis and function of VWF has been well defined, relatively little is understood about the mechanism(s) by which VWF is cleared from the circulation. The intricate linkage between VWF and FVIII complicates the assessment of how the VWF-FVIII complex or its individual components are removed from the circulation and wheras VWF is a determinant of the survival of FVIII, clearance of VWF is independent of FVIII.

The half-life of exogenous VWF in patients with type 3 VWD is approximately 15 hours with some inter-individual variation.(Dobrkovska A 1998, Goudemand, *et al* 2005) The survival of endogenous VWF has also been shown to differ between individuals, as evidenced from studies using DDAVP.(Brown, *et al* 2003, Michiels, *et al* 2002, van Genderen P. J. J 1997) Survival of VWFpp has been shown to be 3-4 fold shorter than for the mature VWF protein, at approximately 2 -3 hours.(Borchiellini, *et al* 1996)

Possible mechanisms by which VWF is cleared from the circulation include mediation by endocytic receptors, proteolysis, renal excretion and extravasation. In view of the molecular size of VWF, the latter two seem unlikely. At the time of this study, it was not known whether VWF clearance was dependent on the distribution of its multimers and therefore related to its proteolysis. Discordance between the half-lives of VWF level and functional activity had been reported following DDAVP,

reflecting a possible effect of rapid proteolysis of VWF and loss of HMW multimers on VWF clearance.(Batlle, *et al* 1987, Casonato, *et al* 2001a, van Genderen P. J. J 1997) Furthermore, the rate of ADAMTS-13 mediated proteolysis had been shown to vary according to ABO blood group,(Bowen 2003)and a founder haplotype in type 1 VWD, the Y1584C mutation,(O'Brien, *et al* 2003) was reported to segregate with increased susceptibility of VWF to ADAMTS-13 proteolysis.(Bowen 2004) While the significance of this increased *in vitro* VWF proteolysis on the *in vivo* clearance of VWF had not been established, the absence of proteolytic fragments in a rat model lacking O-linked VWF carbohydrates and demonstrating increased VWF clearance favoured a receptor-based, rather than a proteolytic-based clearance mechanism.(Stoddart, *et al* 1996) However, in contrast to FVIII, little information was available on the identity of cells and receptors that may mediate the clearance of VWF in humans. It seemed likely that the structural diversity of VWF would facilitate its interaction with a number of endocytic receptors. The liver had been identified as the principal site of VWF clearance in animal models using radiolabelled VWF.(Lenting, *et al* 2004, Roussi, *et al* 1998, Stoddart, *et al* 1996) A detailed discussion of the effect of glycosylation on VWF clearance is presented in Section 1.5: the only known receptor at the time of the study was the asialoglycoprotein receptor (ASGPR), which had been shown mediate the hepatic clearance of hypo-sialylated VWF (Ellies, *et al* 2002, Sodetz, *et al* 1977)or VWF with an altered glycosylation profile in animal studies.(Mohlke, *et al* 1999b)

In addition to the carbohydrate moiety, it is also likely that polypeptide regions of VWF contribute towards its interaction with clearance receptors. Evidence supporting this was accumulating around the time of this study following the observation of increased clearance of VWF released after DDAVP treatment in type 2M Vicenza patients with the R1205H mutation.(Casonato, *et al* 2002) Subsequent *in vivo* clearance studies of recombinant VWF/R1205H in VWF-deficient mice demonstrated the direct causative effect of this mutation.(Lenting, *et al* 2004) This has been followed by reports of other *VWF* mutations in association with increased VWF clearance and a type 1 VWD phenotype.(Haberichter, *et al* 2006a, Schooten, *et al* 2005) Furthermore, several regions within the VWF molecule have now been shown to be contribute towards the recognition and clearance of VWF.(Lenting, *et al* 2004)

1.3 VON WILLEBRAND DISEASE

1.3.1 Prevalence of VWD

VWD is recognised as the most common inherited bleeding disorder in humans. The frequently quoted population prevalence of VWD of 1% derives from two epidemiologic studies (Rodeghiero, *et al* 1987, Werner, *et al* 1993) In contrast, the prevalence of symptomatic VWD presenting to haematologists has been estimated to be approximately 1 in 10 000.(Bloom 1991) In 2001, there were approximately 6000 patients registered in the UK with VWD.(Laffan, *et al* 2004)

1.3.2 Clinical features of VWD

The predominant clinical symptoms of VWD are nosebleeds, bleeding from lesions in the skin, mucosa or gastrointestinal tract, menorrhagia and excessive bleeding following trauma, surgical interventions or childbirth. Patients with severe disease may also bleed into joints or muscles.

1.3.3 Classification of VWD

VWD is a highly heterogeneous disorder resulting from the deficiency and/or dysfunction of VWF, rendering a standardised approach to its diagnosis and subsequent management as particularly problematic. In 1994 a revised classification of VWD was recommended by the VWF Scientific Subcommittee of the International Society on Thrombosis and Haemostasis Scientific and Standardisation Committee on VWF (ISTH) (Table 1.3).(Sadler 1994) This classification is a simplified modification of an earlier classification,(Ruggeri and Zimmerman 1987) and assumes that all cases of VWD arise from mutations within the *VWF* gene.(Sadler 1994) VWD is divided into two broad types based on whether the VWF defect is quantitative, whereby there is a decrease in structurally normal VWF or qualitative, whereby the VWF molecule is abnormal. Type 1 VWD, a partial quantitative deficiency of VWF, is the most common variant and represents around 70% of all cases of VWD. Type 2 variants (subtypes A, B, M and N) are characterised by qualitative defects that correspond with a loss or gain of function. Type 3 VWD is characterised by a severe quantitative deficiency of VWF. Quantitative and qualitative defects are differentiated by means of quantitative and functional laboratory parameters and by analysis of the electrophoretic pattern of VWF multimers.

Table 1.3 Classification of VWD subtypes according to the 1994 ISTH guidelines.(Sadler 1994)

Subtype	Frequency (% of VWD cases) (Colvin 1986)	Clinical Features	Diagnosis	Molecular Basis
Type 3	~6%	Severe bleeding disorder: Autosomal recessive inheritance	Markedly decreased or undetectable VWF:Ag, functional activity and VIII:C	*VWF* gene deletions, nonsense, missense and frameshift mutations
Type 1	~70%	Mild to moderate bleeding, autosomal dominant; incomplete penetrance	VWF:Ag, functional activity and VIII:C all proportionately decreased Normal multimeric distribution	A few missense mutations reported, some cases represent heterozygous form of type 3
Type 2A	~10-15%	Mild to moderate bleeding; usually autosomal dominant, more complete penetrance than type 1	Variably decreased VWF:Ag, platelet-dependent function and FVIII; relative decrease in intermediate and high molecular weight multimers	Missense mutations clustered within VWF- A2 repeat. Two subgroups: a.defect in intracellular transport b. increased proteolysis post-secretion
Type 2B	< 5%	Mild to moderate bleeding; autosomal dominant	Variably decreased VWF:Ag and functional activity and VIII:C; loss of large multimers; enhanced RIPA, thrombocytopenia	Missense mutations clustered in VWF-A1 repeat result in increased affinity of VWF for GPIba, leading to spontaneous platelet binding
Type 2M	rare	Variable bleeding; autosomal dominant	Variably decreased VWF:Ag and platelet-dependent function despite normal multimeric pattern.	Missense mutations and small in frame deletions in VWF-A1 repeat
Type 2N	Uncommon: heterozygotes may be prevalent in some populations	Variable bleeding. Homozygotes (or compound heterozygotes) may resemble autosomal haemophilia A. Coinheritance may modify severity of type 1.	Variable VWF:Ag and activity. Disproportionately low FVIII:C. Generally normal multimers. Decreased or absent VWF binding to FVIII.	Missense mutations in VWF-D' or D3 domains in the N-terminus of mature VWF that interfere with FVIII binding

1.3.4 Testing

The following screening and specific tests used to diagnose and subtype VWD are available:

1) Screening tests

(a) Bleeding time or PFA-100™. Both tests assess primary haemostasis and are not specific for VWD.

(b) Activated partial thromboplastin time (APTT).

(c) Platelet count.

2) Specific tests

(a) FVIII:C: This can be assayed by a one-stage(Simone, *et al* 1967) or two-stage clotting assay and by a chromogenic assay.

(b) VWF:Ag: Immunological methods are used to measure the quantity of VWF:Ag in plasma.(Short, *et al* 1982)

(c) VWF functional assays:

i. VWF ristocetin cofactor activity (VWF:RCo) remains the standard functional assay for VWF.(Macfarlane, *et al* 1975) This test is a measurement of the interaction of VWF with the platelet receptor GPIba. However, there are several drawbacks associated with VWF:RCo, which include considerable inter- and intra-assay variation.

ii. VWF collagen binding activity (VWF:CB) is measured using ELISA and is sensitive to functional variants associated with the loss of high molecular weight (HMW) multimers of VWF.(Brown and Bosak 1986)

iii. VWF activity immunoassay (VWF:Ac) uses monoclonal antibodies that recognise an epitope on VWF involved in its interaction with GPIba.(Murdock, *et al* 1997)

iv. Ristocetin induced platelet aggregation (RIPA). In this assay a range of concentrations of ristocetin (0.2 – 1.5 mg/mL) are added to platelet rich plasma. The amount of platelet aggregation is dependent on the VWF level and the integrity of the VWF – GPIba interaction.

v. VWF multimer analysis. The multimer composition of VWF can be determined by agarose gel electrophoresis in the presence of sodium dodecyl sulphate (SDS).(Ruggeri and Zimmerman 1981) Despite this being the most useful method for the typing

and subtyping of VWD, it is the least suitable for standardisation.(Schneppenheim, *et al* 2001)

vi. FVIII binding assay. This assay measures the ability of VWF to bind FVIII by immobilising the VWF/FVIII complex prior to dissociation of the FVIII using a high concentration calcium chloride. Exogenous FVIII is added to the immobilised VWF and the amount of exogenous FVIII bound by the VWF is measured, utilising either a chromogenic substrate or anti-FVIII antibody.

There has been much debate as to whether the VWF:CB assay should replace VWF:RCo as the measurement of functional activity of VWF. However, limitations in the differentiation of some variants of type 2 from type 1 VWD have been demonstrated,(Riddell, et al 2002) and results have been shown to vary according to the type of collagen used.(Penas, et al 2005) The increasing use of VWF:CB to measure VWF function has led to the detection of subtypes of VWD with isolated collagen-binding defects, which are not classifiable using the 1994 ISTH recommendations. (Ribba, et al 2001) At present VWF:RCo remains the gold standard for diagnostic purposes,(Rodeghiero, et al 1990) although recommended practice in the UK is to use both VWF:RCo and VWF:CB.(Laffan, et al 2004)

1.3.5 Diagnosis of VWD

The diagnosis of VWD is based upon three clinical and laboratory components (Table 1.3). These comprise:

1. a personal history of excessive mucocutaneous bleeding
2. laboratory evaluation consistent with a quantitative and/or qualitiative defect in VWF
3. a family history of excessive bleeding or appropriate *VWF* gene mutation

1.3.6 Molecular genetics of VWD

Types 2 and 3 VWD

There is a significant body of evidence to indicate that types 2A and 2B VWD are almost always autosomal dominant traits due to missense mutations within *VWF*,(Fressinaud, *et al* 2002, Mohlke, *et al* 1999a) and that type 2N and type 3 VWD variants are recessive *VWF* gene traits.(Baronciani, *et al* 2003, Mazurier 1992, Meyer, *et al* 1997, Zhang, *et al* 1992b)

- The majority of type 2A VWD mutations are located within the VWF-A2 domain. The characteristic type 2A phenotype can arise via two distinct mechanisms:
  1. Group 1: *Intracellular processing defects*
     The responsible mutation results in an incorrectly folded protein that is retained in the endoplasmic reticulum (ER). The larger VWF multimers are more likely to contain at least one mutant subunit and are therefore retained in the ER while the smaller multimers are secreted normally.(Lyons, *et al* 1992)
  2. Group 2: *Increased extracellular proteolysis*
     VWF is normally secreted from the cell but has an increased susceptibility to ADAMTS-13 cleavage within the A2 domain resulting in loss of the largest VWF multimers.(Dent, *et al* 1990)

  Other reported causes of HMW deficiency include intracellular proteolysis,(Englender, *et al* 1996) and defective post-translational processing, including defects of dimerisation or multimerisation.(Schneppenheim, *et al* 2001)
- Type 2B VWD is caused by mutations within the VWF-A1 domain that result in increased affinity of VWF for the platelet GPIba receptor. This leads to spontaneous binding of the platelets to the largest VWF multimers and the removal of these complexes from the plasma.
- Type 2M mutations have been described in the VWF-A1 domain that contains the GpIba binding site. The Vicenza subtype, resulting from R1205H in the VWF-D3 domain was classified as a type 2M variant at the time of this study.(Sadler 1994)
- Type 2N VWD is associated with mutations in the D' or D3 domains of VWF that affect VWF binding to FVIII. Co-inheritance of a type 2N mutation with a type 1 VWD allele has been shown to contribute towards the variable expressivity of type 1 VWD.(Mazurier, *et al* 2001)
- Most cases of type 3 VWD are compound heterozygotes, with inheritance of an affected allele from each parent. True homozygotes are also found, usually where consanguinity is common and notably in the family originally described by von Willebrand.(Von Willebrand 1926) The most common mutations in type 3 VWD are total or partial deletions, nonsense, splicing and frameshift mutations found throughout all 52 exons of *VWF*.(Baronciani, *et al* 2003, Mohlke, *et al* 1999a, Zhang, *et al* 1992b)

Type 1 VWD

Despite being the most common subtype of VWD, relatively few mutations causing type 1 VWD had been characterised until very recently. Prior to 2000 a total of 14 mutations had been reported, comprising 11 missense mutations in exons 26, 28 and 52, as well as one nonsense and two frameshift mutations. Furthermore, the mechanism by which a single allele mutation could result in a bleeding disorder was not clear. Two cysteine mutations in the D3 domain, 3445T>C (C1149R) and 3389 G>T (C1130F), were reported as causing an autosomal dominant type 1 phenotype due to a dominant negative effect of the intracellular degradation and impaired secretion of VWF.(Bodo, *et al* 2001, Eikenboom, *et al* 1996) The molecular basis for recessive inheritance has been shown to result from the co-inheritance of a null allele and a mutation on the other allele, commonly one found in type 2N VWD as described above.(Eikenboom, *et al* 1993)

In an attempt to address the molecular and clinical features of type 1 VWD, three multicentre studies were initiated in 2000:

1. European Union (EU) study: Molecular and Clinical Markers for the Diagnosis and Management of Type 1 von Willebrand disease (MCMDM-1VWD)
2. Canadian type 1 VWD study
3. United Kingdom Haemophilia Centre Doctors' Organisation (UKHCDO) type 1 VWD molecular study

The common aim of these studies was to examine the genetic basis of type 1 VWD by analysis of *VWF* in clinically affected patients and to relate the findings to the patient's clinical and laboratory phenotype and response to treatment. The protocols included extensive bleeding histories, comprehensive testing for VWF level and function, linkage analysis to *VWF*, and sequencing of the *VWF* coding region in affected patients.

1.3.7 Difficulties associated with the diagnosis of type 1 VWD

A diagnosis of type 1 VWD is based upon a history of significant mucocutaneous bleeding, laboratory tests compatible with a partial quantitative deficiency of VWF and either a positive family history of type 1 VWD or appropriate *VWF* mutation (Table 1.3).(Sadler 1994) Although strict definitions of VWD subtype are required for the interpretation and aggregation of data and are therefore appropriate for research purposes, the clinical priority is to determine the risk of bleeding in an individual patient and initiate appropriate treatment if indicated, irrespective of whether the patient fulfils a centrally agreed definition. Acknowledgement of the differences in

perspective between the clinician and the scientist may alleviate the frequent problems imposed by strict definitions of VWD in the clinical setting, particularly in the diagnosis of type 1 VWD.

Whether to define type 1 VWD as a disease or a deficiency can pose a challenge to clinicians. It has been proposed that while low VWF levels may identify a risk factor for bleeding, they do not mandate a diagnosis of type 1 VWD.(Sadler 2002) The designation of a cut-off VWF value for the diagnosis of type 1 VWD is arbitrary and considerable overlap is observed between VWF levels in patients and healthy controls.(Sadler 2005) With the exception of severe cases, no strong relationship is generally observed between plasma VWF levels and bleeding manifestations.(Sadler 2003) Furthermore, many factors are known to influence VWF levels, as previously discussed. Other factors that may result in difficulties in the diagnosis of type 1 VWD include the frequent finding of non-linkage between the *VWF* gene and the type 1 phenotype,(Casana, *et al* 2001, Castaman, *et al* 1999) the incomplete penetrance and variable expressivity of mutant *VWF* alleles,(Abildgaard, *et al* 1980, Miller, *et al* 1979a, Miller, *et al* 1979b)and the objective evaluation of a bleeding history or demonstration of a positive family history, such that excessive numbers of healthy individuals are possibly being misdiagnosed with an inherited haemorrhagic disorder.(Rodeghiero and Castaman 2001, Sadler 2002, Sadler 2004) Previous attempts to address these difficulties by classifying some patients as having 'possible' or 'probable' type 1 VWD,(Nitu-Whalley, *et al* 2000, Sadler 2004) have only further added to the confusion. Increasing emphasis has been placed on the utility of the bleeding history in recent years and the usefulness of a quantitative bleeding score in the diagnosis of type 1 VWD has been demonstrated.(Rodeghiero, *et al* 2005)

1.3.8 Type 1 VWD as a complex genetic trait

Although mutation analysis in type 1 VWD had rarely been reported at the outset of this study, the finding of non-linkage between disease phenotype and *VWF* gene as well as the inconsistent correlation between genotype and phenotype in the few identified *VWF* mutations in type 1 VWD, were suggestive of co-existent pathogenic mechanisms in type 1 VWD that may or may not relate to the *VWF* gene. VWF levels were shown to be higher in recombinant variants of VWF/C1149R and VWF/C1130F compared to those observed in the plasma of heterozygous patients,(Bodo, *et al* 2001, Tjernberg, *et al* 2004) a discrepancy that may be explained by the subsequent demonstration of increased clearance of VWF in these *VWF* mutants.(Schooten, *et al* 2005) In addition to the ABO effect on VWF levels,

other as yet unidentified genetic influences account for 40% of the variation in VWF levels in humans.(Levy and Ginsburg 2001) A murine model has been identified in which VWF concentrations have been shown to be significantly altered in response to an aberrantly expressed glycosyltransferase gene that is distinct from the murine *VWF* gene (Mohlke, *et al* 1999b): this model is discussed in detail in Section 1.4.3. In addition, the ST3Gal-IV sialyltransferase has been identified as a possible VWF modifier gene in humans.(Ellies, *et al* 2002) Other studies demonstrate the influence of single nucleotide polymorphisms (SNP) in the promoter region of *VWF* on plasma VWF levels.(Harvey, *et al* 2000) Genome-wide linkage analysis has shown that in addition to the ABO locus on chromosome 9, VWF levels are influenced by regions of chromosomes 1, 2, 5, 6, and 22.(Souto, *et al* 2003)

Therefore, at the time of this study, type 1 VWD was beginning to emerge as a complex genetic trait,(Sadler 2002) with heterogeneity in pathophysiological mechanisms. The likely involvement of a number of modifiers of VWF level, both within and outwith the *VWF* gene was becoming increasingly recognised. These modifiers may have little effect in isolation, but in combination could account for the wide variation in clinical severity and incomplete penetrance of type 1 VWD.

1.4 OLIGOSACCHARIDE STRUCTURES OF VON WILLEBRAND FACTOR

In this section possible pathogenic mechanisms by which the oligosaccharide side chains of VWF may contribute towards a partial quantitative deficiency of the protein are reviewed. The structures of these oligosaccharides and how understanding of their possible functional roles has evolved over the past four decades is also discussed.

1.4.1 Oligosaccharide side chains of VWF

Structure

Oligosaccharides make up approximately 20% of the mass of VWF. N-linked glycosylation occurs co-translationally and the sites of the 12 N-linked and 10 O-linked oligosaccharide side chains have been identified and are distributed throughout the VWF molecule (Appendix 1, Fig.1.4).(Titani, *et al* 1986)

Sialic acids are possibly the most biologically important monosaccharide units of glycoconjugates and are commonly linked to the penultimate galactose (Gal) or N-acetylgalactosamine (GalNAc) on N- and O-glycan branches. The majority of sialic

acids attached to plasma components are a2-3 linked and are produced by up to six of the 18 sialyltransferase enzymes encoded by a2-3 *ST3Gal* genes.

The primary structure of the major N-linked oligosaccharide chain is a monosialylated biantennary complex glycan in which the basic pentasaccharide core has two N-acetyllactosamine residues (Galβ(1→4)GlcNac) added, one of these terminating in sialic acid (Fig.1.5A).(Debeire, *et al* 1983, Samor, *et al* 1982) This structure is identical to that of human lacto-transferrin glycopeptide D,(Spik, *et al* 1982) and also to that of glycopeptide fraction B, derived from secretory immunoglobulin A from human milk.(Pierce-Cretel, *et al* 1982) The primary structure of a tetraantennary glycan has subsequently been described whereby four N-acetyllactosamine residues are added to the basic core in addition to sialic acid.(Samor, *et al* 1986) At present, the structures of 94% of the N-linked oligosaccharides of bi-, tri- and tetra-antennary types are known.(Matsui, *et al* 1992) These include a proportion (~12%) of oligosaccharides containing blood group A, B and H antigenic structures.(Matsui, *et al* 1999, Sodetz, *et al* 1979) Analysis of the O-linked oligosaccharide side chains has shown them to be comprised of mucin-type structures in which GalNAc is linked (α1-3) to either serine or threonine residues (Fig.1.5B).(Samor, *et al* 1989) Blood group antigens have not been demonstrated on these glycans.

Figure 1.4 Positions of the N- and O-linked oligosaccharide side chains on the VWF molecule (amino acid: αα)

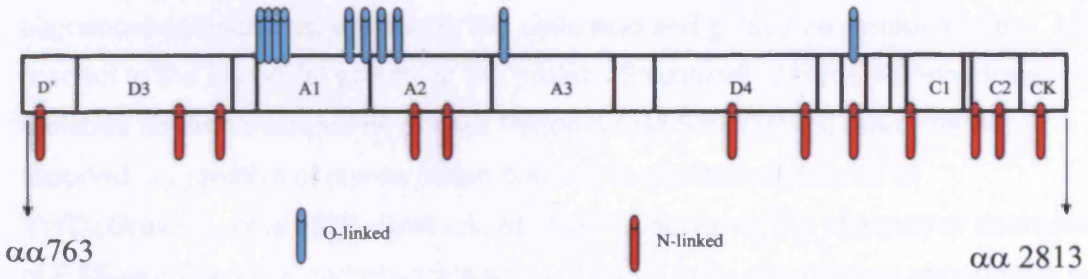
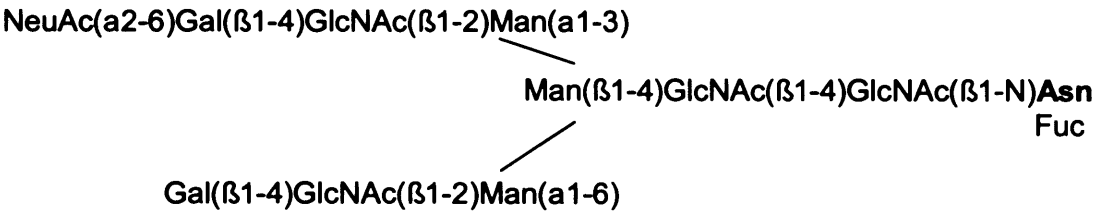


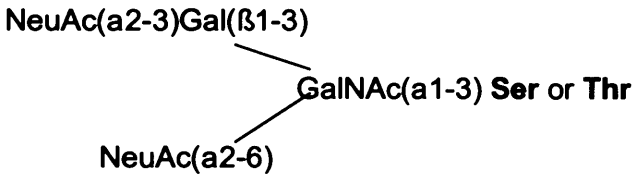
Figure 1.5 Primary structures of the major N-linked (A) and O-linked (B) glycans on VWF

Man - mannose; GlcNAc - N-acetylglucosamine; Gal – galactose; GalNAc - N-acetylgalactosamine; NeuAc - N-acetylneuraminic (sialic) acid; Gal(ß1-4)GlcNAc-N-acetyllactosamine

A. N-linked



B. O-linked



Possible functional roles

In the 1970s, although the structure of the carbohydrate moiety had not yet been elucidated, there was much interest in studying the function of the VWF oligosaccharide chains, especially the sialic acid and galactose residues, with respect to the biological activity of the protein. Structurally altered carbohydrate moieties as demonstrated by absent Periodic Acid Schiff (PAS) reactions were reported, suggestive of glycosylation defects, in qualitative variants of VWD.(Gralnick, *et al* 1976, Gralnick, *et al* 1977) However, the absence or decrease of PAS-reactive VWF carbohydrate was not found to be a consistent abnormality in VWD.(Zimmerman, *et al* 1979) These results should be interpreted in light of the knowledge that PAS is sensitive, but not specific for sialic acid residues. The possible functional roles of the oligosaccharide side chains, in particular, the sialic acid and galactose residues, discussed in this section are summarised in Table 1.4.

Platelet aggregation

The *in vitro* removal of sialic acid from VWF using neuraminidase exposes the penultimate Gal molecule. It is now well established that this in vitro removal does not affect the VWF:Ag concentration, however, its effect on platelet aggregation is disputed in the literature. Some investigators have demonstrated desialylated VWF to be capable of inducing spontaneous platelet aggregation independent of ristocetin, an effect that was abolished by recruitment of GPIba-defective platelets.(De Marco and Shapiro 1981, Vermylen, et al 1974, Vermylen, et al 1973) Others report a resultant incremental loss of VWF:RCo,(Kao, et al 1980, Levy-Toledano, et al 1973, Rosenfeld and Kirby 1979, Sodetz, et al 1978, Sodetz, et al 1977) with further studies reporting no effect on VWF:RCo,(Fukui, et al 1977, Gralnick 1978, Morisato and Gralnick 1980) or platelet aggregation in the presence,(Goudemand, et al 1985, Morisato and Gralnick 1980) or absence,(Rosenfeld and Kirby 1979) of ristocetin. Few studies have tested the direct effects of β-galactosidase or galactose oxidase on intact VWF: these enzymes respectively remove or oxidise only the terminal Gal residues. Treatment of VWF with β-D-galactosidase was shown in one study to result in reduction in RIPA.(Goudemand, et al 1985) Another showed treatment with galactose oxidase, but not β-galactosidase, to result in significant loss of VWF:RCo.(Fukui, et al 1977) In a further study neither galactose oxidase nor β-galactosidase-treated VWF was shown to have any effect on VWF:RCo.(Gralnick, et al 1983) The majority of studies have tested the effects of β-galactosidase or galactose oxidase on desialylated VWF, which results in both the terminal and penultimate Gal residues being released or oxidised. In this state, platelet aggregation was shown to be reduced in both the presence and absence of ristocetin.(Goudemand, et al 1985, Gralnick 1978, Kao, et al 1980, Vermylen, et al 1973) The VWF:RCo was also shown to be reduced.(Gralnick 1978, Gralnick, et al 1983, Morisato and Gralnick 1980, Sodetz, et al 1978)

These *in vitro* studies report discrepant results following the removal of either sialic acid or the terminal Gal residue. However, the studies are in agreement that removal of the penultimate Gal residue results in a loss of VWF:RCo activity. This suggests that intact penultimate Gal moieties on VWF are necessary for its effect on platelet aggregation.

Adsorption to Collagen

Desialylation of intact VWF has been shown not to interfere with the adsorption of VWF to type I collagen.(Kessler, *et al* 1990) However, treatment of desialylated VWF with β-galactosidase or galactose oxidase markedly reduces collagen adsorption suggesting that penultimate galactose moieties are also required for the adsorption of VWF to collagen.(Kessler, *et al* 1990)

Multimeric Structure

It is not clear whether the effect of modified carbohydrates on VWF-platelet and collagen interactions is direct, via altered binding sites, or indirect, via degradation of VWF multimers. Removal or oxidation of the penultimate Gal moiety from desialylated VWF has been shown to significantly affect its multimeric structure with loss of HMW and increase in low molecular weight (LMW) multimers.(Goudemand, *et al* 1985, Gralnick, *et al* 1983, Kessler, *et al* 1990) One of these studies reported a similar loss of HMW multimers upon removal of only the terminal Gal,(Goudemand, *et al* 1985) a finding not confirmed by others.(Gralnick, *et al* 1983) These results suggest that the penultimate and possibly the terminal Gal residues are essential for the maintenance of normal multimeric structure. The effects of inhibiting N-glycosylation in VWF have been studied by treating human umbilical vein endothelial cells (HUVEC) with tunicamycin. The resulting pro-VWF monomers failed to dimerise and accumulated in the endoplasmic reticulum.(Wagner, *et al* 1986) This suggests that initial glycosylation is necessary for VWF multimerisation with carbohydrates providing the VWF monomer with a conformation that favours dimerisation.

Protection from proteolytic degradation

Following the extensive work outlined above, it was shown that rather than being necessary for maintaining multimeric structure and platelet aggregation directly, the carbohydrate moiety protected VWF from proteolytic degradation.(Federici, *et al* 1984) Although a fall in VWF:RCo following treatment with neuraminidase and β-galactosidase was reported in this study, this was to levels which were considerably higher than those previously reported.(Gralnick 1978, Sodetz, *et al* 1978) When VWF was treated with neuraminidase and β-galactosidase in the presence of protease inhibitors, removal of carbohydrate was comparable to that seen in previous studies but there was no associated change in VWF:RCo. Moreover, the multimeric structure remained intact. This would suggest that the oligosaccharide side chains have a role in protecting VWF multimers against traces of one or more plasma proteases. Since traces of one or more proteases may contaminate even

highly purified VWF, removal of the carbohydrate moiety may appear to be directly responsible for causing loss of HMW multimers when it is simply making the protein susceptible to proteolysis by these proteases. Loss of HMW multimers causes a reduction in VWF:RCo and in the absence of protease inhibitors there may have been proteolysis of the HMW multimers resulting in the observed reduction in VWF:RCo. It has subsequently been shown that sialic acid protects VWF by preventing its cleavage in the N-terminal region and thus inhibits the loss of HMW multimers.(Berkowitz and Federici 1988) Studies in other glycoproteins demonstrate similar protective effects by the carbohydrate moiety from both intracellular and extracellular degradation.(Bernard, *et al* 1982)

Table 1.4 Summary of in vitro studies of functional roles of VWF in which the sialic acid (asialo-), terminal galactose (agalacto-) and sialic acid and terminal and penultimate galactose residues (asialo-, agalacto-) have been removed. References denoted by number.
VWF:RCo=ristocetin cofactor activity; VWF:CB=collagen binding activity; HMW=high molecular weight; LMW=low molecular weight; ↑= increased; ↓=decreased; ↔=unchanged.

Treated VWF	Platelet aggregation (ristocetin-independent)	Platelet aggregation (ristocetin-independent)	VWF:RCo	VWF:CB	VWF multimers
Asialo-	↑ (De Marco and Shapiro 1981, Vermylen, et al 1974, Vermylen, et al 1973) ↔ (Rosenfeld and Kirby 1979)	↓ (Kao, et al 1980) ↔ (Gralnick 1978, Morisato and Gralnick 1980)	↓ (Kao, et al 1980, Levy-Toledano, et al 1973, Rosenfeld and Kirby 1979, Sodetz, et al 1978) ↔ (Fukui, et al 1977, Gralnick 1978, Gralnick, et al 1983, Morisato and Gralnick 1980, Zimmerman, et al 1979),	↔ Kessler, et al 1990	-
Agalacto-	-	↓ {Goudemand, 1985 #100}.	↓ (Fukui, et al 1977) ↔ (Fukui, et al 1977, Gralnick, et al 1983)	-	↓ HMW Goudemend et al 1985 ↔ Gralnick et al 1983
Asialo-, agalacto-	↓ (Vermylen, et al 1973)	↓ (Gralnick 1978, Kao, et al 1980, Morisato and Gralnick 1980)	↓ (Gralnick 1978, Gralnick, et al 1983, Kessler, et al 1990, Morisato and Gralnick 1980, Sodetz, et al 1978)	↓ Kessler, et al 1990	↓ HMW Goudemend et al 1985 ↔ Gralnick et al 1983 ↓ Kessler, et al 1990 ↑ LMW

1.4.2 VWF glycosylation and clearance

Clearance of glycoproteins

A primary purpose of glycosylation is to encode the half-life of a glycoprotein. One of the possible reasons for this is the diversity in sugar structures, which results from variety in both constituent oligosaccharides and the linkages that connect them. The carbohydrate moiety of many plasma glycoproteins including erythropoietin, interferon, sex hormone-binding globulin and caeruloplasmin is known to play a key role in their plasma clearance.(Ashwell and Harford 1982, Cousin, *et al* 1999) Desialylation and the subsequent exposure of the penultimate Gal residue has been shown to increase the rate of removal of several glycoproteins from the circulation.(Ain, *et al* 1987, Durocher, *et al* 1975, Morell, *et al* 1971, Nelsestuen and Suttie 1971) This reduction of intravascular half-life has been found when as little as 25% of the sialic acid has been removed. The asialoglycoprotein receptor (ASGPR), a lectin on hepatocytes has been shown to mediate the cellular uptake of a number of desialylated proteins *in vitro* by recognising exposed Gal and GalNAc residues.(Ashwell and Harford 1982, Morell, *et al* 1971, Spivak 1989) The affinity of ASGPR has been shown to be up to 60-fold greater for structures containing GalNAc than for Gal.(Kolatkar, *et al* 1998) However a study using ASGPR-defective mice failed to detect any substantial accumulation of desialylated glycoproteins,(Ishibashi, *et al* 1994) and the role of the ASGPR *in vivo* remains to be determined.

In vitro modification of the carbohydrate moiety of VWF

Asialo-VWF has been shown to bind to the ASGPR and is rapidly removed from the circulation in animal studies.(Sodetz, *et al* 1978, Sodetz, *et al* 1977) In contrast, both intact VWF and asialo-VWF which has been treated with galactosidase exhibit minimal affinity for this receptor.(Sodetz, *et al* 1978) This suggests that exposed penultimate Gal residues may be required for the recognition and subsequent binding of VWF to the hepatic ASGPR in these animal models. As a result of these findings, it was postulated that the increased desialylation and subsequent rapid plasma clearance of VWF may result in a partial quantitative deficiency of the protein *in vivo*.(Sodetz, *et al* 1977)

Although these studies focus on terminal sialic deletion, it is not proposed that this is the only defect; rather that a spectrum of carbohydrate structural modifications could explain the heterogeneity seen in plasma VWF levels. There may be more extensive deletions extending into the structural core of the carbohydrate moiety.

1.4.3 Animal models of a partial quantitative deficiency of VWF

The RIIIS/J mouse was described in 1990 as a model for a partial quantitative deficiency of VWF.(Sweeney, *et al* 1990) The gene responsible, *Mvwf*, is on chromosome 11,(Mohlke, *et al* 1998) distinct from the murine VWF locus on chromosome 6.(Nichols, *et al* 1995b) *Mvwf* is the allele of a previously known glycosyltransferase gene, *N-acetylgalactosaminyltransferase* (*Galgt2*),(Mohlke, *et al* 1999b) the murine expression of which is usually restricted to the kidney and intestine. In RIIIS/J mice, a regulatory mutation causes a switch of this *Galgt2* gene expression to the vascular endothelial cells where plasma VWF is synthesised. Here the VWF undergoes aberrant post-translational modification, with the addition of GalNAc onto the glycans. This abnormal carbohydrate expression in VWF results in a plasma half-life of 25 min as compared with 300 min for unmodified VWF thus reducing the steady state plasma levels of VWF.(Mohlke, *et al* 1999b) Similar phenotypes are observed in homozygous and heterozygous RIIIS/J models, consistent with this gain-of-function hypothesis. The high affinity of the hepatic ASGPR for structures containing subterminal GalNAc suggests that *Galgt2*-modified VWF may be cleared via this receptor. This is supported by the correction of the rapid clearance of the modified VWF following saturation of this receptor with the competitive ligand asialofetuin, although is yet to be definitively shown. Increased VWF clearance has also been demonstrated in other animal models including recombinant VWF defective in O-linked carbohydrates in a rat model,(Stoddart, *et al* 1996) and endogenous VWF in the absence of the sialyltransferase enzyme ST3GalIV in a murine model.(Ellies, *et al* 2002)

1.4.4 ABO blood group

ABH blood group expression on VWF and ABO effect on plasma VWF levels

The relationship between plasma VWF concentration and ABO blood group is well established. Levels of VWF vary by 20 – 30% according to individual ABO blood group with the plasma VWF levels increasing in the order O<A<B<AB.(Gill, *et al* 1987, Orstavik, *et al* 1985) The ABH blood group antigens consist of terminal carbohydrate molecules which are synthesised by the sequential action of glycosyltransferases.(Lowe 1993) These catalyse the addition of specific monosaccharides to a common core precursor antigen (H antigen) to form distinct A and B antigens. A and B antigens differ only by their terminal sugar structure: A antigens terminate in GalNAc, B antigens terminate in Gal, and H in a fucose residue. Individuals with blood group O lack these glycosyltransferases and express only the basic H antigen.

VWF is one of only three glycoproteins shown to express ABH antigens, which are present on 13% of the N-linked oligosaccharide chains of plasma VWF according to the blood group of the individual.(Sodetz, *et al* 1979) ABH antigens are also expressed by FVIII and a2 macroglobulin. Platelet VWF, in contrast, appears to express significantly less ABH antigens,(Brown, *et al* 2002, Matsui, *et al* 1999) and this is consistent with the absence of variation of platelet VWF levels between blood groups.(Sweeney and Hoernig 1992) A possible explanation for this is that intra-platelet VWF is compartmentalised away from the ABO-encoded glycosyltransferases. Alternatively, the carbohydrate side chains of platelet VWF may differ from that of other intra-platelet proteins such that AB antigen addition cannot occur.Although the mechanism for the ABO effect on plasma VWF levels is not known, it seems likely that it is mediated by the presence of the ABH antigenic structures on VWF.

ABH determinants and VWF synthesis and secretion

While it is possible that ABO blood group alters the synthesis or secretion of VWF within endothelial cells, several lines of evidence suggest that this is not the case. Firstly, ABO blood group has not been shown to influence the rate of VWF secretion by the regulated secretory pathway in *in vivo* studies using DDAVP.(Brown, *et al* 2003, O'Donnell and Laffan 2001) Secondly, ABO blood group has not been shown to influence plasma levels of VWFpp or platelet VWF levels. (O'Donnell and Laffan 2001) Finally, transfection of A transferase in a phenotypically group-O endothelial cell line has not been shown to affect the rate of VWF synthesis.(O'Donnell and Laffan 2003) These data suggest that it is unlikely that the ABO effect on VWF levels is mediated by alterations in the biosynthesis and secretion of VWF, and alternative explanations should therefore be sought.

ABH determinants and ADAMTS-13 mediated proteolysis of VWF

It is now a well established view that the one of the functions of the carbohydrate moiety of VWF is to protect the protein from proteolytic degradation, as discussed in Section 1.4.1(Federici, *et al* 1984) Recent studies have demonstrated that the ABO blood group determinants may influence the susceptibility of plasma VWF to ADAMTS-13 proteolysis. The rate of proteolysis was shown to be greater for HMW plasma VWF of group O VWF compared with non-O (in the order O = B > A = AB),(Bowen 2003) and proteolysis of HMW plasma VWF from individuals of the Bombay phenotype was shown to be greater than that for group O.(O'Donnell, *et al* 2005) In addition, measurement of ADAMTS-13 levels has shown these to be higher in blood group O than in non-O individuals.(Mannucci, *et al* 2004) The mechanism

by which ABO blood group may influence the rate of ADAMTS-13 proteolysis of plasma VWF remains unknown. The cleavage site in VWF for the metalloprotease ADAMTS13 is flanked by two N-linked and five O-linked glycosylation sites. In the primary VWF structure, these represent the nearest glycosylation sites, although there may be other nearby glycosylation sites in the tertiary structure. These observations suggest that the presence of ABH antigens on one or more of the glycosylation moieties that flank the VWF cleavage site for ADAMTS-13 may influence proteolysis and through this mechanism influence plasma VWF level. Alternatively, VWF proteolysis and VWF level may not be directly related although both may be influenced by ABO blood group.

ABH determinants and VWF clearance

The hypothesis that terminal carbohydrate moiety expression may determine the rate of VWF clearance is supported by studies of the RIIIS/J mouse,(Mohlke, *et al* 1999b), as described above. Data from other studies has shown infused Factor VIII has a shorter half-life in blood group O compared to group A haemophilia A patients, suggesting the importance of ABH antigens on VWF in determining the rate of clearance.(Vlot, *et al* 2000)

Other blood group antigens

In addition to the ABH blood group antigens, the effect of two other glycosyltransferases on VWF levels has been studied. The Secretor (Se) and Lewis b blood group antigens encode an $\alpha(1,2)$-fucosyltransferase that generates the H blood group antigen in tissues other than red blood cells. An association was found between Se, but not Lewis genotype, and VWF:Ag level with the highest VWF levels found in Se homozygotes.(O'Donnell, *et al* 2002b)

1.5 MANAGEMENT OF VWD

The primary aim of treatment of VWD is to correct the dual defect of haemostasis, namely the platelet adhesive defect and the low level of FVIII. There are currently two main therapeutic options: administration of DDAVP or replacement therapy with plasma-derived concentrates.(Mannucci 2004)

1.5.1 DDAVP

Historical background

In 1772, it was reported by William Hewson that blood clotted rapidly when collected under conditions of stress.(Hewson 1846) Over two centuries later, rapid blood clotting associated with stress was shown to result from the release of adrenaline,(Cannon 1914) which was subsequently explained by the finding of a transient increase in FVIII in rabbits following the administration of adrenaline.(Marcianiak 1957) Reports of raised FVIII following adrenaline infusion in humans soon followed with around two fold increases of FVIII being demonstrated in both haemophilic patients and normal subjects, with no measurable change in other known clotting factors.(Ingram 1961) Investigation of other hormones led to the observation that the antidiuretic hormone vasopressin also led to increased FVIII levels(Mannucci 1972) and the similar effect of its analogue 1-deamino-8-D-arginine vasopressin (DDAVP) was subsequently described.(Cash, *et al* 1974) DDAVP had less vasoactive properties than vasopressin and was used for the first time to treat patients with haemophilia A and VWD in 1977.(Mannucci, *et al* 1977)

Mechanisms of action

DDAVP results in a two to three fold rise in FVIII and VWF levels, with the release of UL VWF multimers that potentiate haemostasis by enhancing the platelet-vessel wall interaction.(Ruggeri, *et al* 1982) DDAVP induces the release of stores of both mature VWF and VWFpp from the Weibel-Palade bodies of endothelial cells via the regulated pathway as discussed in Section 1.2 .2.(Borchiellini, *et al* 1996, Mannucci, *et al* 1976, Tsakiris, *et al* 1995, van Mourik, *et al* 1999) DDAVP is a specific agonist for the vasopressin V2 receptor (V2R) and is known to exert its antidiuretic effect via a rise in cAMP in kidney collecting ducts. However, the cellular mechanism of the haemostatic effects of DDAVP is not clear. Injection of DDAVP into rats has been shown to elicit biological responses that are clearly related to the activation of endothelial cells, including the surface expression of P selectin and subsequent marginalisation of leukocytes.(Kanwar, *et al* 1995) Immunohistochemical

examination of endothelial cells *in vivo* following DDAVP shows a reduction and altered localisation of VWF, with a tendency for the protein to move abluminally towards the cellular basement membrane.(Takeuchi, *et al* 1988) DDAVP has been shown to stimulate VWF secretion in a cAMP-dependent manner in cultured human umbilical vein endothelial cells (HUVEC) following transfection by the V2R.(Kaufmann, *et al* 2000, Moffat, *et al* 1984) Furthermore, V2R mRNA has been demonstrated in human lung microvascular endothelial cells vasopressin.(Kaufmann, *et al* 2000)

Although it was previously hypothesised that the rise in FVIII following DDAVP resulted from its stabilisation in plasma secondary to the release of VWF,(Mannucci and Cattaneo 1992) the finding of comparable FVIII release following DDAVP in type 2N VWD,(Mazurier, *et al* 1994) is suggestive of release of FVIII from as yet unidentified cellular stores. Neither FVIII nor VWF have been shown to be released following chronic administration of exogenous VWF to patients with type 3 VWD, confirming that endogenous synthesis of both VWF and FVIII is required for DDAVP-induced release of FVIII. Furthermore, patients with haemophilia who have undergone liver transplantation for end-stage liver disease have been shown to demonstrate a response in VWF, but not FVIII to DDAVP. (Lamont and Ragni 2005) In contrast, there was a response in both VWF and FVIII in a non-haemophilic transplant recipient. These findings suggest that intracellular co-localisation of FVIII and VWF is necessary for *in vivo* FVIII secretion after DDAVP. Although the storage site(s) of FVIII is not well established, at least some is likely to be released from sinusoidal endothelial cells. In addition, FVIII has been shown to be synthesised in the lung microvascular endothelium(Jacquemin, *et al* 2006)and the co-expression of FVIII and VWF in microvascular endothelial cells has been demonstrated in lung tissue, the significance of which is not yet clear

Use in treatment of VWD

Despite the widely held view that the majority of type 1 VWD patients are responsive to DDAVP, with reported response rates as high as 90%,(Nolan, *et al* 2000) recent results from a multicentre study have suggested that response rates may be much lower, with only 27% of type 1 VWD patients being shown to respond.(Federici, *et al* 2004) The rate of response to DDAVP partly depends upon how response is defined: in the former study, the correction of VWF:Ag, VWF:Ac and FVIII:C into the normal range 30 min following DDAVP infusion constituted a response.(Nolan, *et al* 2000) However, for response to be achieved in the latter study, the attainment of a minimum three fold increase of FVIII:C and VWF:RCo to levels of at least 30 IU/dL and a bleeding time (BT) of less than 12 min was required 2 h after DDAVP

infusion.(Federici, *et al* 2004) The different time intervals used in these studies is likely to account for some of the discrepancy in response rates: as expected, FVIII and VWF:RCo were found to peak at 30 min in the second study and response rates would therefore have been higher had this timepoint been employed. The low proportion of responsive type 1 patients in the latter study may also reflect the severe phenotype of the recruited patients as well as the misclassification of around one third of this patient cohort who showed low VWF:RCo/Ag ratios and normal multimer patterns consistent with type 2M disease.

The heterogeneity of type 2 VWD necessitates consideration of the specific VWF functional defect to determine appropriate treatment: although it has been reported that patients with the subtype 2A show a variable response to DDAVP,(Gralnick, *et al* 1986) none of the type 2A patients in the aforementioned multicentre study were found to be responsive, regardless of the underlying mutation and mechanism of VWF dysfunction.(Federici, *et al* 2004) Other studies have shown no increase in circulating HMW multimers following DDAVP in type 2A disease despite satisfactory increases in VWF and FVIII.(Ruggeri, *et al* 1982) Opinion on the use of DDAVP in type 2B VWD is divided: some clinicians avoid it due to the risk of transient thrombocytopenia and potential adverse events associated with the formation of platelet aggregates,(Federici 2006, Holmberg, *et al* 1983) while others report successful treatment with DDAVP in these patients.(Casonato, *et al* 1990, Fowler, *et al* 1989) Although good responses to DDAVP have been demonstrated in patients with type 2N VWD irrespective of their causative mutation,(Mazurier, *et al* 1994)other data suggest response is limited to patients with the most common mutation, 2561G>A (R854Q).(Federici, *et al* 2004) Because type 3 VWD patients lack releasable stores of VWF, they do not respond to DDAVP,(Ruggeri, *et al* 1982) although DDAVP can be used as an adjunct in these patients.

The obvious advantages of DDAVP include cost and the elimination of risk of blood-bourne pathogen transmission. DDAVP can be administered by intravenous, subcutaneous or intranasal routes at a dose of 0.3 μg kg^{-1}. Respective peak values occur at 30-60 min following intravenous and 90-120 min after subcutaneous and intranasal treatment,(Mannucci 1997)and this response has been shown to be sustained for several hours.(Mannucci 1982) Despite consistency in the response of a given patient on different occasions,(Rodeghiero, *et al* 1989) the therapeutic effect in not predictable in an individual patient and it is recommended that the response to DDAVP is measured in all patients at diagnosis and/or two weeks prior to surgery. The current UK guidelines recommend a trial of DDAVP be performed in patients with types 1 (where baseline VWF levels > 10 IU dL^{-1}), 2A and 2M VWD, and

considered in 2N and 2B disease.(Pasi, *et al* 2004) DDAVP infusions can be repeated every 12 - 24 h depending on the type and severity of the bleeding.(Mannucci, *et al* 1977) However, tachyphylaxis can develop in some patients,(Mannucci, *et al* 1992) and in general, treatment with DDAVP can be usefully repeated between two and four times. The commonest side-effects of DDAVP are facial flushing, headache and tachycardia. Hyponatraemia due to the antidiuretic effect of DDAVP is unusual if excess fluid intake is avoided. In view of the release of UL-VWF multimers, DDAVP is contra-indicated in ischaemic heart disease, although substantiated evidence is lacking.

There are currently no clear objective criteria that define a response to DDAVP. The recommendations in the UK guidelines state that the definition of an adequate response will depend, in part, upon the indication for treatment.(Pasi, *et al* 2004) Although FVIII:C is commonly used as a surrogate marker of the clinical efficacy of DDAVP, it has been shown to be an unreliable guide in VWD patients.(Michiels, *et al* 2002, Nolan, *et al* 2000) The UK guidelines recommend the measurement of both VWF and FVIII levels and in addition to measuring response to DDAVP suggest that levels are repeated at 3 – 6 h post DDAVP to ensure that there is not a rapid fall off in levels.(Pasi, *et al* 2004) Prospective studies on the biological response versus clinical efficacy of DDAVP in VWD types 1 and 2 are in currently in progress.

1.5.2 Plasma concentrates

The second treatment option for VWD relies on the administration of blood products containing concentrated VWF and FVIII and is used in patients unresponsive to DDAVP or in whom DDAVP is contra-indicated. For many years, cryoprecipitate was the mainstay of VWD treatment. Several intermediate-purity and high-purity VWF/FVIII concentrates are now available and have been shown to be effective in both bleeding and surgical settings.(Mannucci 2004) The relative concentrations of VWF and FVIII vary between preparations,(Lethagen, *et al* 2004) and as with any plasma-derived concentrate, there is a risk of transmission of blood-bourne pathogens. Although thrombotic events are rare in VWD patients receiving repeated infusions of concentrates, there is concern that sustained high FVIII levels may increase risk of postoperative venous thromboembolism.(Federici 2006) New VWF products almost devoid of FVIII, as well as recombinant VWF are currently under evaluation.

1.5.3 Other therapies

Other treatments that can be used either alone or adjunctively in VWD include antifibrinolytic agents (mainly tranexamic acid), platelet concentrations and oral oestrogen-progestogen preparations. During recent years initial steps in the development of a gene-based therapy for severe VWD have been taken.(De Meyer 2007, Pergolizzi, *et al* 2006)

Chapter 2

INITIAL AIMS

2.1 OVERVIEW OF PREVIOUS STUDIES OF VWF CLEARANCE

Despite the 1994 classification of VWD being based upon the assumption that all VWD arose from mutations of the *VWF* gene,(Sadler 1994) by the time of this study it was becoming increasingly accepted that a partial quantitative deficiency of VWF may represent a complex genetic trait.(Sadler 2002) In contrast to other VWD types, very few mutations in *VWF* had been linked to type 1 VWD.(Allen, *et al* 2000, Bodo, *et al* 2001, Eikenboom, *et al* 1996) Although some type 1 VWD phenotypes had been linked to defects within *VWF*, others were not shown to cosegregate with genetic markers at the *VWF* locus.(Casana, *et al* 2001, Castaman, *et al* 1999) Furthermore, variation in resting VWF levels in obligate heterozygous carriers of VWD type 3 alleles had been demonstrated.(Sadler 2002) Other than ABO blood group, factors contributing towards the genetic variability in VWF levels were largely unknown. The increasing recognition of modifiers of plasma levels of VWF that were independent of *VWF* supported the view that while some case of type 1 VWD may result from mutations within *VWF*, the remainder could result from the mutation of a gene separate from *VWF* or the interaction of several genes. Type 1 VWD was therefore becoming more widely viewed as a continuum, with low levels of VWF associated with a bleeding phenotype resulting from heterogeneous pathogenic mechanisms.

The half-life of VWF following the administration of the vasopressin analogue DDAVP was studied during the early development of DDAVP as a therapeutic agent.(Mannucci, *et al* 1977) Initial observations suggested that there was no significant difference in the half-lives of VWF released following DDAVP infusion between patients with 'classical' VWD and either a normal control group or individuals with mild haemophilia A.(Mannucci 1982) However, the rapid return to baseline of plasma FVIII:C, VWF:Ag and VWF:RCo following DDAVP infusion in type 1 VWD patients was subsequently observed,(de la Fuente, *et al* 1985) and half-life values for these parameters were found to be reduced in type 1 VWD subjects.(Rodeghiero, *et al* 1988) Latterly, the half-life of VWF released following DDAVP was determined in patients with type 1 VWD as well as mild haemophilia A patients and normal controls.(Brown, *et al* 2003, Michiels, *et al* 2002, van Genderen P. J. J 1997) These studies showed significant reductions in VWF half-life in a proportion of patients with type 1 VWD and it was proposed that increased plasma clearance of VWF may contribute towards the pathogenesis of type 1 VWD in some patients.(Brown, *et al* 2003)

Increased steady-state plasma ratios of VWF propeptide (VWFpp) to the mature VWF protein were reported in some patients with VWD, in particular in patients with type 2A VWD in whom increased sensitivity to proteolysis had been demonstrated.(de Romeuf and Mazurier 1998) However this ratio was not found to reliably discriminate between types 1 and 2 VWD, consistent with the theory that increased VWF clearance may also underlie some cases of type 1 VWD. Few studies had examined VWFpp in type 1 VWD.

No common mechanism had been identified that could account for the increased clearance of VWF observed in some patients with type 1 VWD following DDAVP. Clearance mechanism(s) of VWF were poorly defined, although data supportive of both receptor mediated and proteolytic-based mechanisms had been published.(Batlle, *et al* 1987, Bowen 2003, Casonato, *et al* 2001a, Stoddart, *et al* 1996, van Genderen P. J. J 1997) Defective proteolysis by the metalloprotease ADAMTS-13 had been characterised as the pathogenic mechanism in thrombotic thrombocytopenic purpura.(Moake, *et al* 1982) However, little was known about the possible effects of increased ADAMTS-13 proteolysis on circulating VWF levels, and no studies had been published examining the relationship between proteolysis of VWF by ADAMTS-13 and VWF plasma clearance.

It was not known whether increased VWF clearance in humans could result from either alteration in the primary structure of VWF or its post-translational modifications, including glycosylation as demonstrated in the RIIIS/J murine model. There was a paucity of mutational data in type 1 VWD and no studies had been published examining the molecular basis of VWF clearance. Data from the 1970s had demonstrated binding of asialo-VWF to the hepatic ASGPR and its subsequent rapid removal from the circulation in animal studies,(Sodetz, *et al* 1978, Sodetz, *et al* 1977) suggesting that exposed galactose residues may be required for the binding of VWF to the ASGPR. This was supported by the demonstration of increased clearance of endogenous VWF in a murine model in the absence of the sialyltransferase ST3GalIV, an enzyme that mediates attachment of sialyl groups to terminal galactose residues. The finding that VWF released from endothelial storage organelles following DDAVP infusion differed from circulating VWF in terms of multimer composition and expression of A antigen (Brown, *et al* 2002, Ruggeri, *et al* 1982) suggested that there may be other structural differences between stored and circulating VWF, which could include other variations in glycosylation pattern. While it had been proposed that the ABH glycans may influence the rate of VWF clearance,(Mohlke, *et al* 1999b, O'Donnell, *et al* 2002a, Vlot, *et al* 2000) no direct

examination of the effect of ABO blood group on VWF clearance had been performed. Furthermore, no studies had investigated the influence of exposure of galactose residues of VWF on VWF clearance in humans.

2.2 INITIAL AIMS AND APPROACH

The aim of this study was to investigate whether increased VWF clearance is a possible pathogenic mechanism in type 1 VWD, and to identify determinants that may affect it. The plan of investigation was to recruit patients with type 1 VWD, to measure steady-state levels of VWF and related parameters, and to stimulate VWF release from the endothelial pool by infusion with DDAVP. The half-life of the released VWF would be determined, structural features, such as VWF glycosylation and collagen binding ability investigated and susceptibility to cleavage and ADAMTS-13 levels determined. Thus criteria that reflect and may directly affect VWF life-cycle and clearance would be monitored. Discrete areas of the *VWF* gene were to be sequenced in order to identify causative mutations underlying the VWD. A control group of patients with haemophilia A was chosen, as by definition these patients would have normal VWF synthesis and structure.

This approach has the limitation that VWF clearance being examined in both patient and control groups is not VWF released by constitutive secretion, but rather the stored pool in Weibel Palade bodies of endothelial cells. However, this approach was safe and involved the study of human clearance systems, including proteolysis and clearance receptors. In addition, valuable information for individual patient treatment would be gathered.

Chapter 3

STANDARD MATERIALS AND METHODS

3.1 PATIENT MATERIAL

Patients and controls studied were registered at the Katharine Dormandy Haemophilia Centre, Royal Free Hospital. All patients had been previously diagnosed with VWD and controls with mild Haemophilia A. Patient material was collected with informed patient consent and the study was conducted with ethical committee approval.

The diagnosis of type 1 VWD was based upon a personal and family history of bleeding, and a concordant reduction in VWF:Ag level and VWF:RCo activity in accordance with the 1994 ISTH recommendations.(Sadler 1994) Genotypic analysis had not been performed previously. Phenotypic data of the 43 type 1 VWD and 17 Haemophilia A patients investigated are shown in Appendix 2. Three of the recruited VWD patients were subsequently excluded from the study (Patients 3, 20 and 43) based on the finding of abnormal ratios of VWF:CB to VWF:Ag (< 0.7), which is not consistent with 'a normal VWF which is quantitatively decreased'.(Sadler 1994) These patients are further investigated in Chapter 8.

The diagnosis of type 2M VWD was based on a personal and family bleeding history, reduced VWF:Ag and VWF:RCo with a discordant VWF:RCo/VWF:Ag ratio of <0.7 in the presence of HMW VWF multimers.(Federici 1998) Data for these patients is presented in Chapter 9.

Whole blood samples were collected by venepuncture into 1 mL trisodium citrate, 0.106 $molL^{-1}$ (Sarstedt Monovette, Germany) in a ratio of 1 part anticoagulant to 9 parts blood for phenotypic analysis. Plasma samples were double centrifuged at 2000g, separated and stored at –80°C until assayed.

3.2 COAGULATION INVESTIGATIONS

Initial investigations and resulting diagnoses were in general performed by laboratory staff at the Haemophilia Centre, Royal Free Hospital. Further coagulation tests prior to and over 6 h following infusion of DDAVP were performed by myself and Ms.Anne Riddell, Senior Research and Development Scientist.

3.2.1 Chemicals and Reagents

Common chemicals were supplied by BDH Chemical Ltd, and Sigma-Aldrich Chemical Company, Dorset, UK unless otherwise stated.

3.2.2 von Willebrand factor antigen (VWF:Ag) ELISA

Chemicals and Reagents

1. Rabbit anti-human VWF antibody (Dako, Cambridgeshire, UK).
2. Horseradish peroxidise (HRP)-conjugated rabbit anti-human VWF antibody (Dako, Cambridgeshire, UK).
3. Pooled normal plasma: Cryocheck (Precision Biologic, Canada) calibrated against 9th British Standard reference plasma with known [VWF] (NIBSC, Potters Bar, UK)
4. Abnormal reference plasma: Trol-P (Dade Behring. Germany)
5. Bicarbonate coating buffer 0.05 M, pH 9.6
    a. Na_2CO_3 0.79 g
    b. $NaHCO_3$ 1.47 g
    c. Distilled H_2O 500 mL
6. Wash buffer: Phosphate Buffered Saline (PBS) with Tween 20 (Tw 20), 0.2%, 0.155M, pH 7.0 (10 x working strength)
    a. $NaH_2PO_4.2H_2O$ 1.95 g
    b. $Na_2HPO_4.12H_2O$ 13.4 g
    c. NaCl 141.1g
    d. 10 mL Tween 20
    e. Distilled H_2O 5 L
7. Dilution buffer: 15 g Polyethylene glycol (PEG) 8000 in 500 mL working strength PBS-Tw20
8. Substrate buffer, 0.1 M, pH 5.0
    a. Citric acid 3.65 g
    b. $Na_2HPO_4.12H_2O$ 11.94 g
    c. Distilled H_2O 500 mL
9. O-Phenylenediamine (OPD) tablet: 10 mg
10. 1.5 M H_2SO_4
11. Patient platelet poor plasma
12. 30% Hydrogen Peroxide (BD, UK)

Equipment

1. Maxisorb 96 well microtitre plates (NUNC; Life Technologies, Paisley, UK)
2. Sealing film
3. Plate washer (Dynex MRW, West Sussex, UK)
4. Plate reader (Dynex MRX, West Sussex, UK)
5. Gilson 401 dilutor
6. Plate shaker (Amersham International plc, Bucks, UK)
7. Pipettes and tips
8. Waterbath 37°C

Method

The 96 well plate was coated with the rabbit anti-human VWF antibody, diluted 1/1000 in 0.05 $molL^{-1}$ carbonate/bicarbonate buffer (pH 9.6) and 100 μL of the diluted coating antibody was added to each well of the plate. The plate was then sealed and incubated at 4 °C overnight. The plate was washed five times with the wash buffer, then inverted and gently blotted on absorbent paper.

The standard curve consisted of dilutions of pooled normal plasma (Cryocheck) from 1/80 to 1/1600. The patient plasma samples and reference samples were diluted 1:100 and 1:200 in dilution buffer. Each dilution was dispensed in duplicate, with 100 μL of sample per well. The plate was sealed and incubated on a plate shaker (400-500 oscillations per minute) at room temperature for one hour, and then washed five times with the wash buffer. The HRP-conjugated rabbit anti-human VWF antibody was diluted 1/8000 in dilution buffer, and 100 μL was added to each well. The plate was again sealed and incubated for one hour at room temperature on the plate shaker. Prior to the end of incubation the substrate solution was prepared by dissolving one OPD tablet in 15 mL of substrate buffer. This was brought to room temperature in a waterbath during which time the plate was washed for a final five times. Immediately prior to use, 7 μL of 30% Hydrogen Peroxide was added to the substrate solution. 100 μL of substrate was added to each well and the plate was incubated on the bench for 8-10 min to allow optimal colour development. The reaction was terminated by adding 100 μL of 1.5 M H_2SO_4 to each well at the same time interval as the substrate was added and gently shaking the plate to mix.

The plate optical density (OD) at 490 nm was then read on a spectrophotometric plate reader. The standard curve was derived from a plot of the OD_{490nm} against VWF concentration on a semi-log plot. The 1/100 dilution of the reference plasma

was taken as 100%. The VWF:Ag levels of the samples were extrapolated from the standard curve and the mean values were calculated. The coefficient of variation (CV) was < 5%.

3.2.3 Ristocetin Co-factor activity (VWF:RCo)

Chemicals and Reagents

1. Lyophilised platelets (Alpha Laboratories, UK) reconstituted with 4.0 mL Tris Buffered Saline (TBS)
2. Ristocetin A SO_4 (Lundbeck, Germany)
3. Distilled H_2O
4. Citrate saline diluent: 2 mL 33.3 gL^{-1} sodium citrate solution made up to 20 mL with sodium chloride 0.9% (Baxter, UK)
5. Pooled normal plasma: Cryocheck (Precision Biologic, Canada) calibrated against 9th British Standard reference plasma with known [VWF] (NIBSC, Potters Bar, UK)
6. Abnormal reference plasma (Dade Behring. Germany)/ SYSMEX Trol-P
7. 9^{th} British Standard reference plasma (NIBSC, Potters Bar, UK) with known VWF:RCo: reconstituted with 1mL distilled water
8. Patient platelet-poor plasma

Equipment

1. PAP-4 Aggregometer (Alpha Laboratories, UK)
2. Pipettes
3. Plastic coated stir bars (Alpha Laboratories, UK)
4. Glass tubes (Alpha Laboratories, UK)

Method

Doubling dilutions of standard (Cryocheck) were prepared from 1/2 to 1/32 for use as the standard curve. Normal and abnormal controls were prepared using reconstituted British standard and SYSMEX Trol-P respectively and prepared at dilutions of 1/2 and 1/4 in citrate saline. Patient samples were thawed and initial dilutions of 1/4 in citrate saline were prepared. The assay was performed on a PAP-4 platelet aggregometer connected to a pen recorder. The 100% baseline was calibrated as 125 μL lyophilised platelets + 125 μL TBS. Cuvettes containing 200 μL lyophilised platelets and 25 μL ristocetin were prepared and inserted into the test well and the test baseline (0%) set for each of the four channels. 25 μL plasma

dilutions of standard or test was added to each tube. Following completion of agglutination, the reading was stopped (180 secs). The standard curve was plotted on log-linear paper (2 cycles) with agglutination in divisions on the linear scale and U/dL co-factor on the log scale. The patient VWF:RCo was read off the standard curve.

3.2.4 Factor VIII:C (One stage clotting assay: Semi-automated)

Chemicals and Reagents

1. Owren's buffered saline (OBS), pH 7.35:

Owren's Barbiturate Buffer	200 mL
NaCl (0.9%)	800 mL

Owren's Barbiturate Buffer:

a.	Sodium Diethylbarbiturate (Barbitone Sodium)	5.88 g
b.	Sodium Chloride	7.34 g
c.	1 M Hydrochloric Acid	21.5 mL
d.	Distilled H_2O	1 L

2. Lyophilised FVIII deficient plasma (Technoclone, UK) reconstituted with 1 mL distilled H_2O
3. Patient platelet-poor plasma
4. Pooled normal plasma: Cryocheck (Precision Biologic, Canada) calibrated against 9th British Standard reference plasma with known [FVIII] (NIBSC, Potters Bar, UK)
5. APTT Lyophilised silica (Instrumentation laboratories, Warrington, UK): 1 L reconstituted with 9 mL distilled H_2O.
6. 25 mM $CaCl_2$

Equipment

1. ACL 3000 (Instrumentation Laboratories, Warrington, UK)
2. Gilson 401 dilutor
3. ACL cuvettes
4. Pipettes and tips
5. Waterbath 37°C

Method

The samples and standard were prepared in OBS: samples were diluted 1/10 – 1/40 and the standard 1/10. The reconstituted FVIII-deficient plasma, APTT reagent and $CaCl_2$ were placed in the ACL 3000 and one cuvette per sample

dilution were placed consecutively from position 1. In addition, one sample cuvette was placed in 'pool' and 'dil' positions: diluted (1/10) standard was added to the cuvette in 'pool' position and OBS to the 'dil' position. The diluted patient samples were added to each cuvette in turn (minimum 250 μL). The *Single Factor* option was selected for FVIII followed by *High Curve* and the value of the standard was entered. A fresh rotor was place in the ACL Rotor compartment, the sample numbers entered onto a loadlist and the run commenced. The results were calculated automatically.

3.3 VON WILLEBRAND FACTOR MULTIMER ANALYSIS

Chemicals & Reagents

1. Electrode Buffer, pH = 8.45
   - a. 100 mM Tris 60.55 g
   - b. 15 mM Glycine 22.52 g
   - c. 1 g/L SDS 50 mL @ 10%
   - d. Distilled H_2O 4950 mL

   NB Dilute 1:2 for anode
2. Sample Buffer, pH 6.7
   - a. 70 mM Tris 0.085 g
   - b. 4 mM EDTA 0.015 g
   - c. 24g/L SDS 0.24 g
   - d. Urea 0.54 g
   - e. Distilled H_2O 10 mL
3. Separating gel buffer, pH 9.6
   - a. 200 mM Tris 6.05 g
   - b. 100 mM Glycine 1.88 g
   - c. 4 g/L SDS 10 mL @10)%
   - d. Distilled H_2O 250 mL
4. Stacking gel buffer, pH 6.7
   - a. 70 mM Tris 2.118 g
   - b. 4 mM EDTA 0.373 g
   - c. 4 g/L SDS 10 mL @ 10%
   - d. Distilled H_2O 250 mL
5. Transfer Buffer: PBS (3.2.2)

6. Tween Tris Buffered Saline (TTBS), pH= 7.5
   a. Tris Base 60.55 g
   b. NaCl 45 g
   c. Distilled H_2O 5000 mL
   Adjust pH with HCL
   d. Tween 20 10 mL
7. 0.1 M Tris HCL (Ph = 9.5)
   a. Tris base 1.21 g
   b. Distilled H_2O 100 mL
   Adjust pH with 0.1 M HCL
8. Rabbit anti-human VWF antibody (Dako, Cambridgeshire, UK).
9. Vectastain Kit - Alkaline phosphatase Rabbit IgG (Vector Laboratories, UK) AK5001
10. Alkaline phosphatase substrate kit IV BCIP/NBT (Vector Laboratories, UK) SK 5400
11. Skimmed Milk Powder (MARVEL) Chivers, Ireland Ltd, Dublin, Ireland

Equipment

1. Gel Tray
2. Glasswear
3. BIORAD Electrophoresis Tank
4. Power Pack
5. 2 x 3 MM wick (Whatman Gel Blot Paper, Fisher Scientific, UK)
6. Cotton Lint
7. Cling Film
8. Whatman 3MM chromatography paper
9. Tissues
10. Nitrocellulose Paper (Hybond C)
11. Perspex Tray for Blotting
12. Thick glass plate
13. Orbital Mixer
14. Weight
15. ImageStore 7500

Method *(Ruggeri, et al 1982)*

1. Prepare gel tray and pre-warm in incubator at 55°C.
2. Preparation of Separating Gel
   Dissolve 0.60 g agarose in 40 mL Separating Gel Buffer (1.4%) by bringing to boil, allow to cool to 55°C and set in tray at 4°C for a minimum of 1 h.

3. Preparation of Stacking Gel:
   Remove a 18 mm strip of agarose from the top edge of the separating gel. Dissolve 0.16 g agarose in 20 mL Stacking Gel Buffer (0.8%). Allow stacking gel to cool to 55°C then, using the 10 mL syringe, add 7.5-8.0 mL into the separating gel. Insert the comb allowing a 1.5mm gap between the bottom of the comb teeth and the gel tray. Leave to set for minimum 20 min.
4. Fill the electrophoresis tank, reservoirs with electrode buffer, dilute buffer 1:2 or anode
5. Gently remove the comb and tape from the gel and place the gel into the electrophoresis tank.
6. Position 3 x3 MM wicks on each end of the gel, ensuring full contact across the width of the gel.
7. Prepare the controls and samples by adding 10 μL sample to 90 μL sample buffer. Vortex briefly to ensure mixing
8. Add 20 μL of appropriate sample to each well. Switch unit to voltage and run at 280V to run the samples out of the wells. Switch to Amps and reduce to 0.02A
9. Preparation of Blot:
   1 tray
   1 Gel tray
   2x3MM 3 sided wick
   exclusion frame
   Nitrocellulose paper
   2x3MM wick
   10 cm stack of blue towelling
   glass plate
10. After electrophoresis remove the gel and briefly wash in transfer buffer before inverting onto capillary blotting unit.
11. Leave the blotting unit overnight, take down blot carefully, remove nitrocellulose paper and block with 100 mL milk solution (minimum 15 min)
12. Decant the milk solution and wash in 100 mL TBS (10 min)
13. Decant the PBS and carefully roll nitrocellulose paper, insert into wide necked 500 mL bottle, Carefully unroll the paper ensuring there are no air bubbles between the paper and bottle
14. Wash again with 50 mL TBS for 5 min
15. Decant TBS and add first antibody to the front of the membrane: 80 μL anti-VWF in 100 mL PBS; incubate for 3 h.
16. Decant and wash with 50 mL TBS for 5 min X 3

17. From Vectastain kit, for each gel dilute 66 μL of biotinylated anti-rabbit antibody in 20 mL of TTBS.
18. Add to 500 mL bottle containing blot and incubate for 2 h.
19. Decant and wash with 50 mL TBS for 5 min X 3.
20. From the Vectastain kit, add 3 drops of reagent A to 15 mL of TTBS, then add 3 drops of reagent B. Incubate the membrane in reagent A+B for 30 minutes on the orbital mixer at room temperature.
21. Wash 3Xs in TTBS
22. Carefully re-roll nitrocellulose paper and remove from bottle, unroll into tray
23. Prepare colour reagents: Vectastain Kit.
24. Visualisation: Using the alkaline phosphatase substrate kit, for each membrane make up the substrate solution as shown.
25. 5 mL 0.1 mL 0.1 M Tris HCl. Add 3 drops of solution reagent 1, 2 & 3. Mix well and then pour onto the nitrocellulose blot.
26. Allow to develop so that multimeric pattern can be seen.
27. Wash with distilled water
28. When developed dry flat on absorbent paper.
29. Make a permanent record by photographing using the photocopier (Fig. 8.1)

3.4 GENOTYPIC ANALYSIS

3.4.1 Preparation of deoxyribonucleic acid (DNA)

Chemicals and Reagents

1. Cell lysis buffer (CLB):

Sucrose	110 g
Triton x 100	10 mL
1M $MgCl_2$	5 mL
Tris-HCl 1M	10 mL
Deionised H_2O:	to 1000 mL

2. TE Buffer, pH 8.0
   10mM Tris pH 7.6
   1mM EDTA pH 8.0

3. Nuclear lysis buffer (NLB):

Lithium acetate	3.5 g
Lithium dodecylsulphate	2 g
Disodium EDTA 0.1M pH 8.0	1 mL
Tris-HCl 1M	1 mL
Distilled H_2O:	to 100 mL

4. Phenol:Chloroform:Isoamylalcohol (25:24:1)
5. Chloroform
6. Ice cold ethanol

Equipment

1. Standard pastettes
2. 10 mL capped polypropylene tubes
3. 50 mL Falcon tubes
4. Sealed glass Pasteur pipettes
5. 1 mL Sarsted screw top tubes and caps
6. Benchtop centrifuge
7. Rotary mixer

Method

Buffy coats from 10 mL whole blood were thawed at room temperature and decanted into a 50 mL Falcon tube. 50mL CLB (stored at 4°C) was added and the tube that contained the buffy coat was rinsed with CLB to remove adherent cells. The Falcon tube containing buffy coat and CLB was mixed and placed on ice for 20 min. It was then centrifuged at 2400 rpm for 20 min. The supernatant, containing the lysed red blood cells was discarded. The white blood cell pellet was re-suspended in 1 mL TE pH 8.0 and to this, 2 mL NLB was added and mixed on an orbital shaker for 5 min. The sample was then transferred into 10 mL polypropylene tube and 1 mL phenol:chloroform:isoamylalcohol was added. Following ten inversions of the tube to mix, the sample was spun at 2400 rpm for 5 min. The aqueous layer was removed to a fresh tube and 1 mL chloroform was added. This was again mixed and centrifuged. The aqueous layer was removed and the DNA precipitated by adding 2.5 volumes of absolute ethanol (stored at -20 °C) and slowly inverting the tube. The DNA was collected onto a sealed Pasteur pipette and resuspended in a Sarsted tube for 24 h in 250-500 µL TE buffer. Genomic DNA was then ready to use.

3.4.2 Quantification of DNA

Equipment

1. Glass microcapillary tubules
2. GeneQuant pro spectrophotometer

Method

The spectrophotometer was zeroed using a microcapillary with TE buffer prior to drawing up the DNA sample. The absorbance of the DNA sample was read at 260 nm and 280 nm. The ratio of the absorbance at 260 nm to that at 280 nm was used to give an indication of the purity of the DNA.

3.4.3 Polymerase chain reaction (PCR)

(For amplification of exon 28 (28B) encoding A2 domain)

Chemicals and Reagents

1. 10 x NH_4 reaction buffer*
2. 1.25 mM dNTPs (Helena Biosciences, Sunderland, UK)
3. 50 mM $MgCl_2$*
4. 20 mM primer mix**
5. Bio*Taq* polymerase buffer (Bioline, London, UK)
6. Sterile H_2O

**supplied with Taq*

*** PRIMERS FOR AMPLIFICATION OF 682 bp product of EXON 28*: primers K2A and K1B

forward: 5'TGGTTCTGGATGTGGCGTTC3'

reverse: 5'TCTTGGCAGATGCATGTAGC3'

Equipment

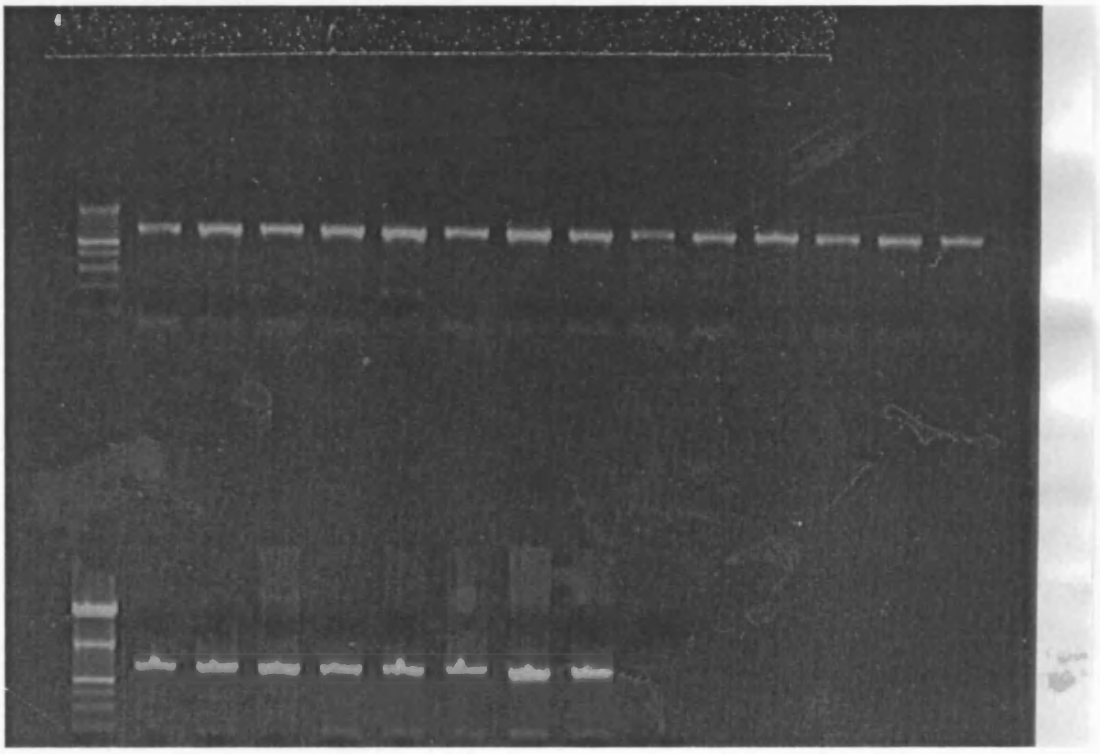
GeneAmp PCR system 9700 (Applied Biosystems, Warrington, UK)

Eppendorf tubes

Genomic DNA was amplified in a 50 μL mixture containing: 5 μL 10 x NH_4 reaction buffer, 5 μL 1.25 mM dNTPs, 1.5 μL 50 mM $MgCl_2$, 1 μL primer mix, 0.25 μL Bio*Taq* polymerase buffer and 35.25 μL sterile H_2O. A megamix was prepared with the primers, dNTPs, polymerase buffer and water to a volume calculated on the total number of PCR reactions. Finally the Bio*Taq* polymerase was added to the megamix (from ice), which was aliquotted into 48 μL samples in 0.5 mL Eppendorf tubes, to which 2 μL of DNA was added and mixed/centrifuged.

40 cycles of PCR (94 °C/30 sec; 58 °C/30 sec; 72 °C/30 sec) were performed in GeneAmp PCR system 9700 thermal cycler. These were preceded by 5 min incubation at 94°C and followed by 10 min incubation at 72°C. Each PCR experiment included a 'no template control'. PCR products (5 µL) were electrophoresed with gel-loading buffer (2 µL) at 100V for 1 h through the agarose gel (1.5% w/v) and visualised under UV light using ethidium bromide staining (Fig.3.1).

Figure 3.1 Products obtained following PCR amplification of exon 28B of *VWF* gene using primers K2A and K1B. PCR products were electrophoresed at 100 V for 1 h through a 1.5% w/v agarose gel and visualised under UV light with ethidium bromide staining.
Lane 1 of each row: marker (MXIV, Roche Diagnostics, Mannheim, Germany), Lanes 2 – 15: genomic DNA, lane 10 of lower row: negative control (blank).



Lane:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

3.5 MEASUREMENT OF PLASMA CLEARANCE OF VWF FOLLOWING ADMINISTRATION OF DDAVP

3.5.1 Principle of method used to determine VWF:Ag half-life (Somosgyi 1999)

VWF:Ag levels are measured at baseline and and at hourly time intervals over 6 h following DDAVP. The baseline VWF:Ag level is subtracted from the subsequent VWF:Ag levels and linear regression analysis is used to determine the slope of the semi-logarithmic plot of VWF:Ag against time. From this slope, the first-order rate constant for the elimination of released VWF **(k)** is derived, based on the formula $\mathbf{C_t = C_0 e^{-kt}}$, which describes the changes in concentration of a substance with a clearance demonstrating first-order kinetics. C_t = plasma concentration of VWF:Ag at time point t after DDAVP infusion, C_0 = concentration of VWF at time O, *e* = base for natural logarithms.The apparent half-life of VWF:Ag (VWF:Ag $t_{1/2}$) is then calculated from the equation: $\mathbf{t_{1/2} = \ln 2/k}$.

Calculation of the VWF:Ag $t_{1/2}$ by this method assumes the following:

1. VWF clearance demonstrates first order kinetics.(Mannucci 1982)
2. The lack of an extravascular compartment for VWF.(Meriane, *et al* 1993)
3. A distribution phase of VWF of one hour and therefore the VWF:Ag level 1 h following DDAVP represents the beginning of the elimination phase.

This method was selected to determine VWF:Ag $t_{1/2}$ for two reasons. Firstly, it is an accepted and published method of calculating the VWF:Ag $t_{1/2}$, which enables comparison of the results from this study to be made with previously published data.(Brown, *et al* 2003, Mannucci 1982, Michiels, *et al* 2002, van Genderen P. J. J 1997) Secondly, unlike pharmacokinetic data in severe factor deficiencies, the determination of the baseline VWF:Ag level is problematic due to variability in the steady-state VWF level over a protracted time period. In a pharmacokinetic study of a Factor VIII concentrate in patients with severe haemophilia A, the standard practice would be to take regular samples over a time period that is equivalent to at least two or three times the half-life. The derivation of the first-order rate constant for the elimination of VWF from the slope of the semi-logarithmic plot of VWF concentration against time surmounts problems of both a fluctuating basal VWF level and quantity of VWF released by DDAVP.

3.5.2 Validation of method

The method used to determine VWF:Ag $t_{1/2}$ in this thesis has previously been compared to the pharmacokinetic evaluation of VWF:Ag data up to 48 h post DDAVP.(Brown 2003) As expected, significant variability was found amongst VWF:Ag values following return to steady-state levels, illustrating the difficulty in determining the correct cut off for a return to the baseline VWF:Ag level. However, no significant difference was found between VWF:Ag $t_{1/2}$ values calculated using a pharmacokinetic software programme (PK Analyst) for the 48 h data with those derived over 6 h by the method used in this study.(Brown 2003).

3.5.3 Validation of control group

Performing DDAVP studies in normal volunteers is not possible on an ethical basis. The selection of a control group of haemophila A patients in this study was based on data from previously published studies: the method used to estimate VWF:Ag $t_{1/2}$ has previously been used in DDAVP studies of normal individuals and patients with mild haemophilia A, as well as VWD patients.(Brown, *et al* 2003, Mannucci 1982, Michiels, *et al* 2002, van Genderen P. J. J 1997) These studies show that calculated VWF:Ag $t_{1/2}$ values obtained in haemophilia A patients are comparable to normal controls with respective median values and 95% confidence intervals of 9.4 h (8.2 – 10.5 h, n = 7, (Mannucci 1982) and 11.8 h (10.5 – 14.3 h, n = 9,(van Genderen P. J. J 1997) in normal controls and 9.9 h (8.5 – 11.2 h, n = 9,(Mannucci 1982) and 9.5 h (4.3 – 17.4 h, n = 7, (Brown, *et al* 2003) in patients with mild haemophilia A.

3.5.4 Subjects

Inclusion criteria: Type 1 VWD study group

Forty three patients (23 males, 20 females) from 40 kindred with a historical diagnosis of type 1 VWD were re-evaluated and recruited into the study on satisfaction of the following criteria, based on the 1994 ISTH recommendations: (Sadler 1994)

1. Personal/family history of bleeding
2. Age > 18 years and < 60 years
3. VWF:Ag level < 50 $IUdL^{-1}$
4. VWF:RCo activity < 50 $IUdL^{-1}$
5. VWF:RCo/VWG:Ag ratio > 0.7
6. Normal VWF multimer profile

Phenotypic data for the patient cohort are shown in Appendix 2. Genotypic analysis had not previously been performed in these patients.

Inclusion criteria: Haemophilia A control group

VWF clearance was measured in a control group of 17 male patients:

1. Age > 18 years and < 60 years
2. FVIII:C levels between 5 and 50 $IUdL^{-1}$.

Phenotypic data for the control group are shown in Appendix 2.

Exclusion criteria (study and control groups)

1. Known arterial disease, hypertension or history of thrombosis
2. Impaired renal or liver function
3. FVIII or VWF inhibitor, past or present
4. Regular medication
5. Previous vasovagal syncope due to venesection or DDAVP

3.5.5 Method

6 h DDAVP study

DDAVP was infused intravenously over 15 min at a dose of 0.3 μgkg^{-1}. Venous blood samples were taken prior to the start of the DDAVP infusion (time 0, T_0) and hourly for 6 h from the start of the infusion (T_1 –T_6). Whole blood samples were collected into 3.8% trisodium citrate (109 $mmolL^{-1}$, 10% v/v). Platelet poor plasma was prepared and stored at –80°C until assayed. The VWF:Ag was assayed using ELISA.

Calculation of VWF:Ag half-life

Concentration of VWF:Ag (T_0) was subtracted from VWF:Ag concentrations at T_1 – T_6. Values obtained from the logarithmic transformation of the change in VWF:Ag at each timepoint were plotted against time and and the slope of the best-fit line was determined by regression analysis. The k value was calculated by multiplying the slope by -2.303 and VWF:Ag $t_{1/2}$ calculated by dividing the natural logarithmic value of 2 by the k value (ln 2/k).(Somosgyi 1999).

Chapter 4

CLEARANCE OF VWF IN TYPE 1 VWD

4.1 INTRODUCTION

The quantitative decrease in VWF levels characteristic of type 1 VWD may result from defects in VWF synthesis or secretion and/or increased clearance of VWF. Indeed a shortened half-life of VWF has been reported in some patients with a partial quantitative VWF deficiency.(Brown, *et al* 2003, Michiels, *et al* 2002, van Genderen P. J. J 1997) DDAVP induces the release of intracellular stores of VWF, and the measurement of VWF half-life in previous studies was based on the time taken for VWF released following DDAVP to return to steady-state concentrations.

Levels of VWF vary by 20 - 30% according to individual ABO blood group, with plasma VWF levels increasing in the order O<A<B<AB.(Gill, *et al* 1987, Shima, *et al* 1995) Although the mechanism for the ABO effect on plasma VWF levels is not known, it appears likely that it is mediated by the ABH antigenic structures present on the N-linked chains of VWF.(Matsui, *et al* 1992, Sodetz, *et al* 1979) Data from previous studies do not suggest an effect of ABO determinants on the synthesis and/or secretion of VWF.(Brown, *et al* 2003, O'Donnell and Laffan 2001, O'Donnell and Laffan 2003) The hypothesis that ABH antigen expression may influence the rate of VWF clearance is supported by studies of the RIIIS/J mouse.(Mohlke, *et al* 1999b). Indirect evidence is provided by the observation that infused FVIII disappears more rapidly in blood group O than in non-O haemophilia patients.(Vlot, *et al* 2000) Furthermore, recent *in-vitro* studies have shown a variation in susceptibility of VWF cleavage by the metalloprotease ADAMTS-13 according to blood group, with VWF associated with blood group O being the most susceptible to cleavage.(Bowen 2003)
The ratio of FVIII to VWF:Ag has been shown to provide useful information on the steady state kinetics of VWF. While defects in VWF synthesis or secretion have been shown to result in increased ratios of greater than one, this ratio has not shown to be affected by increased VWF clearance in some patients with type 2 VWD.(Eikenboom, *et al* 2002)

4.2 AIMS

The objectives of this study were to investigate the release and clearance of endothelial derived VWF in a group of type 1 VWD patients and haemophilia A controls following infusion of DDAVP. The possibility that expression of ABH antigens on VWF may influence the rate of plasma clearance of VWF was examined in these subjects. Finally, the relationship between VWF clearance and the ratio of FVIII:C to VWF:Ag was investigated in this cohort of type 1 VWD patients.

4.3. MATERIALS AND METHODS

4.3.1 Patient and controls

The patients recruited into the study and control groups are described in Chapter 3, Section 3.1 (Appendix 2).

4.3.2 Phenotypic data

VWF:Ag and FVIII levels were measured as described in Chapter 3, Section 3.2.

4.3.3 Measurement of plasma clearance of VWF

The method used to calculate VWF:Ag half-life (VWF:Ag $t_{1/2}$) is described in Chapter 3, Section 3.5.

4.3.4 Data and statistical analysis

Analyses of data were performed using GraphPad Prism (GraphPad Prism version 4.0, GraphPad Software, San Diego, USA). Results are expressed as median values with range. Data was analysed using Spearman's rank correlation and the Mann-Whitney-Wilcoxon and Kruskal-Wallis tests.

Table 4.1. Phenotypic data for type 1 VWD patients and haemophilia A controls at diagnosis

Data is presented as median with range.

Values are based on the mean of 3 separate measurements

number of subjects (n)

*$P < 0.001$

	FVIII:C ($IUdL^{-1}$) 50-150	**VWF:Ag ($IUdL^{-1}$) 50-150**	**VWF:RCo ($IUdL^{-1}$) 50-150**	**VWF:RCo/ VWF:Ag**
Type 1 VWD (n = 40)	60 (7 - 131)	36* (9 - 50)	36* (9 - 50)	0.97 (0.72 - 2.29)
Haemophilia A (n = 17)	17 (4 - 38)	105 (45 - 238)	106 (48 - 300)	1.00 (0.71 - 1.78)

4.4 RESULTS

4.4.1 Phenotypic data

Diagnostic phenotypic data for the 40 patients with type 1 VWD (study group) and 17 patients with mild haemophilia A (control group) are shown in Appendices 1 and 2 and summarised in Table 4.1. As expected, the respective median concentrations of VWF:Ag were significantly lower in the type 1 VWD patients than haemophilia controls ($P < 0.001$). The median age of the VWD patients was 39 years (21 – 60) compared to 41 years (19-58) in the haemophilia A controls. No significant difference was found in VWF:Ag concentration between type 1 VWD patients of blood group O (median 36 $IUdL^{-1}$, $P = 0.4$, n = 22) and blood group A (30 $IUdL^{-1}$, n = 15)

4.4.2 One hour response to DDAVP

The increase in VWF:Ag from pre-DDAVP (T_0) to 1 h post DDAVP (T_1) in individual study patients and controls are shown in Appendix 3. VWF:Ag levels at T_0 were found to be greater than 50 $IUdL^{-1}$ in a proportion of patients who, on previous repeated testing, including recruitment samples, had fulfilled the criteria for type 1 VWD.(Sadler 1994)

The absolute 1 h VWF:Ag response was greater in the controls than the study group, $P < 0.01$ with respective median increases of 138 $IUdL^{-1}$ (30 – 202, n = 17) and 103 $IUdL^{-1}$ (14 – 234, n = 40, Table 4.2). However, the relative 1 h response was found to be less in the controls than the VWD patients, $P < 0.001$, with respective median increases of 2.5 fold (1.2 – 4.4, n = 17) and 3.6 fold (2.3 – 12.5, n = 40, Table 4.2). The absolute and relative 1 h responses plotted against VWF:Ag levels at T_0 are shown in Fig. 4.1 and Fig. 4.2. The absolute increase in VWF:Ag was shown to correlate with VWF:Ag level at T_0 in the whole group (r = 0.47, $P < 0.001$, n = 57, Fig.4.1). Although this correlation was shown to be significant in the VWD patients (r = 0.57, $P < 0.001$, n = 40), this was not found in the haemophilia A controls (r = -0.02, $P = 0.9$, n = 17). Within the VWD study group, no significant difference in absolute 1 h increase in VWF:Ag was found between patients of blood group O and A, $P = 0.35$ with respective median increases of 119 $IUdL^{-1}$ (28 – 188, n = 22) and 100 $IUdL^{-1}$ (14 – 234, n = 15).

The inverse correlation demonstrated between VWF:Ag level at T_0 and the relative increase in VWF:Ag in the whole group (r = -0.6, $P < 0.001$, n = 57, Fig.4.2), was largely attributable to the haemophilia A controls (r = -0.9, $P < 0.001$, n = 17); as

expected, such inverse correlation was not seen in the type 1 VWD patients (r = -0.28, P = 0.08, n = 40)

Table 4.2. 1 h DDAVP response and calculated half-life values of VWF:Ag in type 1 VWD patients and haemophilia A controls.

Data is presented as median with range; * $P < 0.0001$; ** $P < 0.02$

Response is defined as the difference between plasma concentration of VWF:Ag at baseline (T_0) and 1 h following DDAVP (T_1).

	1 h absolute increase in VWF:Ag ($IUdL^{-1}$)	**1 h relative increase in VWF:Ag (fold)**	**VWF:Ag $t_{1/2}$ (h)**
Type 1 VWD (n = 40)	103* (14 - 234)	3.6* (2.3 – 12.5	4.2** (1.3 – 11.4)
Haemophilia A (n = 17)	138 (30 – 202)	2.5 (1.2 – 4.4)	10.1 (4.1 to greater than 30)

Figure 4.1. Relationship between baseline VWF: Ag levels and absolute increase in VWF:Ag 1 h following DDAVP. VWF:Ag prior to the administration of DDAVP (T_0) and increase in VWF:Ag 1 h post DDAVP are shown for patients with type 1 VWD (◆, n = 40) and haemophilia A controls (△, n = 17). Correlation was found between baseline VWF:Ag levels and absolute 1 h increase in VWF:Ag in the study and control groups, r = 0.47, P < 0.001, n = 57 and the study group alone, r = 0.57, P < 0.001, n = 40 but not in the controls, r = -0.02, P = 0.9, n = 17.

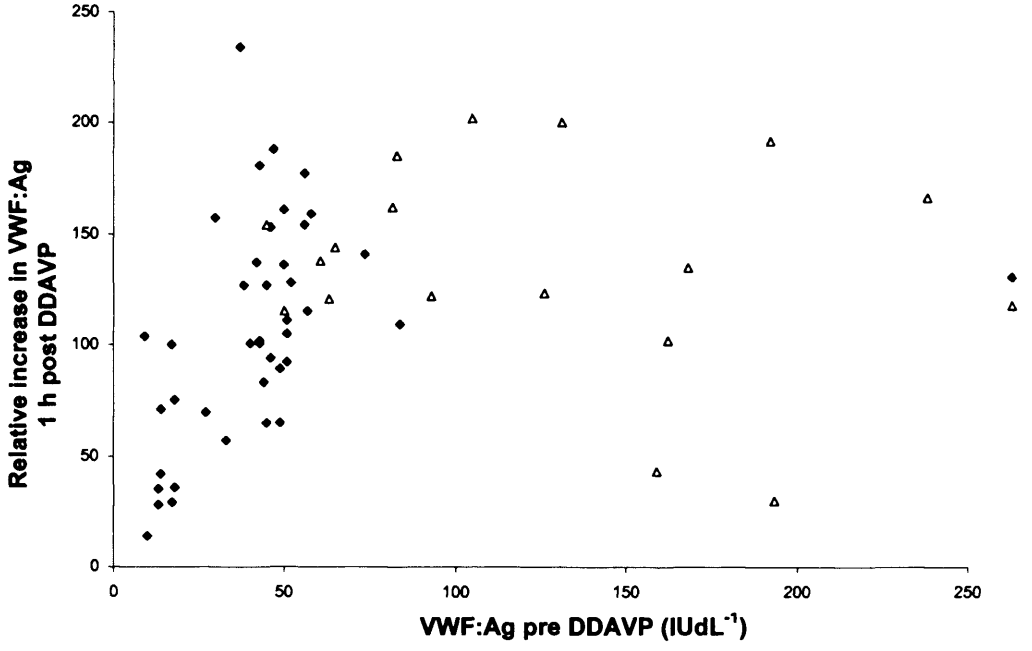
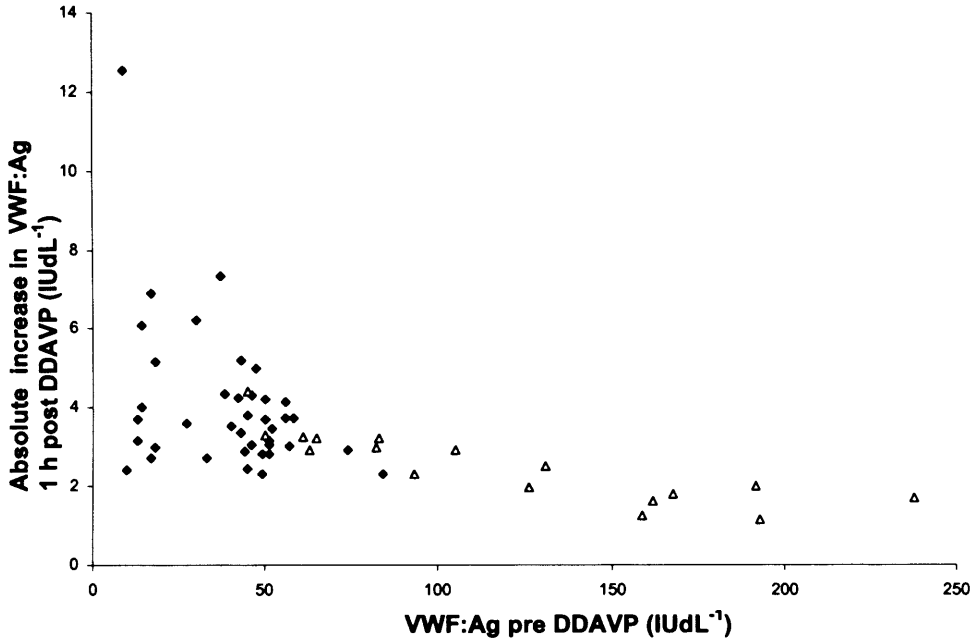


Figure 4.2. Relationship between baseline VWF: Ag levels and relative increase in VWF:Ag 1 h following DDAVP. VWF:Ag prior to the administration of DDAVP and relative rise in VWF:Ag 1 h post DDAVP are shown for patients with type 1 VWD (◆,n = 40) and haemophilia A controls (△ , n = 17). Inverse correlation was found between baseline VWF:Ag levels and relative 1 h increase in VWF:Ag in the study and control groups, r = -0.6, P < 0.001, n = 57 and the control group alone, r = -0.9, P < 0.001, n = 17 but not in the study group, r = -0.28, P = 0.08, n = 40.

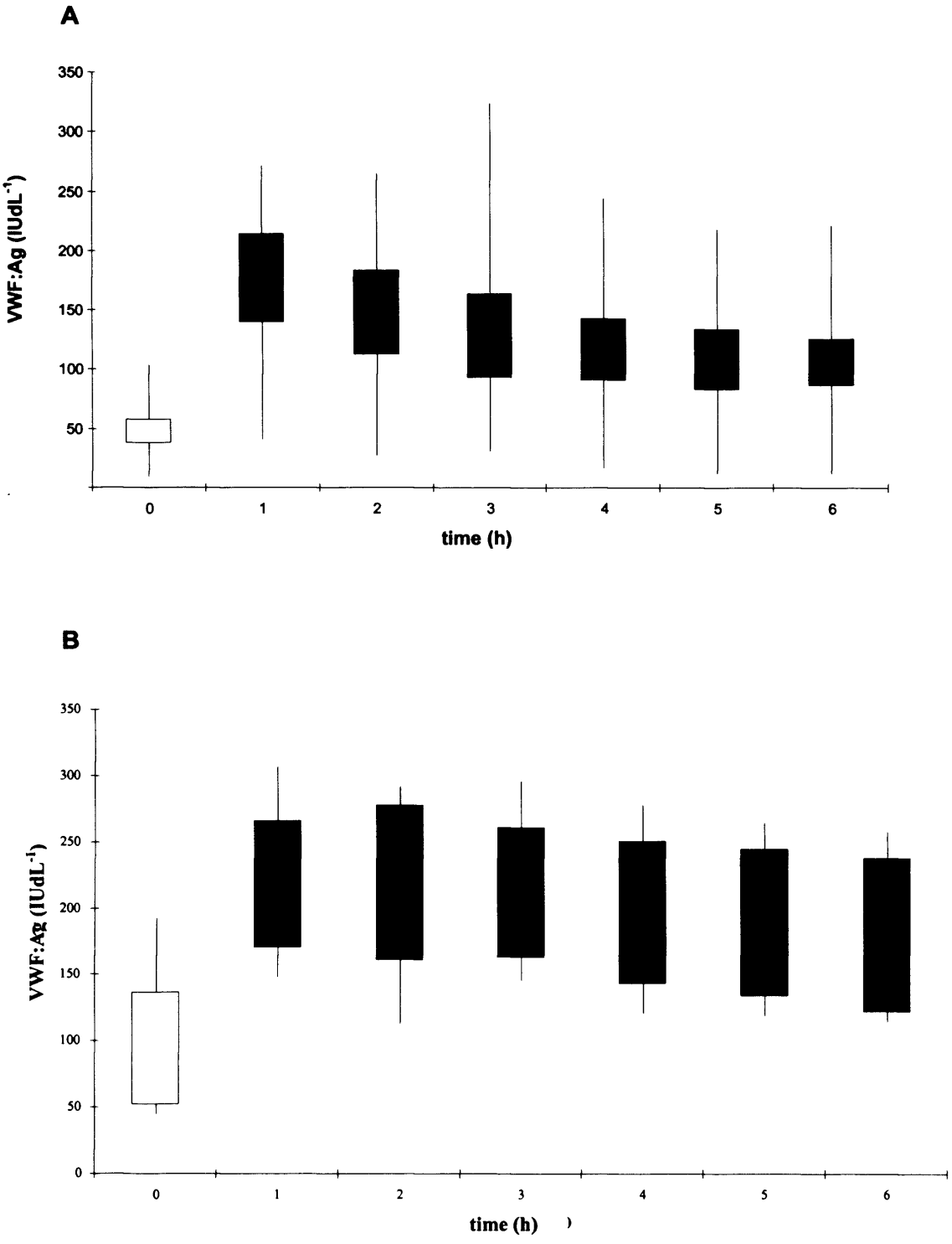


4.4.3 VWF:Ag levels pre- and over 6 hours post-DDAVP

VWF:Ag levels at T_0 - T_6 in the type 1 VWD study patients and haemophilia A controls are shown in Fig. 4.3. Despite the greater 1 h increase in VWF:Ag in the control group, the rate of return of VWF:Ag to steady-state levels was greater in the study patients. Furthermore, the interquartile range was found to be wider in the control group than VWD patients.

Figure 4.3. VWF:Ag values prior to and over 6 h following DDAVP administration in type 1 VWD patients (n = 40) and haemophilia A controls (n = 17).

Data is presented as 25th and 75th centiles with range. Normal range 50 – 150 $IUdL^{-1}$



4.4.4 Plasma clearance of VWF:Ag post DDAVP infusion

Individual VWF:Ag $t_{1/2}$ values are shown in Appendix 3. The VWF:Ag $t_{1/2}$ values in the type 1 VWD patients were significantly shorter (median 4.2 h, 1.9 – 11.4, n = 40) $P < 0.001$ than the controls (10.1 h, 4.1 - >30, n = 17, Fig. 4.4). In two of the control subjects, the 1 h rise in VWF:Ag concentration was sustained over the 6 h time course and a first-order rate constant for the elimination phase of released VWF (*k* value) was not obtainable. The VWF:Ag $t_{1/2}$ values for these subjects is shown as greater than 30 h. Significant correlation was found between the VWF:Ag level at T_0 and the VWF:Ag $t_{1/2}$ when the study and control groups were analysed together (r = 0.58, $P < 0.001$, n = 57, Fig.4.5). However, when the study and controls groups were analysed separately, this correlation was shown to be weak (r = 0.26, $P = 0.08$, n = 40 and r = 0.46, $P = 0.06$, n = 17, respectively). The VWF:Ag $t_{1/2}$ values for family members within the two families recruited with type 1 VWD are shown in Table 4.3.

Figure 4.4. Calculated half-life values VWF:Ag in type 1 VWD patients and haemophilia A controls. VWF:Ag was measured in plasma samples taken prior to and at hourly intervals over 6 h following intravenous administration of DDAVP. The plasma clearance of VWF:Ag was increased in the type 1 VWD patients (median 4.2 h, $P < 0.001$, n = 40) compared to the controls (10.1 h, n = 17). In two of the control subjects, the 1 h rise in VWF:Ag concentration was sustained over the 6 h time course and a *k* value was not obtainable. The VWF:Ag $t_{1/2}$ values for these subjects is shown as being greater than 30 h.

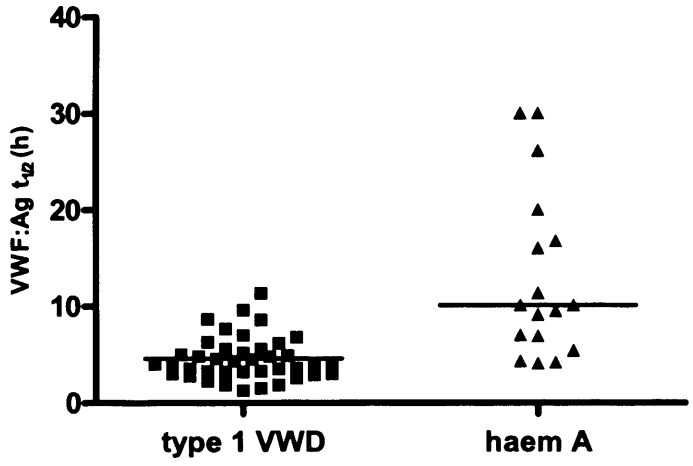


Figure 4.5. Relationship between baseline VWF:Ag concentration and half-life of released VWF. VWF:Ag concentration prior to the administration of DDAVP (T_0) and calculated VWF:Ag $t_{1/2}$ values are shown for patients with type 1 VWD (◆, n = 40, and haemophilia A controls (△., n = 17). Correlation was found between baseline VWF:Ag and VWF:Ag $t_{1/2}$ in the study and control groups (r = 0.58, $P <$ 0.001, n = 57) but not in the study or control groups alone (r = 0.26, P = 0.08, n = 40; r = 0.46, P = 0.06, n = 17 respectively).

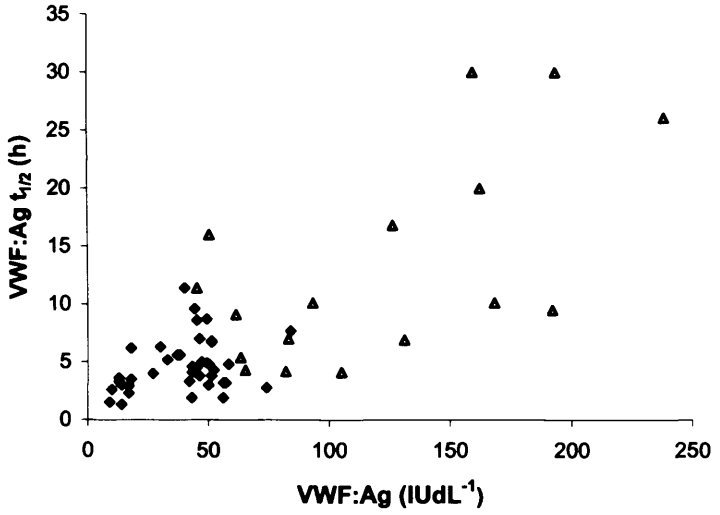


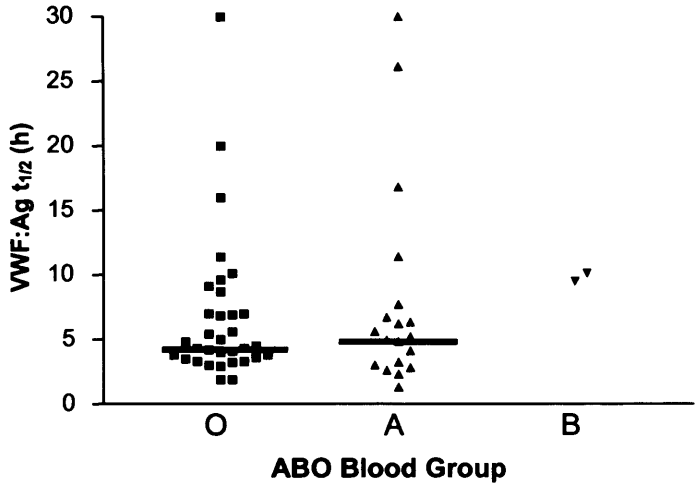
Table 4.3. VWF:Ag half-life values within type 1 VWD kindred.
Generation is indicated in superscript.

Kindred	Patient No.	ABO blood group	Age	VWF:Ag ($IUdL^{-1}$)	VWF:Ag $t_{1/2}$ (h)
8^2	8	A	36	29	3.0
8^1	11	A	60	36	6.8
8^2	13	A	31	36	3.2
10^2	10	A	21	21	3.2
10^1	25	A	45	13	5.6

4.4.5 Clearance of VWF:Ag and ABO blood group

The VWF:Ag $t_{1/2}$ values for the study and control groups according to ABO blood group are shown in Fig.4.6. Although the median VWF:Ag $t_{1/2}$ was shorter in subjects of blood group A (5.2 h, n = 18) compared to blood group O (4.5 h, n = 34), no significant difference in VWF:Ag $t_{1/2}$ values was found between ABO blood groups, $P = 0.3$. Similarly, no significant difference in VWF:Ag $t_{1/2}$ values was found between ABO blood groups when the study and control groups were analysed separately ($P = 0.7$, n = 37; $P = 0.4$, n = 17 respectively, data not shown).

Figure 4.6. Calculated VWF:Ag half-life values for study and control groups according to ABO blood group. Median values are shown: blood group O: 4.5 h (n = 34); blood group A: 5.2 h (n = 18); blood group B: 9.8 h (n = 2). No significant difference in VWF:Ag $t_{1/2}$ values between ABO blood groups was found, $P = 0.3$.



4.4.6 Clearance of VWF:Ag and ratio of FVIII to VWF:Ag

The median FVIII:VWF ratio in the type 1 VWD patients was 1.68 (0.43 - 3.14), n = 40. No correlation was shown between VWF:Ag $t_{1/2}$ and the ratio of FVIII to VWF:Ag in the type 1 VWD patients (r = 0.08, $P = 0.6$, n = 40, Fig. 4.7). Inverse correlation was found between the FVIII:VWF ratio and the absolute 1 h increase in VWF:Ag , r = -0.36, $P < 0.02$, n = 40, Fig. 4.8.

Figure 4.7. Relationship between half-life values of VWF and circulating plasma ratio of FVIII:C to VWF:Ag in patients with type 1 VWD. No correlation was found between the FVIII/VWF ratio and VWF:Ag $t_{1/2}$, r = 0.08, *P* = 0.6, n = 40.

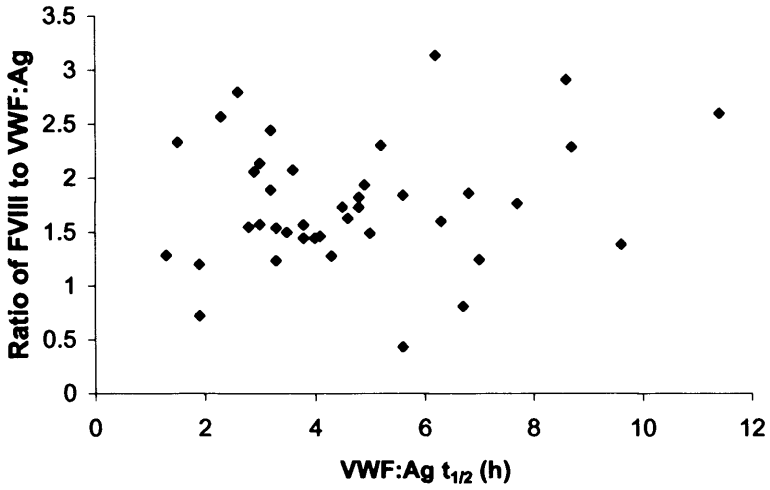
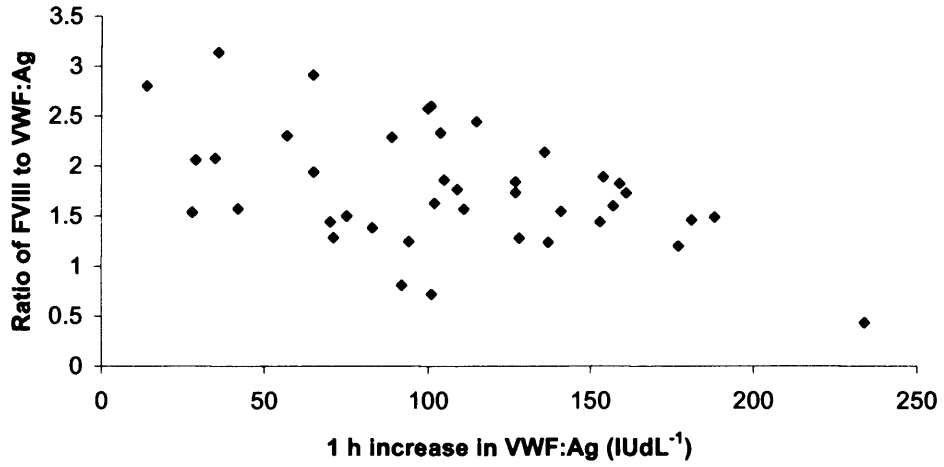


Figure 4.8. Relationship between ratio of FVIII:C to VWF:Ag and 1h increase in VWF:Ag in type 1 VWD patients. Inverse correlation was found between FVIII/VWF:Ag ratio and 1 h increase in VWF:Ag following DDAVP , r = -0.36, *P* < 0.02, n = 40.



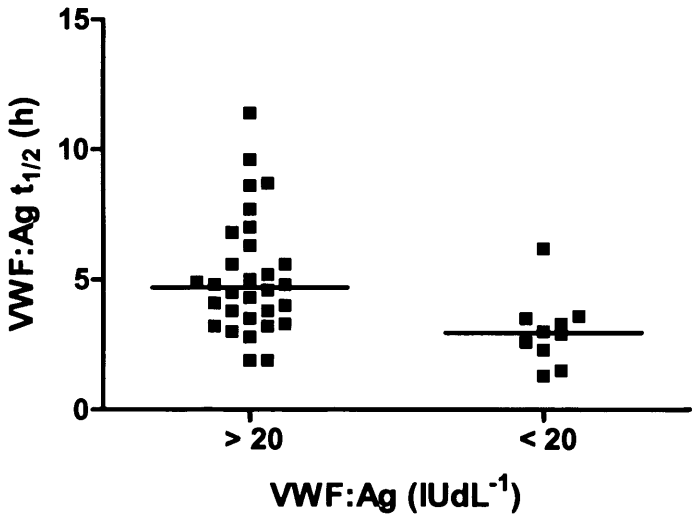
4.4.7 Analysis of type 1 VWD patients according to disease phenotype

It has been demonstrated that type 1 VWD patients with more severe phenotypes, as defined by VWF:Ag < 20 $IUdL^{-1}$ are more likely to demonstrate causative mutations within the *VWF* gene.(Eikenboom, *et al* 2006) Therefore a partial quantitative deficiency of VWF resulting from composite factors distinct from deleterious mutations within *VWF* is more likely to be of relevance in milder type 1 VWD phenotypes. In view of the likely heterogeneity in pathogenic mechanisms within this study cohort, type 1 VWD patients were subclassified into categories of 'severe' or 'mild' phenotype, defined using a cut-off VWF:Ag level of 20 $IUdL^{-1}$. Patients were analysed according to whether VWF:Ag level was greater (n = 30) or less (n = 10, Table 4.4) than 20 $IUdL^{-1}$. ABO blood groups were found to be more evenly distributed within the severe patients (five blood group A and four blood group O). This differed from the group with milder phenotype in whom a greater number of patients were blood group O than A (22 and 15 respectively). The 1 h increase in VWF:Ag level following DDAVP was variable within both subgroups, with neither demonstrating significant correlation between VWF:Ag level at T_0 and absolute 1 h increase in VWF:Ag (r = 0.19, $P = 0.6$, n = 10; r = 0.19, $P = 0.3$, n = 30 respectively). VWF:Ag $t_{1/2}$ values in patients with VWF:Ag levels < 20 $IUdL^{-1}$ were found to be shortened (median 2.9 h, $P < 0.005$, n = 10) as compared to patients with VWF:Ag levels > 20 $IUdL^{-1}$(4.7 h, n = 30, Fig. 4.9, Table 4.4) although both subgroups demonstrated significantly shorter VWF:Ag $t_{1/2}$ values than the haemophilia A controls, $P < 0.001$. No significant correlation between VWF:Ag level at T_0 and VWF:Ag $t_{1/2}$ was found in either subgroup (r = 0.4, $P = 0.24$, n = 10; r = -0.3, $P = 0.09$, n = 30). While the circulating ratio of FVIII to VWF:Ag was found to be higher in the more severe patients (median 2.07, n = 10) than the milder patients with type 1 VWD (1.6, n = 30) this was not shown to be statistically significant ($P = 0.08$).

Table 4.4. 1 h DDAVP response and half-life values of VWF:Ag in type 1 VWD patients with steady-state VWF:Ag levels of less than 20 IUdL^{-1} (n = 10)

Patient No.	ABO Blood group	VWF:Ag T_0 (IUdL^{-1})	VWF:Ag T_1 (IUdL^{-1})	VWF:Ag $t_{1/2}$ (h)	Ratio of FVIII:C to VWF:Ag
7	A	17	117	2.3	2.6
14	O	13	41	3.3	1.5
22	A	18	54	6.2	3.1
27	A	10	24	2.6	2.8
28	O	17	46	2.9	2.1
31	O	18	93	3.5	1.5
32	NT	9	113	1.5	2.3
38	O	13	48	3.6	2.1
40	A	14	85	1.3	1.3
43	A	14	56	3.0	1.6

Figure 4.9. Calculated VWF:Ag half-life values for type 1 VWD patients according to disease phenotype. VWF:Ag $t_{1/2}$ values for patients with VWF:Ag levels < 20 IUdL^{-1} are shorter (median 2.9 h, $P < 0.005$, n = 10) than for patients with VWF:Ag levels > 20 IUdL^{-1} (4.7 h, n = 30). The VWF:Ag $t_{1/2}$ values in both groups were shorter than the haemophilia A controls, $P < 0.001$.



4.5 DISCUSSION

In this study, the release and clearance of VWF released following treatment with DDAVP has been examined in patients with type 1 VWD and haemophilia A controls. In keeping with previous reports, DDAVP resulted in the correction of the VWF levels into the normal range in most of the type 1 VWD study patients,(Nolan, *et al* 2000) and all of the controls, with approximately two to three fold increases in FVIII and VWF levels observed.(Ruggeri, *et al* 1982) A partial quantitative deficiency of VWF can result from reduced or impaired intracellular transport or secretion of VWF, or increased plasma clearance or a combination of both mechanisms. This study demonstrates increased plasma clearance of VWF in a significant proportion of patients with type 1 VWD, a finding that is consistent with previous reports.(Brown, *et al* 2003, Michiels, *et al* 2002, van Genderen P. J. J 1997) Together, these observations suggest that decreased survival of VWF in plasma may be a mechanism underlying low levels in some patients with type 1 VWD.

The half-life of VWF:Ag showed only a weak correlation with circulating plasma levels in the patients with type 1 VWD suggesting that reduced steady state VWF levels are either not, or only in part, due to increased clearance of VWF; a defect in intracellular transport and secretion by endothelial cells may also contribute towards the deficiency of VWF in these patients. Another possible explanation for these observations is that because of structural differences between VWF released by the regulated pathway following DDAVP administration and plasma VWF, the clearance kinetics of plasma and newly released VWF may differ. Indeed, in terms of multimer composition and expression of A antigen VWF released following DDAVP infusion differs from circulating VWF.(Brown, *et al* 2002, Ruggeri, *et al* 1982) A third explanation for the apparent discrepancy between plasma VWF levels and increased VWF clearance could be that the clearance rate is dependent on the plasma VWF concentration. However as VWF clearance has been shown to demonstrate first order kinetics, (Mannucci 1982) higher steady state levels of VWF:Ag can be interpreted as a consequence, rather than a cause, of longer VWF half-life values in this study. This is supported by the previous finding of normal VWF half-lives of infused cryoprecipitate and high purity VWF concentrates in patients with type 3 VWD.(Meriane, *et al* 1993, Over, *et al* 1978, Sultan, *et al* 1976) It therefore appears unlikely that the relationship between VWF:Ag half-life and VWF:Ag level can be explained by saturation of the clearance pathway.

In view of these findings, caution should be exercised in extrapolating post-DDAVP clearance kinetics to steady state plasma VWF levels.

The co-existence of impaired VWF synthesis or secretion and increased VWF clearance resulting in the reduced VWF levels in some type 1 VWD patients in this study is supported by several further findings. Firstly, a restricted VWF response to DDAVP was found in a significant proportion of type 1 VWD patients, in particular those with a more severe phenotype. As synthesis and secretion of VWF is normal in patients with haemophilia A, the observation that plasma VWF levels appear to be independent of the amount of VWF released is expected in this group.
Secondly, the ratio of circulating FVIII to VWF:Ag was found to be increased (a suggested indicator of impaired VWF synthesis/secretion) in the majority of type 1 VWD patients, irrespective of VWF:Ag half-life, a finding that was also more pronounced within the more severe VWD patients. This ratio was shown to inversely correlate with the response in VWF:Ag to DDAVP in the type 1 VWD cohort, with greater restriction in VWF response resulting in higher ratios of FVIII to VWF:Ag. VWF and FVIII circulate in plasma as a non-covalent complex, with only 1-2% of available VWF monomers being occupied by FVIII.(Vlot, *et al* 1998) Although the plasma molar ratio of 1:1 refers to the respective measurement of levels of FVIII and VWF in plasma in standardised units based on assumed levels of normality (100 $IUdL^{-1}$), the circulating molecular weight ratio of FVIII to VWF is around 1:50. It has been demonstrated that the molar ratio of FVIII to VWF:Ag can vary in VWD according to the underlying pathogenic mechanism: while defects in VWF synthesis or secretion have been shown to be associated with an increase in this ratio, FVIII/VWF:Ag ratios appear unchanged in cases of type 2 VWD with increased VWF clearance.(Eikenboom, *et al* 2002) A ratio of FVIII to VWF:Ag of less than one was found in a type 1 VWD patient known to have a FVIII binding defect as well as a known carrier of haemophilia A with type 1 VWD (Appendix 2). These patients are further discussed in Chapter 8.

Higher heritability of VWD has previously been reported in patients with lower VWF levels.(Eikenboom, *et al* 2006) In this study, patients with steady-state VWF levels reduced to < 20 $IUdL^{-1}$ were found to be less likely to achieve satisfactory responses to DDAVP and more likely to display higher ratios of FVIII to VWF:Ag. While both of these findings are suggestive of defective synthesis or secretion of VWF, no correlation was found between the concentrations of circulating and released VWF in this subgroup.

Furthermore, many of the patients with more severe phenotype displayed shortened VWF $t_{1/2}$ values, which appeared to contribute significantly towards the relationship between half-life of VWF:Ag and circulating plasma levels observed in the whole type 1 VWD group. These findings suggest that some cases of a partial quantitative deficiency of VWF may result from mutations within *VWF* that could, at least in part, be causative of increased VWF clearance. Indeed, recent studies have demonstrated *VWF* mutations that cause both increased clearance of VWF and impaired VWF synthesis or secretion. (Bodo, *et al* 2001, Schooten, *et al* 2005, Tjernberg, *et al* 2004) Molecular analysis of the patients in this study, including the limited family data is discussed in Chapter 8.

On the other hand, deleterious mutations within the *VWF* gene are less likely to be found in patients with higher VWF levels.(Eikenboom, *et al* 2006) Milder phenotypes are therefore more likely to result from the interplay of multiple factors, which include ABO blood group and polymorphisms. Indeed, around 60% of the type 1 VWD patients in this study were of blood group O as compared to 45% in the normal population, a finding that is well established.(Gill, *et al* 1987) Such enrichment of blood group O in this study was limited to the patients displaying a milder phenotype. To date, ABO blood group is the only genetic factor that has been clearly linked to VWF plasma concentration, accounting for around one third of the genetic variability of plasma VWF levels,(Orstavik, *et al* 1985) although the mechanism of ABO effect has not been established. ABH antigens are present within the oligosaccharide component of VWF according to the blood group of the individual,(Matsui, *et al* 1992) although detection of the antigens on VWF varies between plasma and intracellular VWF in humans.(Brown, *et al* 2002) .In view of possible confounding influences on VWF level in the type 1 VWD study patients, it is likely that any ABO effect would be more apparent in the control patients in this study and for this reason the study and control groups were analysed together. No significant difference in VWF response was found between blood group O and non-O individuals in this study (data not shown) consistent with previous observations that ABO group does not affect the rate of secretion by the regulated pathway.(O'Donnell and Laffan 2001, O'Donnell, *et al* 2000) Despite the finding that VWF half-lives were shorter in blood group O than blood group A subjects, this was not found to be statistically significant. Although these findings do not suggest a direct relationship between ABO blood group and the plasma clearance of VWF, this could be due to insufficient numbers of study and control patients and therefore this study may be under-powered and limited in its ability to demonstrate a significant difference in VWF clearance between blood groups. Furthermore, the effect of ABO

type may be modest because a difference in clearance rate of only 25 – 35% would account for the average difference in VWF level between blood groups.

The indirect evidence supporting increased clearance of VWF with respect to ABO blood group derives from the effect of aberrant glycosylation of murine VWF with resultant increased clearance,(Mohlke, *et al* 1999b) as well as the observation that infused FVIII disappears more rapidly in blood group O than in non-O haemophilia patients.(Vlot, *et al* 2000) More recently, the ratio of VWFpp to VWF:Ag, a suggested marker of increased clearance, has been shown to be higher in blood group O subjects than their non-O counterparts.(Haberichter, *et al* 2006b, Nossent, *et al* 2006) This is further addressed in Chapter 5. As discussed in Chapter 1, a receptor-based basis formed the favoured mechanism of VWF clearance at the time of this study although no receptors had yet been identified. The ASGPR has a high affinity for GalNAc and Gal, which are the respective terminal sugar residues on the A and B blood group antigenic structures, in contrast to the O antigenic structures, which terminate in a fucose residue. Therefore the lowest clearance rates (and resultant highest VWF plasma levels) would be expected in individuals of blood group O were the ASGPR to significantly contribute towards uptake of VWF in humans. Although the effect of ADAMTS-13 mediated proteolysis on VWF clearance is not known, ABO blood group determinants have been shown to influence the susceptibility of plasma VWF to ADAMTS-13 proteolysis.(Bowen 2003, O'Donnell, *et al* 2005) The relationship between ADAMTS-13 mediated proteolysis and the clearance of VWF within this patient cohort, including that in relation to ABO blood group, is examined in Chapter 6.

The rationale for the use of patients with mild haemophilia A as controls in this study has been discussed in Chapter 3. The distribution of the pre- and post-DDAVP VWF:Ag values was found to be wider in the controls compared to the type 1 VWD patients, a finding most likely to reflect normal biological variation. Similarly, there was more variation in the half-life values in the controls than the type 1 VWD patients, although significant differences between the respective medians/means has consistently been shown in both this and previous studies.(Brown, *et al* 2003)

Finally, the findings in this study illustrate the potential limitations of judging the effectiveness of DDAVP treatment by the initial rise of VWF alone and support the recommendation of measuring VWF levels at several time points following DDAVP.(Pasi, *et al* 2004) Few studies have addressed the clinical efficacy of DDAVP, in particular as the sole therapeutic agent.(Rodeghiero, *et al* 1996) The

data in this study suggest that if normal VWF levels are required to prevent or stop bleeding, treatment with DDAVP at intervals of 12 hours, as is the current standard practice, may be insufficient in some type 1 VWD patients. However, the rapid fall in VWF levels post-DDAVP in some type 1 VWD patients may be compensated by the other haemostatic effects of DDAVP.(Cattaneo, *et al* 1989) To address this, studies of the efficacy of DDAVP are required that analyse clinical response in relation to the VWF half-life.

In summary, increased clearance of VWF released following DDAVP is a frequent finding in type 1 VWD patients in this study, the largest of its kind to date. This increased clearance does not appear to consistently reflect the lowered steady state plasma VWF levels in these patients and possible limitations in the use of this method to study clearance of VWF following its constitutive secretion have been discussed. The findings in this study illustrate the need for a better understanding of the *in vivo* mechanisms of VWF clearance both intrinsic and extrinsic to VWF. Further studies in this thesis attempt to identify a common mechanism as a cause of the increased VWF clearance found in these patients.

Chapter 5

VWF PROPEPTIDE

5.1 INTRODUCTION

The propeptide of VWF (VWFpp) was first reported in 1978,(Montgomery and Zimmerman 1978) when it was found to be absent from the platelets and plasma from individuals with severe VWD. Although the *VWF* gene encodes both VWFpp and the mature VWF protein,(Ginsburg, *et al* 1985) VWFpp is cleaved prior to secretion of VWF.(Wagner, *et al* 1987) Secretion of both VWFpp and mature VWF is via the constitutive and regulated pathways and the two proteins circulate independently in plasma. VWFpp has been shown to be important for the intracellular trafficking and processing of VWF.(Borchiellini, *et al* 1996, Haberichter, *et al* 2003, Haberichter, *et al* 2002, Rosenberg, *et al* 2002, van Mourik and Romani de Wit 2001, Wise, *et al* 1988)

In normal individuals VWFpp is found to circulate at around one tenth of the molar concentration of mature VWF.(Borchiellini, *et al* 1996) Despite the demonstration of near-equimolar release of VWFpp and VWF,(Borchiellini, *et al* 1996, Wagner, *et al* 1987) the respective half-lives have been shown to differ by 3 – 4 fold,(Borchiellini, *et al* 1996) thus accounting for the difference in steady-state concentrations. Measurement of the plasma concentration of VWFpp and VWF:Ag in patients with a partial quantitative deficiency of VWF may therefore provide insight into the underlying pathogenic mechanism. Indeed, it has been proposed that an increased ratio of VWFpp to VWF:Ag may be a useful marker of plasma VWF clearance.(de Romeuf and Mazurier 1998)

5.2 AIMS

The aims of this study were to investigate the release and clearance of endothelial derived VWFpp following administration of DDAVP in patients with type 1 VWD and to compare these findings with the data previously obtained from studies of the mature VWF protein. To examine whether measurement of the relative plasma concentrations of VWFpp and VWF:Ag is predictive of increased plasma clearance of VWF, the relationship between the half-life of VWF released following DDAVP and the steady-state ratio of VWFpp to VWF:Ag was investigated. Finally, to further investigate the mechanism of ABO effect on plasma VWF levels, the ratios of VWFpp to VWF:Ag were examined for ABO-related differences.

5.3 MATERIALS AND METHODS

5.3.1 Patients and controls

Subgroups of type 1 VWD patients (n = 26) and haemophilia A controls (n = 10) from the cohorts recruited to the VWF clearance study in Chapter 4 were investigated (Appendix 3). Baseline plasma samples of 20 normal controls were used to derive a reference range for the plasma concentration of VWFpp. Five of the type 1 VWD patients had steady-state VWF:Ag levels reduced to < 20 $IUdL^{-1}$. Diagnostic phenotypic data for patient and control groups is summarised in Table 5.1.

5.3.2 Measurement of VWFpp by ELISA

Principle

The method employed in this study is based on a previously described method.(Borchiellini, *et al* 1996) A monoclonal antibody directed to human VWFpp, coupled to a polystyrene microtitre plate, is incubated with the plasma sample to be analysed. The immune complex formed is then incubated with a second anti-propeptide antibody covalently coupled to horse radish peroxidise (HRP). The amount of bound HRP relates to the concentration of propeptide antigen in the sample.

Chemicals, reagents, controls, equipment and method were as described for the VWF:Ag ELISA (Chapter 3, Section 3.2.2) using the following modifications:

1. The standard curve consisted of doubling dilutions of pooled normal plasma from 1/10 to 1/1280.
2. Coating antibody: CLB – pproIgG (Sanquin Research, University of Amsterdam, Netherlands) stored at 30°C, diluted to 5 μgmL^{-1} in bicarbonate coating buffer. Incubation was at 4°C.
3. All subsequent incubations were performed at 37°C (incubator).
4. Dilution buffer: PBS/Tw-20/1% BSA (Sigma-Aldrich, Dorset, UK)
5. Following overnight incubation with coat buffer, free binding sites were blocked with dilution buffer for 2 h, 200 µL per well. This was then washed x 5 before addition of plasma patient and reference samples.
6. Plasma samples were incubated for 2 h.
7. Capture antibody: HRP-CLB – pro 14.3, stored at 30°C, diluted 1/4000 (Sanquin Research, University of Amsterdam, Netherlands)

8. Measurements were determined against a plasma pool standard containing 6.3 nmolL^{-1} propeptide and 50 nmolL $^{-1}$ VWF (monomer concentration) and results were expressed in molar concentrations.
9. CV for the assay was 6.8%.

5.3.3 Measurement of molar concentration of VWF:Ag

Because endothelial cell release reactions link VWF and its propeptide relative to their molar concentrations, the use of relative unitage concentrations has the potential to cause confusion when comparing different data. VWF:Ag data were therefore also expressed as molar concentrations in these studies. Molar concentrations of VWF:Ag were derived from the VWF:Ag levels measured in Chapter 4 (IU dL^{-1}) as previously described.(Borchiellini, *et al* 1996)

5.3.4 Measurement of release and clearance of VWFpp and VWF:Ag following DDAVP

Platelet poor plasma samples were analysed prior to (T_0) and over 6 h following DDAVP administration (T_1 - T_6). The decay curves of VWFpp and VWF:Ag were extrapolated to the time point T = 30 min (linear regression of semi-log plot), the calculated values were corrected for baseline values and the respective 30 min responses of VWF and propeptide were plotted, and the slope determined. The method used to calculate VWF:Ag $t_{1/2}$ and VWFpp $t_{1/2}$ is described in Chapter 3, Section 3.5.

5.3.5 Data and statistical analysis

Analyses of data were performed using GraphPad Prism (GraphPad Prism version 4.0, GraphPad Software, San Diego, USA). Data was analysed using the Mann-Whitney-Wilcoxon, Kruskall-Wallis tests and paired t- test and correlation analysis was performed using Spearman's rank. Data are presented as median values with range.

Table 5.1. Phenotypic data of patients with type 1 VWD and haemophilia A and normal controls.

	FVIII:C ($IU\ dL^{-1}$) 50 - 150	VWF:Ag ($IU\ dL^{-1}$) 50 - 150	VWF:RCo ($IU\ dL^{-1}$) 50 - 150	VWF:RCo / VWF:Ag
Type 1 VWD (n = 26)	60 (13 – 80)	36 (13 – 50)	36 (11 – 52)	0.97 (0.7 – 1.4)
Haemophilia A (n = 10)	18 (5 – 38)	94 (45 – 193)	100 (50 – 158)	0.94 (0.7 – 1.3)
Normal controls (n = 20)	-	115 (66 – 194)	-	-

Data are shown as median (range).

5.4 RESULTS

5.4.1 Pre-DDAVP

Molar concentrations of VWFpp and VWF:Ag at T_0 in the type 1 VWD patients (n = 26) and the haemophilia A controls (n = 10) are given in Appendix 3 and circulating VWF:Ag levels in 20 normal controls are given in Appendix 4. Data is shown in Fig.5.1 and summarised in Table 5.2. The respective median concentrations of VWF:Ag and VWFpp were lower in the type 1 VWD patients than both the haemophilia A and normal control groups, $P < 0.001$. The VWFpp/VWF:Ag ratio was found to be increased in five patients with type 1 VWD (Fig. 5.1C). However, VWFpp/VWF:Ag ratios did not differ between the VWD patients and haemophilia A and normal control groups, $P = 0.4$ (Fig. 5.1C).

5.4.2 Relationship between baseline VWF:Ag concentration and ratio of VWFpp to VWF:Ag

Significant inverse correlation was found between circulating VWF:Ag levels at T_0 and VWFpp/VWF:Ag ratio in the type 1 VWD study and haemophilia A control groups ($r = -0.38$, $P < 0.02$, n = 36, Fig.5.2) and VWD patients alone ($r = -0.58$, $P < 0.01$, n = 26) but was not observed in the haemophilia A controls ($r = -0.45$, $P = 0.2$, n = 10) Four of the patients with increased VWFpp/VWF:Ag ratios had T_0 VWF:Ag levels < 20 $IUdL^{-1}$ (<10 nmol L^{-1}, Fig. 5.2).

5.4.3 One hour response to DDAVP

One hour responses in VWFpp and VWF:Ag did not differ between the VWD patients and haemophilia A controls, $P > 0.2$. Median increases in VWFpp from T_0 to T_1 were 28.4 and 40.0 $nmolL^{-1}$ respectively (Table 5.3). However, in both the VWD and control groups, the increase in VWF:Ag from T_0 to T_1 was significantly higher than the VWFpp increase, $P < 0.005$ (Table 5.3). In addition, the comparative release of VWFpp and VWF:Ag 30 min following DDAVP was examined in the type 1 VWD patients: VWFpp and VWF:Ag responses were derived at time point T = 30 min. The slope of the VWFpp vs VWF:Ag response was 0.48 (Fig. 5.3), representing the molar ratio of released VWFpp to VWF:Ag. The VWFpp/VWF:Ag ratios were approximately 3 fold higher at T_1 compared to T_0, in the VWD patients, and around 2 fold higher in Haem A, $P < 0.001$ (Table 5.3). No difference was found in the VWFpp/VWF:Ag ratio at T_1 between the VWD patients and control groups, $P = 0.8$.

Table 5.2 Molar concentrations of VWF:Ag and VWFpp in patients with type 1 VWD and haemophilia A controls prior to administration of DDAVP.
In addition, circulating plasma levels are given for normal controls.

	VWF:Ag (nmol L^{-1}) 25 - 75	**VWFpp (nmol L^{-1})**	**VWFpp/ VWFAg ratio**
Type 1 VWD (n = 26)	23.5* (5.5 – 42)	2.9 * (1.4 – 10.9)	0.13 n.s. (0.06 – 0.52)
Haemophilia A (n = 10)	47 (22.5 – 96.5)	8.2 (3.3 – 22.0)	0.17 n.s. (0.06 – 0.70)
Normal controls (n = 20)	57.5 (33 – 97)	7.4 (4.5 – 17.4)	0.12 (0.07 – 0.30)

*$P < 0.001$; n.s. not significant; data is presented as medians (range)
Normal ranges: VWFpp 4.5 – 17.4 nmol L^{-1}(as shown); VWF:Ag 25 – 75 nmol L^{-1}.

Table 5.3 1 h DDAVP response of VWF:Ag and VWFpp in patients with type 1 VWD and haemophilia A controls.

	VWF:Ag (nmol L^{-1})	VWFpp (nmol L^{-1})	VWFpp/VWF:Ag ratio at T_1
Type 1 VWD (n=26)	56.5 *# (14 – 117)	28.4* (9.7 – 46.7)	0.42 (0.16 – 0.77)
Haemophilia A (n=10	61.0# (15 - 101)	40.0 (0 – 52.7)	0.37 (0.07 - 0.57)

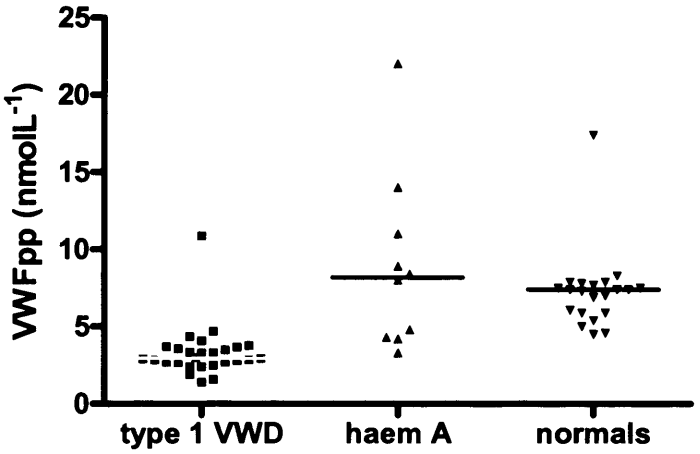
Response is defined as the difference between molar concentration of VWF at baseline (T_0) and 1 h following DDAVP (T_1);

* no significant difference between 1 h increase in VWF:Ag or VWFpp between study and control groups, $P > 0.2$;

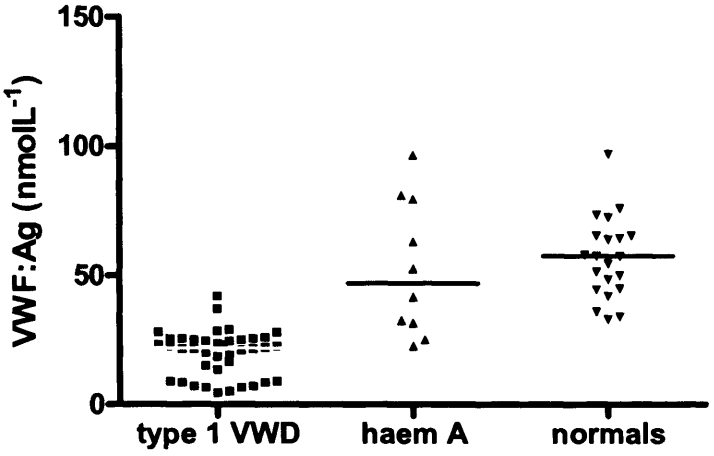
the 1 h increase in VWF:Ag from was significantly higher than the propeptide increase in both study and control groups, $P < 0.005$; data is presented as medians (range)

Figure 5.1. Molar concentrations of VWFpp (A) and VWF:Ag (B) and VWFpp/VWF:Ag ratios (C) at baseline in type 1 VWD patients (n = 26), haemophilia A controls (n = 10), and normal controls (n = 20). Median values are given in Table 5.2. Normal ranges: VWFpp 4.5 – 17.4 nmol L^{-1} (as shown); VWF:Ag 25 – 75 nmol L^{-1}. VWF:Ag and VWFpp levels were shown to be reduced in the type 1 VWD patients compared to both control groups, $P < 0.001$. No difference in VWFpp/VWF:Ag ratios was found between the groups, $P = 0.4$

A



B


150
100
50
0
VWF:Ag (nmolL-1)
type 1 VWD
haem A
normals

C

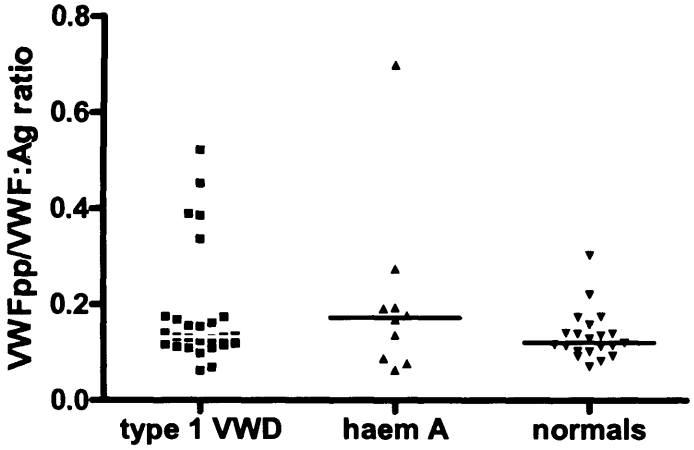

0.8
0.6
0.4
0.2
0.0
VWFpp/VWF:Ag ratio
type 1 VWD
haem A
normals

Figure 5.2. Relationship between baseline VWF:Ag molar concentration and ratio of VWFpp to VWF:Ag. VWF.Ag concentration and the ratio of VWFpp to VWF:Ag prior to the administrations of DDAVP are shown for patients with type 1 VWD (◆ ,n = 26) and haemophilia A controls (△ ,n = 10). Significant inverse correlation was found between VWF:Ag and VWFpp/VWF:Ag ratio in the study and control groups (r = -0.38, $P < 0.02$, n = 36) and VWD patients alone (r = -0.58, $P < 0.01$, n = 26) but not in the haemophilia A controls (r = -0.45, $P = 0.2$, n = 10)

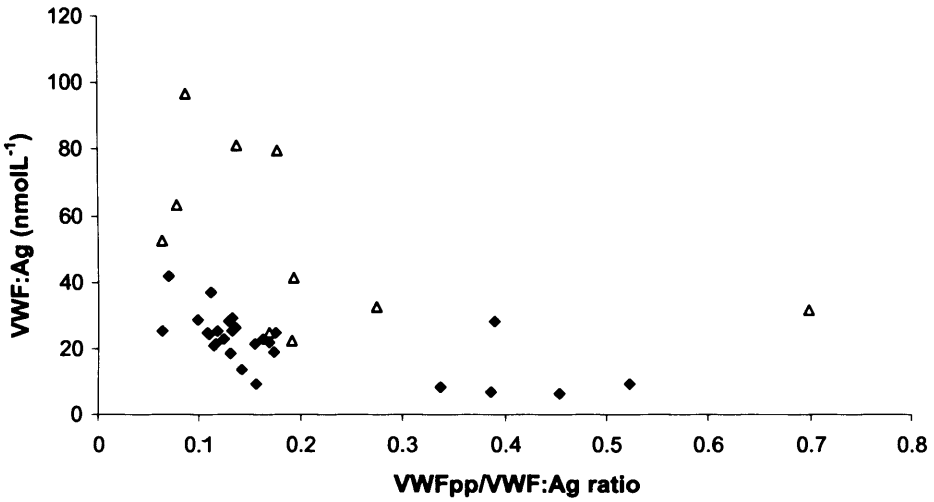
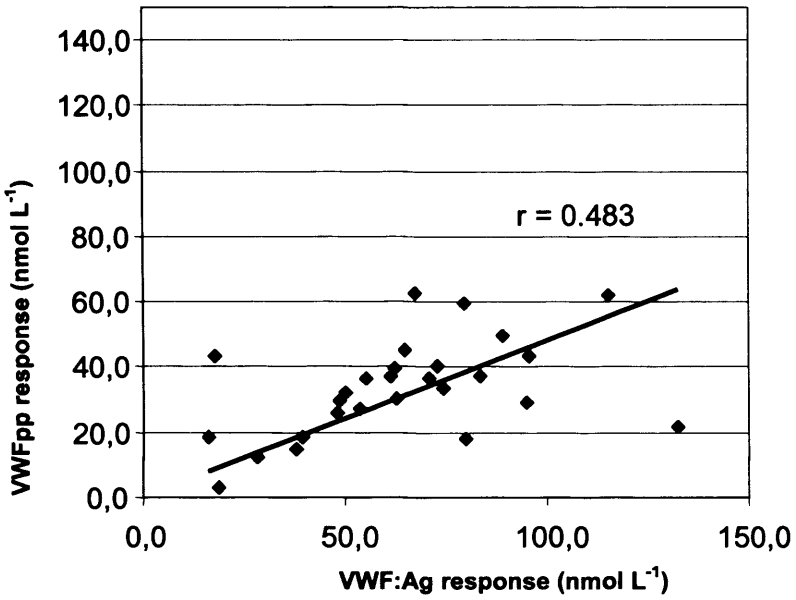


Figure 5.3. Increase in molar concentration of VWF:Ag and VWF propeptide 30 min after DDAVP administration in patients with type 1 VWD (n=26). The slope (r value) of 0.48 represents release of approximately two moles of VWF per mole VWFpp.



5.4.4 Clearance of VWFpp and VWF:Ag

The molar concentrations of VWFpp and VWF:Ag over the time course T_0 - T_6 are shown for the type 1 VWD patients and haemophilia A controls in Figs 5.4 and 5.5 respectively. As was found for the type 1 VWD study cohort in Chapter 4, VWF:Ag $t_{1/2}$ values were significantly shorter in this subgroup of type 1 VWD patients (median 3.8 h, $P < 0.001$, n = 26) than the haemophilia A controls (median 13.7 h, n = 10, Table 5.4, Fig. 5.6). In contrast, there was no difference in VWFpp $t_{1/2}$ values between the study and control groups, $P = 0.8$, with respective median values of 2.5 and 2.3 h (Table 5.4, Fig.5.6).

5.4.5 Relationship between VWF half-life and ratio of VWFpp to VWF:Ag

No correlation was found between the circulating plasma VWFpp/VWF:Ag ratio and VWF:Ag $t_{1/2}$ either in the type 1 VWD study and haemophilia A control groups (r = -0.16, $P = 0.4$, n = 36, Fig. 5.7), or the study patients (r = -0.32, $P = 0.1$, n = 26). Similarly, no correlation was found between the VWFpp/VWF:Ag ratio at T_1 and VWF:Ag $t_{1/2}$, $P = 0.2$ (data not shown). However, shortened VWF:Ag $t_{1/2}$ values were observed in the five type 1 VWD patients with increased VWFpp/VWF:Ag ratios (Fig. 5.7).

Table 5.4 Calculated half-life values of VWF:Ag and VWFpp in type 1 VWD patients and haemophilia A controls.

	VWF:Ag $t_{1/2}$ (h)	VWFpp $t_{1/2}$ (h)
Type 1 VWD (n = 26)	3.8* (1.9 – 9.6)	2.5n.s (1.7 – 8.0)
Haemophilia A (n = 10)	13.7 (4.1 - >30)	2.3 (0.8 – 8.5)

*P < 0.001; n.s. not significant; data is presented as medians (range)

Figure 5.4. Molar concentrations of VWFpp (A) and VWF:Ag (B) prior to and over 6 h following DDAVP administration in type 1 VWD patients (n = 26).
Data is presented as 25th and 75th centiles with range. Normal ranges: VWFpp 4.5 – 17.4 nmol L^{-1}; VWF:Ag 25 – 75 nmol L^{-1}.

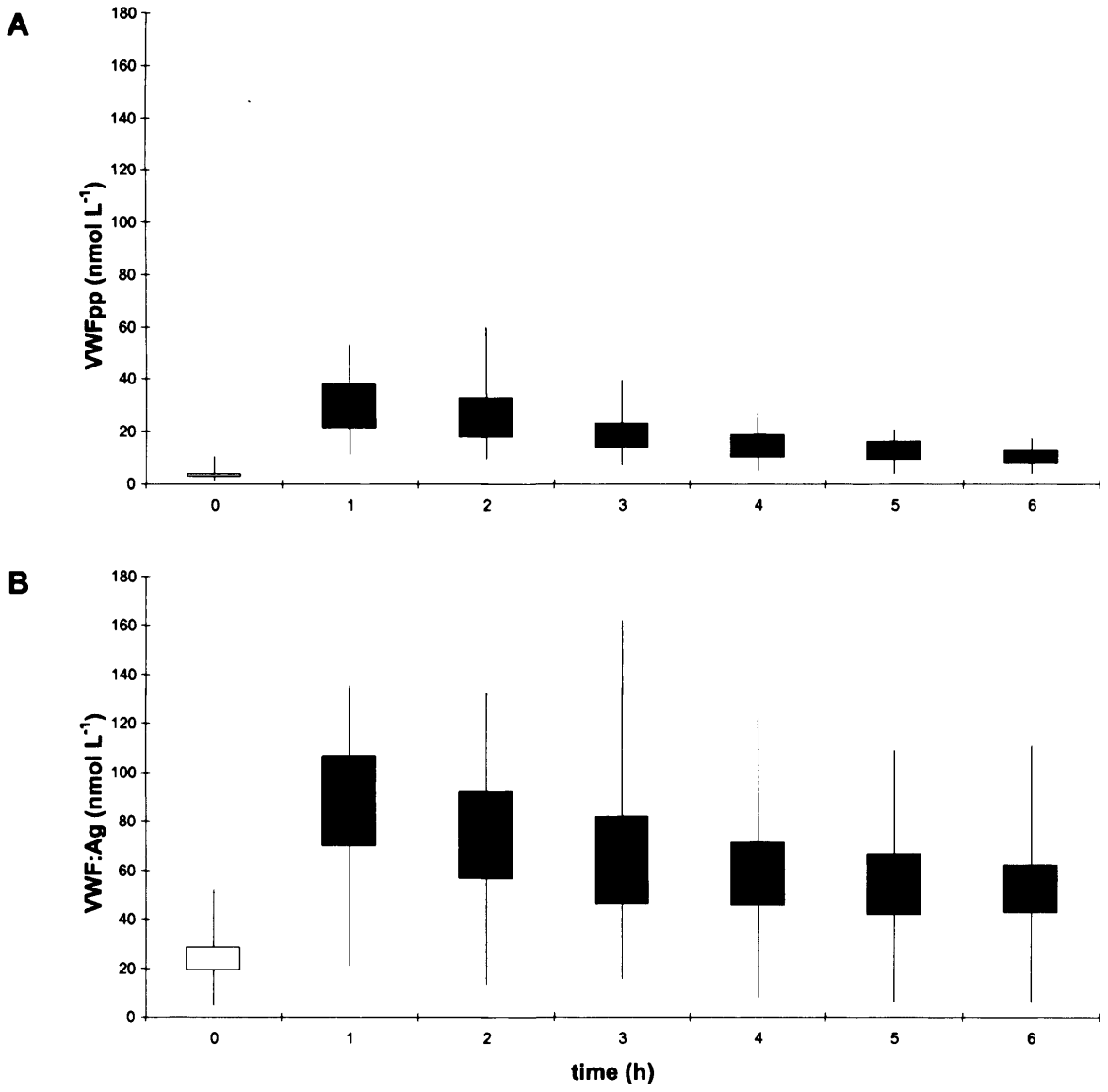
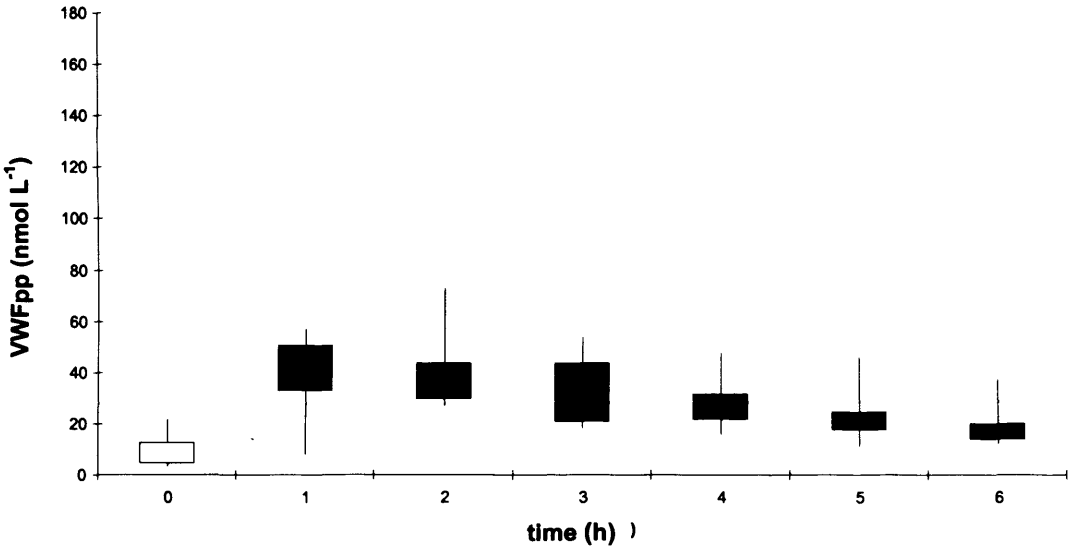


Figure 5.5. Molar concentrations of VWFpp (A) and VWF:Ag (B) prior to and over 6 h following DDAVP administration in haemophilia A controls (n = 10). Data is presented as 25th and 75th centiles with range. Normal ranges: VWFpp 4.5 – 17.4 nmol L^{-1}; VWF:Ag 25 – 75 nmol L^{-1}.

A



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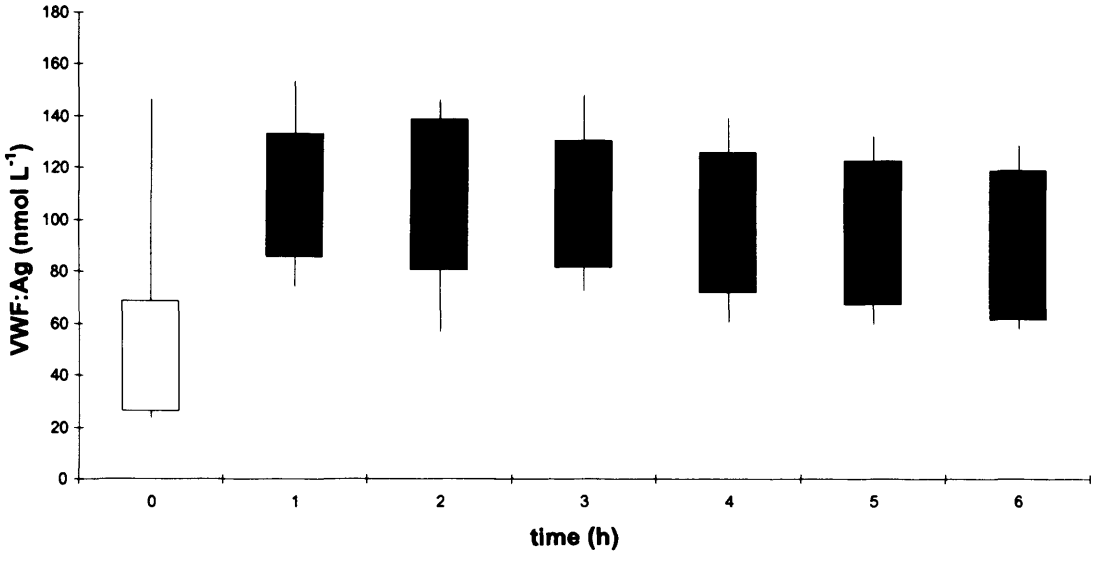


Figure 5.6A. Calculated half-life values of VWF:Ag in type 1 VWD patients and haemophilia A controls. VWF:Ag $t_{1/2}$ values were significantly shorter in type 1 VWD patients (median 3.8 h, $P < 0.001$, n = 26) than the controls (13.7 h, n = 10).

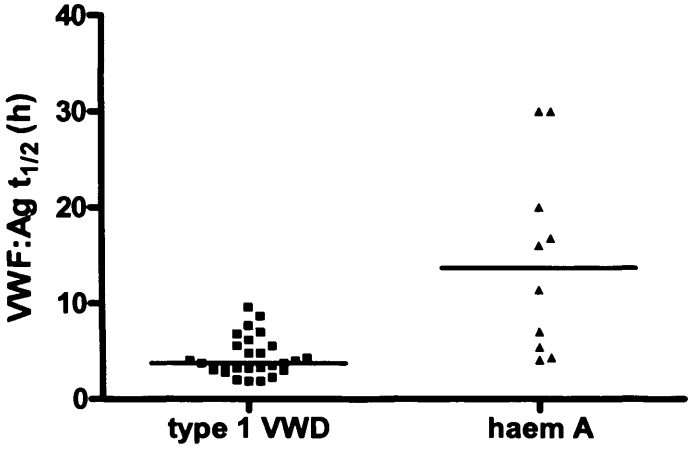


Figure 5.6B. Calculated half-life values of VWFpp in patients with type 1 VWD and haemophilia A controls.:No difference in VWFpp $t_{1/2}$ was found between the study (median 2.5 h, $P = 0.8$, n = 26) and control groups (2.3 h, n = 10)

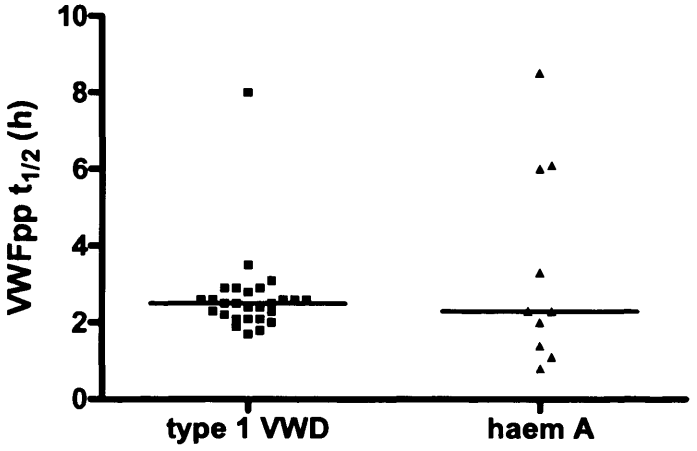
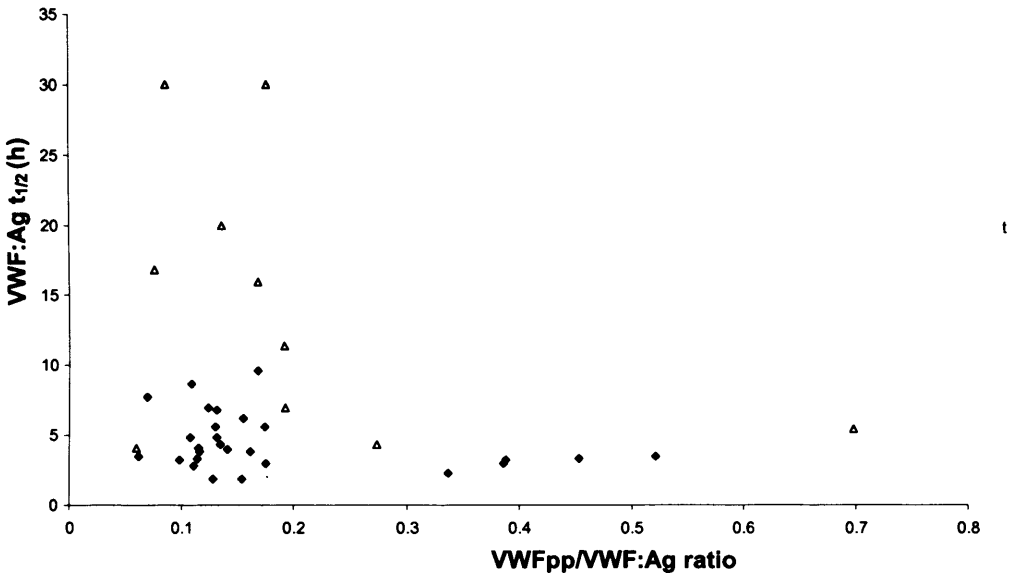


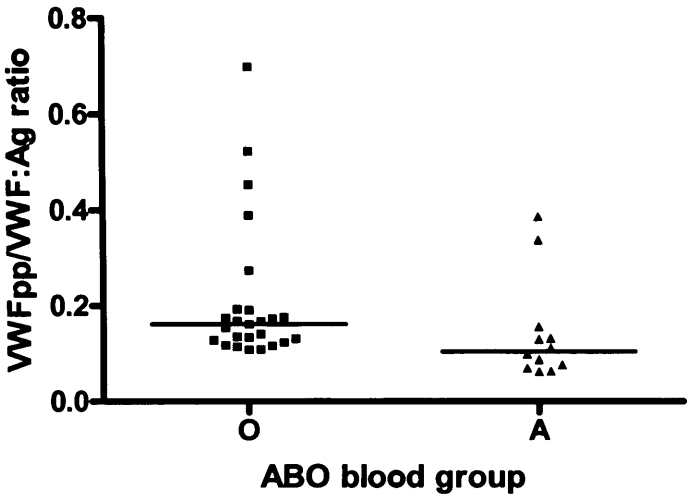
Figure 5.7. Relationship between VWF:Ag half-life and ratio of VWFpp to VWF:Ag. VWF:Ag $t_{1/2}$ values and the ratio of VWFpp to VWF:Ag in circulating plasma are shown for patients with type 1 VWD (◆,n = 26) and haemophilia A controls (△ , n = 10). No correlation was found between the VWFpp/VWF:Ag ratio and VWF:Ag $t_{1/2}$, both in the study and control groups (r = -0.16, *P* = 0.4, n = 36) and the VWD patients alone (r = -0.32, *P* = 0.1, n = 26).



5.4.6 Relationship between ABO blood group and ratio of VWFpp to VWF:Ag

Analysis of the type 1 VWD study and haemophilia A control groups together showed the VWFpp/VWF:Ag ratios to be significantly increased in blood group O subjects (median 0.16, 0.11 – 0.70, *P* < 0.01, n = 24, Fig. 5.8) compared to blood group A subjects (0.10, 0.06 – 0.39, n = 12). Within the VWD patients, no significant difference was found in VWFpp/VWF:Ag ratios between blood group O and A patients, *P* = 0.2.(data not shown).

Figure 5.8. Ratio of VWFpp to VWF:Ag in study and control groups according to ABO blood group The VWFpp/VWF:Ag ratio was found to be significantly increased in blood group O subjects (median 0.16, $P < 0.01$, n = 24) compared to blood group A subjects (median 0.10, n = 12).



5.5 DISCUSSION

In this study, having confirmed the previous finding of increased plasma clearance of VWF released following DDAVP in the type 1 VWD patient subgroup, steady-state levels of VWFpp and VWF were examined and the release and clearance of VWFpp investigated. In addition to demonstrating reduced VWF levels, the type 1 VWD study group were also shown to have lower steady-state concentrations of VWFpp than both haemophilia and normal control groups, consistent with defective VWF synthesis or secretion. Data from previous studies indicate that the plasma clearance of VWFpp is independent of VWF clearance and its shorter plasma survival has been shown to account for the lower steady steady-state concentrations of VWFpp compared to mature VWF in normal individuals (VWFpp/VWF:Ag molar ratio ~0.1). (Borchiellini, *et al* 1996) However, despite the increased VWF:Ag clearance and preservation of normal VWFpp survival in the type 1 VWD patients in this study, no difference was found in VWFpp/VWF:Ag ratios between the study and control groups. Differences between the kinetics of VWFpp and VWF:Ag and their disappearance following DDAVP administration could underlie this unexpected finding. Pertinent to this is that, unlike in cultured endothelial cells(Wagner, *et al* 1987) and previous DDAVP studies in healthy individuals(Borchiellini, *et al* 1996), equimolar release of VWFpp and VWF:Ag was not demonstrated, either in the control group or in the patients with type 1 VWD. This was reflected by restricted

DDAVP-induced increases in VWFpp/VWF:Ag ratio compared to previous studies.(Borchiellini, *et al* 1996) and indicates that not all VWFpp secreted after DDAVP administration is directly released into the systemic circulation. It is likely that a proportion of newly secreted VWFpp is extracted by other compartments (e.g. the extra-cellular matrix of the endothelium, circulating blood cells). Therefore, the multi-compartment distribution of VWFpp (and possibly VWF) could complicate the interpretation of clearance kinetics. Thus, the findings in this study suggest that, as was shown for mature VWF in Chapter 4, measurement of the propeptide of VWF following administration of DDAVP may also not reflect its steady state plasma levels.

It has previously been hypothesised that measurement of plasma levels of VWFpp in conjunction with VWF:Ag may provide insight into the underlying mechanism resulting in VWD.(de Romeuf and Mazurier 1998) While it has been proposed that concordant reductions in VWF:Ag and VWFpp resulting in preservation of the VWFpp/VWF:Ag ratio may reflect defects in VWF synthesis and intracellular transport prior to the cleavage of VWFpp, it has been suggested that alterations in VWF following its secretion (such as increased clearance) could result in an increase in the VWFpp/VWF:Ag ratio. Indeed, increased VWFpp/VWF:Ag ratios have recently been reported in association with increased VWF clearance in a homogeneous population of type 1 VWD patients with cysteine mutations in the VWF-D3 domain,(Schooten, et al 2005) as well as in affected type 1 VWD individuals within four families with mutations in the D3 and D4 domains.(Haberichter, *et al* 2006a)

Although increased plasma clearance of VWF was not shown to be consistently associated with an increase in the ratio of VWFpp to VWF:Ag in this study, significantly increased VWF clearance was observed in the five type 1 VWD patients with increased ratios of VWFpp to VWF:Ag. Of these five patients, four displayed a more severe VWD phenotype as defined by steady-state VWF:Ag concentrations of less than 20 $IUdL^{-1}$, which contributed significantly towards the overall finding of inverse correlation between VWFpp/VWF:Ag ratio and VWF:Ag level. These findings suggest that while an increased VWFpp/VWF:Ag ratio may be a useful surrogate marker for the increased plasma clearance of VWF, increased clearance of VWF released following DDAVP is frequently found in the context of a normal ratio. The apparent dissociation of VWF:Ag half-life from the VWFpp/VWF:Ag ratio further supports the likelihood of co-existent pathogenic mechanisms in these type 1 VWD patients, in particular those with milder phenotypes as discussed in Chapter 4.

The findings in this study cast doubt on whether the VWFpp/VWF:Ag ratio can usefully predict increased clearance of VWF in heterogeneous patient populations and challenge the findings from limited studies published in a few families, (Haberichter, *et al* 2006b) highlighting the problem of generalising a study of small numbers of patients from selected type 1 VWD families. The subgroup of VWD patients of more severe phenotype and increased VWFpp/VWF:Ag ratios is further considered in relation to findings of molecular analysis of *VWF* in Chapter 8.

The final aim of this study was to examine for ABO-related differences in the ratio of VWFpp to VWF:Ag. The suggestion that VWF released following DDAVP may not necessarily be a good measure of clearance of VWF released following constitutive secretion in the steady-state is further supported by the finding of increased VWFpp/VWF:Ag ratios in association with blood group O in this study. This is despite the lack of relationship previously demonstrated between ABO blood group and clearance of VWF following DDAVP (Chapter 4). In view of the lack of consistency shown between VWF:Ag clearance following DDAVP and VWFpp/VWF:Ag ratio, such a discrepancy is not unexpected. The ratios of VWFpp to VWF:Ag in the blood group A subjects were similar to the normal controls, fluctuating around a median of 0.1. The finding that the ratio was increased in blood group O subjects is consistent with recent findings from other studies (Haberichter, *et al* 2006a, Nossent, *et al* 2006) and supports the hypothesis of an ABO blood group effect on the rate of VWF clearance.(Mohlke, *et al* 1999b, Vlot, *et al* 2000)

The VWFpp immunoassay is a robust, reproducible assay. However, monoclonal antibodies to VWFpp were not commercially available at the time of this study. In laboratories where antibodies have been raised to VWFpp, the measurement of VWFpp has been successfully used to support the diagnosis of some cases of VWD, including acquired VWD. It has also been suggested that VWFpp may be useful in distinguishing between cases of acute and chronic endothelial cell perturbation.(van Mourik, *et al* 1999) Although the VWFpp assay was not being performed routinely at the time of this study, its use is likely to be widened following the recent development of commercial assays. In this study, the overall reduction in VWFpp levels in type 1 VWD patients illustrates the potential utility of this assay in the diagnosis of type 1 VWD. However, its value in differentiating causative mechanisms of the type 1 phenotype appears to be limited, as shown in this heterogeneous type 1 VWD cohort. As the VWFpp assay is not currently standardised, studies are required to evaluate inherited and acquired defects of VWF and recruitment into an ISTH/SSC multicentre study is underway.

Chapter 6

ADAMTS-13, VWF:CB and CLEARANCE OF VWF

6.1 INTRODUCTION

VWF multimer size is controlled by several mechanisms in the circulation including proteolysis at a single peptide bond Tyr ^{1605} –Met ^{1606} in the VWF-A2 domain. This cleavage is the function of the plasma metalloprotease ADAMTS-13, first identified in 1996,(Furlan, *et al* 1996, Tsai 1996) and further characterised in 2001.(Levy, *et al* 2001, Zheng, *et al* 2001) Defective ADAMTS-13 proteolysis results in the accumulation of uncleaved or partially cleaved unusually or ultra-large (UL) multimers of VWF, resulting in systemic thrombotic microangiopathies such as thrombotic thrombocytopenic purpura (TTP).(Moake, *et al* 1982) The possible effects of increased ADAMTS-13 proteolysis on circulating VWF levels are less clear. *In-vitro* studies have shown a variation in susceptibility of VWF to ADAMTS-13 mediated proteolysis according to blood group,(Bowen 2003) although whether this relates to lowered VWF levels has yet to be determined. Furthermore, increased susceptibility of VWF to proteolysis by ADAMTS-13 has been shown to associate with the Y1584C mutation,(Bowen and Collins 2004) reported as a founder haplotype among patients with type 1 VWD.(O'Brien, *et al* 2003)

Circulating plasma VWF in patients with type 1 VWD demonstrates normal functional activity and multimeric pattern. At the time of this study, the clearance pathway of VWF was poorly understood and it had not been established whether removal of VWF multimers was size-dependent.

High molecular weight (HMW) multimers of VWF preferentially bind to collagen(Santoro 1983) and therefore their function can be measured using the VWF collagen-binding assay (VWF:CB)(Brown and Bosak 1986). An inverse relationship has been demonstrated between VWF-related parameters and ADAMTS-13 activity over a wide range of VWF concentrations including following DDAVP, suggestive of consumption of ADAMTS-13 following the release of HMW multimers of VWF.(Mannucci, *et al* 2004, Reiter, *et al* 2003)

6.2 AIMS

The aim of this study was to examine the relationship between VWF multimer size and the release and clearance of VWF by comparison of VWF level and collagen binding activity prior to and following DDAVP infusion. ADAMTS-13 activity was measured to determine whether this related to VWF clearance and/or VWF levels in this group of patients with type 1 VWD. It is also possible that released VWF multimers are more susceptible to proteolysis by ADAMTS-13 and that this may account for the increased plasma clearance of VWF observed in some type 1 VWD patients. To investigate this, susceptibility of HMW multimers of VWF to ADAMTS-13 mediated proteolysis was determined in the type 1 VWD patients.

6.3 MATERIALS AND METHODS

6.3.1 Patients and controls

Subgroups of type 1 VWD patients (n = 26) and haemophilia A controls (n = 10) from the cohorts recruited to the VWF clearance study in Chapter 4 were investigated.(Appendix 3) Diagnostic phenotypic data for the patient and control groups is summarised in Table 5.1. For the proteolysis studies, plasmas from a homozygous Y1584Y individual and a heterozygous Y1584C individual were used as controls. Both individuals had haemostatic parameters within the normal range.

6.3.2 VWF collagen binding ELISA (VWF:CB)

Principle

Collagen binds to VWF with preferential binding for HMW multimers of VWF. The ELISA used to measure VWF:CB activity in this study is based on the originally described immunoassay. (Brown and Bosak 1986).

Chemicals, reagents, controls, equipment and method

Chemicals, reagents, controls, equipment and method were as described for the VWF:Ag ELISA (Chapter 3, Section 3.2.2) using the following modifications:

1. The standard curve consisted of doubling dilutions of pooled normal plasma from 1/160 to 1/3200.
2. Coating antibody: human type III collagen (Cambridge Bio Science, UK) diluted in CB coat buffer 3 μgmL^{-1} (w/v), pH 7.3. Overnight incubation was at room temperature.

3. CB Coat buffer, pH 7.3 :
    a. NaCl 8.0 g
    b. $Na_2HPO_4.12H2O$ 2.11 g
    c. $NaH2PO_4.H2O$ 0.2 g
    d. KCL 0.2 g
    e. Distilled H_2O 5 L
4. Dilution buffer: PBS/Tw-20/1% BSA (Sigma-Aldrich, Dorset, UK)
5. Following incubation with coat buffer, free binding sites were blocked with dilution buffer for 1 h, 150 μL per well, room temperature. This was then washed x 5 before addition of plasma patient and reference samples.
6. Capture antibody (HRP-conjugated rabbit anti-human VWF) diluted 1/3000 in dilution buffer
7. CV for the assay was 7%.

6.3.3 Measurement of release and clearance of VWF:CB and VWF:Ag following DDAVP

Plasma samples were analysed prior to (T_0) and over 6 h following DDAVP administration (T_1 - T_6). The method used to calculate VWF:CB $t_{1/2}$ and VWF:Ag $t_{1/2}$ is described in Chapter 3, Section 3.5.

6.3.4 Measurement of ADAMTS-13 activity

Analysis was performed by Dr Richard Starke, University College London

ADAMTS-13 activity was measured using a method based on the preferential binding of HMW forms of VWF to collagen, as previously described.(Gerritsen, *et al* 1999) The diluted plasma sample to be tested was added to normal human plasma in which protease activity had been abolished. The VWF present in the protease-depleted plasma was digested by the ADAMTS-13 in the test plasma. VWF:CB activity of the LMW multimers resulting from the proteolytic degradation of VWF was measured. ADAMTS-13 activity was quantitated using a calibration curve obtained from the incubation of VWF-substrate with serial dilutions of normal plasma.
CVs for the assay were 3.5% (intra-plate) and <5% (inter-plate). Plasma samples were analysed prior to (T_0) and over 6 h following DDAVP administration (T_1 - T_6).

6.3.5 *Susceptibility of VWF to proteolysis*

Analysis was performed by Dr Derrick Bowen, School of Medicine, Cardiff University

Cryoprecipitate was prepared from plasma as previously described,(Bowen and Collins 2004) and dissolved in 50 μL of Tris-HCl (5 mmol L^{-1}, pH 8.0). VWF:Ag of the dissolved cryoprecipitate was determined using ELISA. Proteolysis was performed using a minor modification of a previously described method(Gerritsen, *et al* 1999): dissolved cryoprecipitate was used as the source of VWF, and group O cryodepleted plasma was used as the source of ADAMTS-13. Both the quantity of VWF:Ag and the volume of group O cryodepleted plasma were identical for all proteolysis incubations. The same group O cryodepleted plasma was used for all experiments. Proteolysis reactions were incubated at 37°C for 3 hours and the extent of proteolysis was assessed using VWF multimer analysis (Chapter 3, Section 3.3) applied to aliquots taken at the start and end of the proteolysis incubation. The susceptibility of VWF to proteolysis was measured in patient samples at T_0 and T_1.

6.3.6 *Genotypic analysis of the Y1584C mutation*

Genomic DNA was extracted as described in Chapter 3, Section 3.4.1. A 682-bp fragment of exon 28 of *VWF* was amplified by PCR using primers K2A and K1B primers as described in Section 3.4.3. The Y1584C amino acid mutation in the A2 domain of VWF results from the single nucleotide polymorphism (SNP) 4751A>G in exon 28 of the *VWF* gene. This SNP affects a *Kpn*I restriction site (GGTACC, A = *Kpn*I$^+$ and encodes tyrosine, G = *Kpn*I$^-$ and encodes cysteine) Thus, the 682 bp K2A-K1B PCR product was genotyped at codon 1584 by incubating an aliquot (15 μL) for 2 hs with 40 U of *Kpn*I restriction enzyme (Roche, East Sussex, UK). *Kpn*I$^+$ yielded fragments of 276 and 406 bp, for *Kpn*I$^-$ the PCR product remained undigested at 682 bp. 1.5 % agarose gel was prepared (2.25 g agarose, 150 mL TBS, 8 μL ethidium bromide) and 8 μL of restricted product was run with 3 μL loading buffer. Marker XIV was used (2 μL MXIV, 15 μL H_2O, 4 μL loading buffer). Unrestricted PCR product was used as the negative control (blank).

6.3.7 *Data and statistical analysis*

Analyses of data were performed using GraphPad Prism (GraphPad Prism version 4.0, GraphPad Software, San Diego, USA). Data was analysed using the Mann-Whitney-Wilcoxon test and correlation analysis was performed using Spearman's rank. Data are presented as median values with range.

6.4 RESULTS

6.4.1 Collagen binding activity and VWF release

VWF:CB activities, VWF:Ag levels and ratios of VWF:CB to VWF:Ag at T_0 and T_1 in type 1 VWD patients (n = 26) and haemophilia A controls (n = 10) are summarised in Table 6.1. As expected, VWF:CB activities were significantly lower in the VWD study patients than controls ($P < 0.05$) at both T_0 and T_1 and no significant difference was found between VWF:CB/VWF:Ag ratios in the study (median 0.9, $P > 0.1$, n = 26) and control groups (0.96, n = 10, Table 6.1).

In the type 1 VWD patients, the ratio of VWF:CB to VWF:Ag at T_1 was significantly higher than at T_0, $P < 0.01$ (Fig.6.1), with respective median values of 1.1 (0.5 – 1.5) and 0.9 (0.6 – 1.4). No significant difference was found between the median ratios at T_0 and T_6 (median 0.97, $P = 0.2$). VWF:CB/VWF:Ag ratios were also increased in the haemophilia A controls at T_1 (median 1.1, 0.9 – 1.4, Fig.6.2) compared to T_0 (0.95, 0.8 – 1.3) although this was not shown to be statistically significant ($P = 0.08$). Within the VWD study group, 20 patients demonstrated an increase in VWF:CB/VWF:Ag ratio following DDAVP, reflecting the release of HMW multimers of VWF. Of the six VWD patients in whom the VWF:CB/VWF:Ag ratio was reduced at T_1 compared to T_0, two patients (Patients 10 and 25) from one kindred (Kindred 10) displayed significant reduction in T_1 VWF:CB/VWF:Ag ratios compared to the median, with respective T_0 values of 0.9 and 0.9 falling to 0.6 and 0.5 at T_1. In the control group, a reduction in VWF:CB/VWF:Ag ratio between T_0 and T_1 was shown in three subjects, although the values at T_1 did not vary significantly from the median.

Table 6.1. VWF:Ag, VWF:CB and VWF:CB/VWF:Ag ratios prior to and 1 h following administration of DDAVP in type 1 VWD patients and haemophilia A controls. ADAMTS-13 activity is also shown in the type 1 VWD patients.

*$P < 0.005$; ** $P < 0.05$; n.s. not significant; NT. not tested;

	pre DDAVP (T_0)				1h post DDAVP (T_1)			
	VWF:Ag ($IUdL^{-1}$) 50 - 150	**VWF:CB ($IUdL^{-1}$) 50 - 175**	**VWF:CB/ VWF:Ag ratio**	**ADAMTS-13 activity (%) 66 - 126**	**VWF:Ag ($IUdL^{-1}$)**	**VWF:CB ($IUdL^{-1}$)**	**VWF:CB/ VWF:Ag ratio**	**ADAMTS-13 activity (%)**
Type 1 VWD (n=26)	47* (11 – 84)	43* (9 - 102)	0.91 n.s. (0.60 – 1.37)	89 (51 – 127)	175** (41 – 271)	178* (41 – 265)	1.10 n.s. (0.50 – 1.50)	75 (42.7 – 111)
Haemophilia A (n=10)	94 (45 -193)	95 (49 – 186)	0.96 (0.85 – 1.35)	NT	216 (165 – 307)	246 (154 – 300)	1.12 (0.89 – 1.36)	NT

Figure 6.1. Ratio of VWF:CB to VWF:Ag pre- and post-DDAVP in patients with type 1 VWD (n = 26). VWF:CB/VWF:Ag ratios are shown at baseline (T_0) and 1 h and 6 h following DDAVP (T_1, T_6). The VWF:CB/VWF:Ag ratio at T_1 was found to be significantly increased (median 1.1, $P < 0.01$) as compared to T_0 (0.9). Median ratios at T_0 and T_6 were not found to differ significantly (median 0.97, $P = 0.2$).

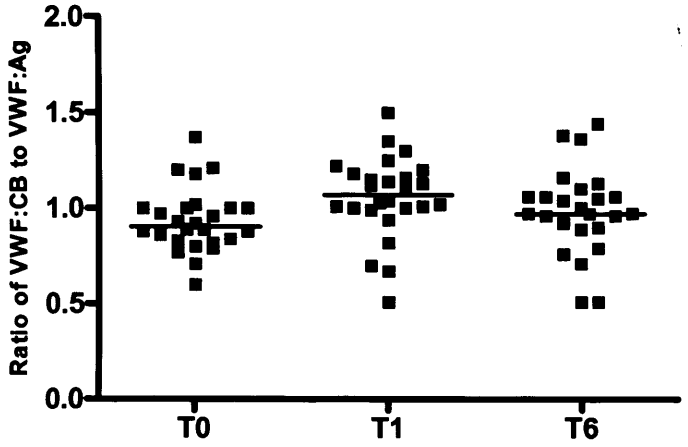
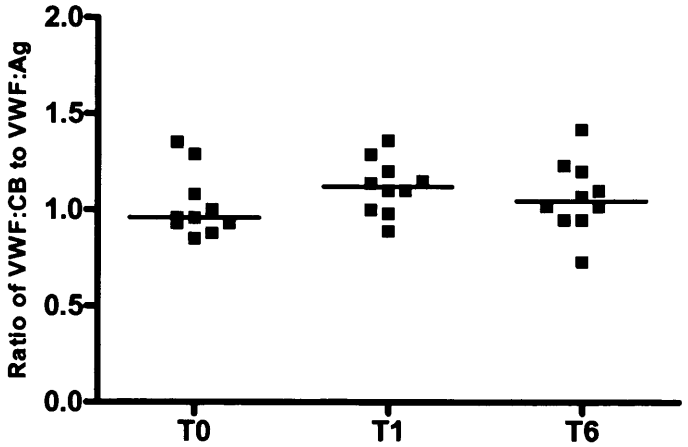


Figure 6.2. Ratio of VWF:CB to VWF:Ag pre- and post-DDAVP in haemophilia controls (n = 10). Increased ratios of VWF:CB/VWF:Ag were found at T_1 (median 1.1) compared to T_0 (0.9), however this was not statistically significant, $P = 0.08$.



6.4.2 Collagen binding activity and VWF clearance

Calculated VWF:CB $t_{1/2}$ values for the type 1 VWD patients and haemophilia A controls are given in Appendix 3 and shown in Fig.6.3. As was shown for VWF:Ag $t_{1/2}$, VWF:CB $t_{1/2}$ values in the type 1 VWD study patients were significantly shorter (median 4.4 h, $P < 0.005$) than the controls (median 8.2 h, Table 6.2). Similarly, correlation was found between VWF:CB at T_0 and VWF:CB $t_{1/2}$ in the combined study and controls groups ($r = 0.43$, $P < 0.05$, $n = 36$), but not in either group when analysed separately ($r = 0.24$, $P = 0.2$, $n = 26$; $r = 0.13$, $P = 0.7$, $n = 10$). In addition, VWF:CB $t_{1/2}$ correlated significantly with VWF:Ag $t_{1/2}$ on analysis of both groups together ($r = 0.77$, $P < 0.0001$, $n = 36$, Fig.6.4) as well in each group alone ($r = 0.66$, $P < 0.005$, $n = 26$; $r = 0.79$, $P < 0.005$, $n = 10$).

Table 6.2. Calculated half-life values of VWF:Ag and VWF:CB in type 1 VWD patients and haemophilia A controls.

Data are presented as medians (range). *$P < 0.005$

	VWF:Ag $t_{1/2}$ (h)	**VWF:CB $t_{1/2}$ (h)**
Type 1 VWD (n=26)	3.8* (1.9 – 9.6)	4.4* (1.7 – 10.3)
Haemophilia A (n=10)	13.7 (4.1 - >30)	8.2 (3.4 – 35.9)

Figure 6.3. Calculated VWF:CB half-life values in type 1 VWD patients and haemophilia A controls). Median VWF:CB $t_{1/2}$ values in the VWD patients (4.4 h, $P < 0.005$, $n = 26$) were significantly shorter than the controls (8.2 h, $n = 10$).

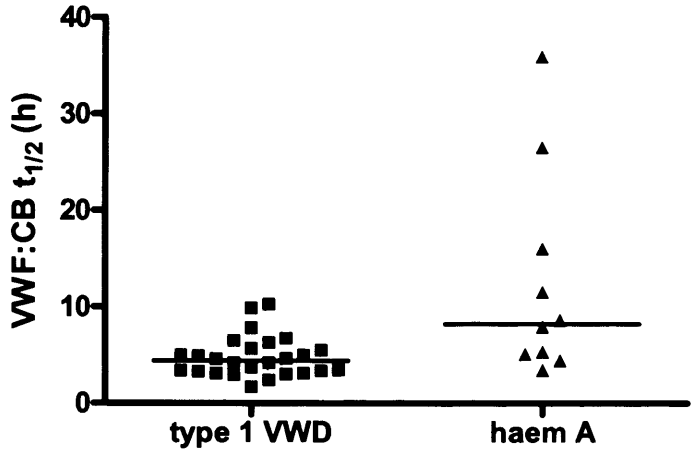
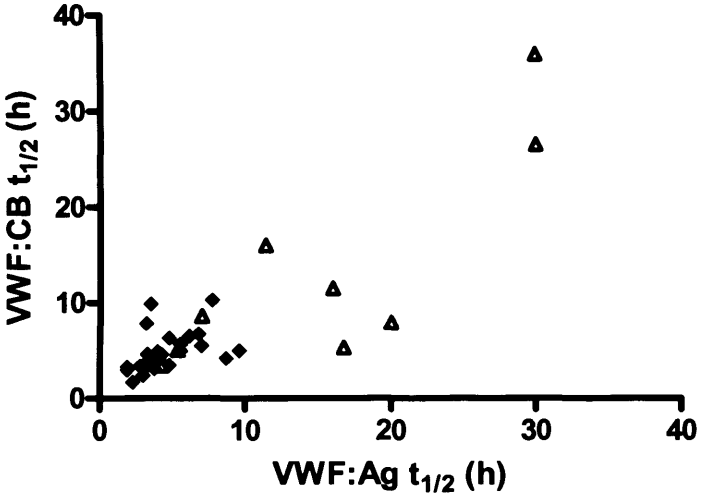


Figure 6.4. Relationship between half-life values of VWF:Ag and VWF:CB in type 1 VWD patients and haemophilia A controls. Values of VWF:Ag $t_{1/2}$ and VWF:CB $t_{1/2}$ are shown for patients with type 1 VWD (◆ ,n = 26) and haemophilia A controls (△ , n = 10). Correlation between VWF:Ag $t_{1/2}$ and VWF:CB $t_{1/2}$ was found on analysis of the study and control groups together (r = 0.77, $P < 0.0001$, n = 36) and study patients (r = 0.66, $P < 0.005$, n = 26) and control groups alone (r = 0.79, $P < 0.005$, n = 10).



6.4.3 ADAMTS-13 activity and VWF clearance

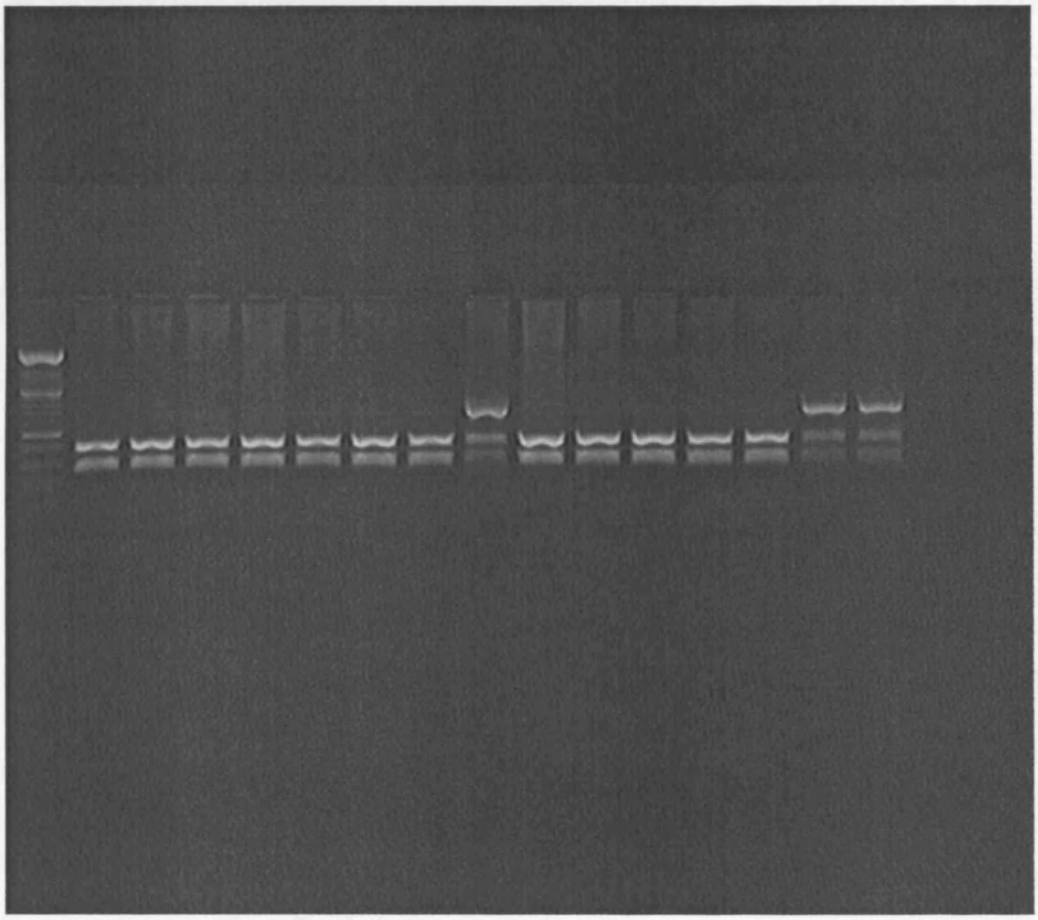
ADAMTS-13 activities were measured pre- and post-DDAVP (T_0 - T_6) in type 1 VWD patients (n = 26). ADAMTS-13 levels at T_0 were within normal limits in 22 of the type 1 VWD patients; the remaining four had reductions in ADAMTS-13 of up to 15% (Fig. 6.5). DDAVP infusion resulted in an overall decrease in ADAMTS-13 levels (Fig. 6.5, Table 6.1). The fall in ADAMTS-13 activity from T_0 to T_1 correlated significantly with the increase in VWF:Ag and VWF:CB (r = -0.58, $P < 0.005$; r = -0.32, $P < 0.0005$, data not shown). However, no inverse correlation was found between absolute ADAMTS-13 activity and either VWF:Ag level or VWF:CB activity prior to or over 6 h following DDAVP, $T_0 - T_6$ ($P > 0.1$, data not shown). In addition no correlation was found between baseline ADAMTS-13 activity (T_0) and either VWF:Ag $t_{1/2}$ (r = -0.03, $P = 0.9$) or VWF:CB $t_{1/2}$ (r = 0.12, $P = 0.6$). ADAMTS-13 activity was found to be higher in VWD patients of blood group A (median 99.7% (80.2 – 126.6), n = 8, $P < 0.01$) than blood group O (80.3% (50.8 – 95.8) n = 16, data not shown).

Figure 6.5. ADAMTS-13 activity pre- and post-DDAVP in patients with type 1 VWD (n = 26). ADAMTS-13 activities are shown at baseline (T_0) and 1 h and 6 h following DDAVP (T_1, T_6 respectively). ADAMTS-13 activity was reduced at both T_1 (median 75%, $P < 0.05$) and T_6 (76%, $P < 0.05$) as compared to T_0 (89%).

6.4.4 Y1584C mutation and VWF clearance

Genotypic analysis for 4751A>G was performed in type 1 VWD patients (n = 26) and haemophilia A controls (n = 10) Heterozygosity for the A and G alleles was demonstrated in one study patient (Patient 12) and one control (Control 11). (Fig.6.6) Respective VWF:Ag $t_{1/2}$ values in these heterozygous subjects were 4.8 and 16 h compared to median values of 3.8 and 11.4 h in the homozygous 4751A VWD patients (n = 25) and haemophilia A controls (n = 9).

Figure 6.6. Genotypic analysis for 4751A>G performed as described in Materials and Methods. *KpnI*$^{+}$ yielded fragments of 276 and 406 bps, for *KpnI*$^{-}$; the PCR product remained undigested at 682 bps. Lane 16: positive control (Y1584C) showing undigested and digested fragments; Lane 17 Blank. Subjects in lanes 9 (Patient 12) and 15 (Control 11) can be seen to be heterozygous for the A and G alleles (heterozygous Y1584C).



Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

6.4.5 Susceptibility of VWF to proteolysis by ADAMTS-13; relation to VWF clearance.

The susceptibility of VWF to ADAMTS-13 mediated proteolysis was measured pre- and 1 h post DDAVP infusion (T_0, T_1) in the type 1 VWD patients study group (n = 26).

Multimer profiles of cryo-precipitated VWF at T_0 and pre-proteolysis

All samples gave a multimer profile essentially comparable with normal. In addition, all samples showed a relative increase in a band, which had the fastest electrophoretic mobility on the blot (Fig.6.7).

Proteolysis of cryo-precipitated VWF, T_0

The VWF of only one sample, Patient 12 (Y1584C as shown above), showed a significantly increased susceptibility to proteolysis compared with a homozygous Y1584Y control. Susceptibility was equivalent to that observed for a heterozygous Y1584C control (Fig 6.8).

Multimer profiles of cryo-precipitated VWF at T_1 and pre-proteolysis

1. Patient samples showed the full range of multimers from LMW to HMW (Fig. 6.8).
2. All T_1 samples showed an increase in the low and intermediate weight multimers, suggesting an increased overall level of proteolysis (Fig. 6.7).

Comparison of multimer profiles of cryo-precipitated VWF at T_0 and T_1 and pre-proteolysis

1. There was a greater representation of HMW multimers in Patients 18, 21 and 26 at T_1 compared with T_0. This is not unexpected in view of the action of DDAVP.
2. The relative enrichment noted above for the fastest migrating band at T_0 was apparent, but to a lesser extent, at T_1 (Fig.6.7).

Comparison of proteolysis of cryo-precipitated VWF at T_0 and T_1

1. Patient 12 (Y1584C), who showed an increased susceptibility to proteolysis at T_0 (Fig.6.8A), also showed increased susceptibility to proteolysis at T_1 (Fig. 6.8B), and the magnitude was similar for both.
2. All remaining patients showed an equivalent susceptibility to proteolysis at T_1 compared with T_0.

3. The fast migrating band that appeared increased prior to proteolysis in both pre- and post-DDAVP samples, was no longer enriched after proteolysis.

In summary, for a given patient, plasma VWF showed a similar extent of proteolysis at T_0 and T_1, irrespective of the Y1584C phenotype (Fig.6.8B)
Although the T_1 multimer profiles indicate that there is an increased level of proteolysis of VWF in the circulation at T_1, the proteolysis studies show that this is not associated with an increased susceptibility of VWF to proteolysis.

The significance of the fast migrating band is uncertain: although this increase has not been observed previously on screening of normal samples, because it immuno-stained with polyclonal anti-VWF, it must be related to VWF. The increase in this band was very evident at T_0, less evident at T_1 and, following proteolysis of each sample, the band was no longer enriched, possible because it was masked by a product of VWF proteolysis.

Figure 6.7 Multimeric analysis of VWF pre- and post- DDAVP, before and after VWF proteolysis. Respective pre- and post-DDAVP samples are placed side-by-side. The gel shows the fast-migrating band which is increased in all samples, the increase in the satellite bands of each triplet post-DDAVP and the equivalent proteolysis of each pre- and post-DDAVP pair.

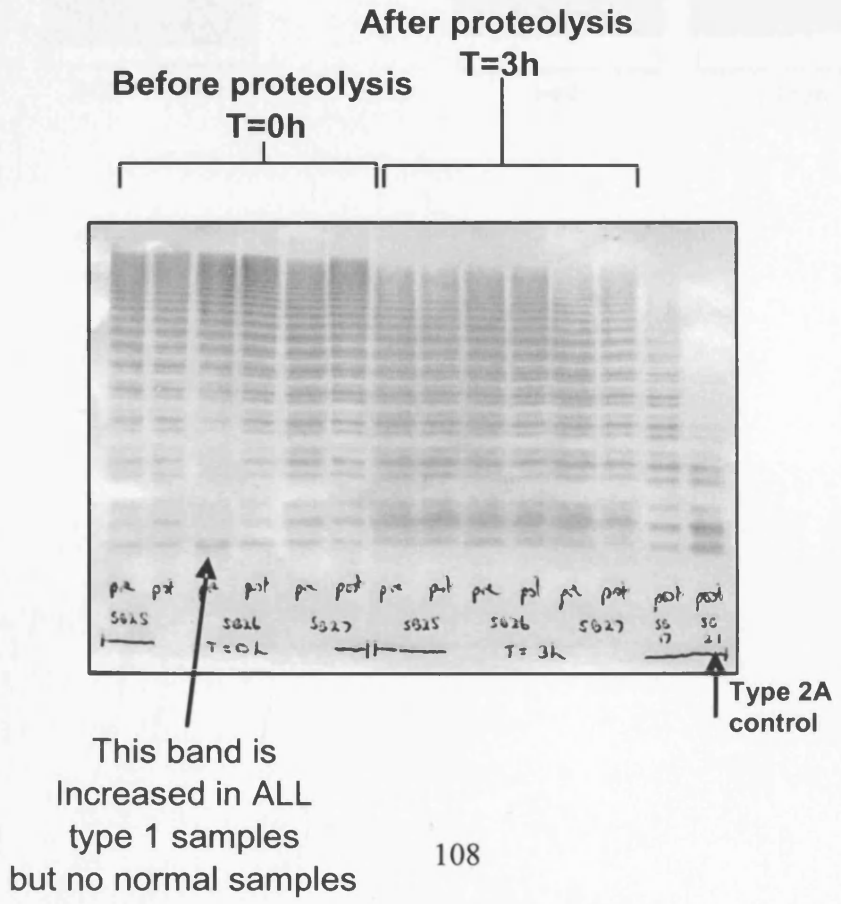
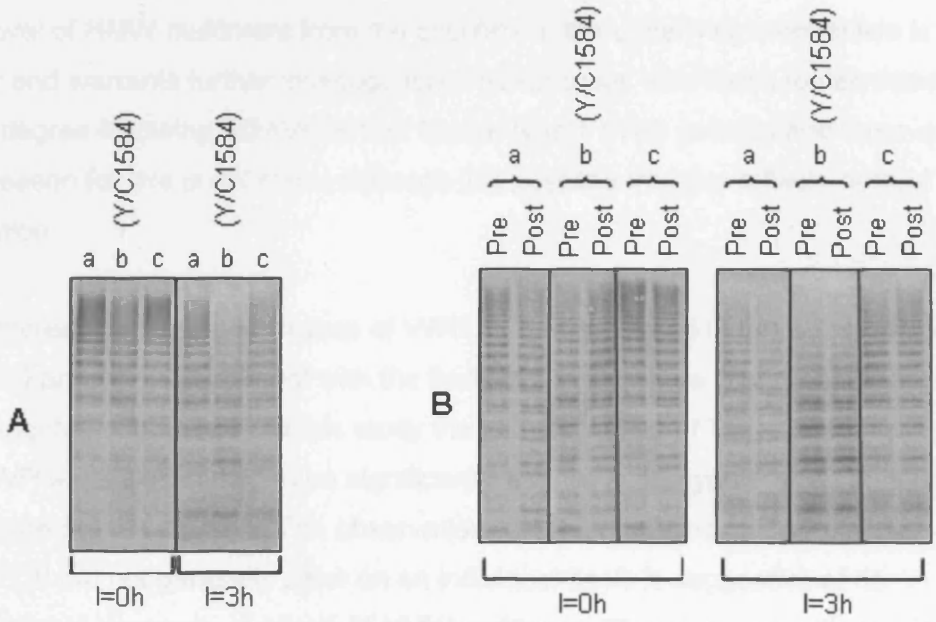


Figure 6.8 Assessment of the extent of proteolysis of plasma VWF using multimer analysis

A. Plasma VWF from 3 patients (a, b, c) with type 1 VWD was cryoprecipitated and incubated with group O cryodepleted plasma as a source of ADAMTS-13. Comparison of the multimer profiles immediately before proteolysis (I = 0h) and at the end of the proteolysis incubation (I = 3h) reveals a loss of HMW forms and an increase in LMW forms, together with a relative increase in the satellite bands. Proteolysis was considerably greater in the case of patient 'b', who is heterozygous for Y1584C (Patient 12)

B. The analysis is as described for panel A, however it includes both pre- and post-DDAVP plasma VWF (T_0 and T_1)



6.5 DISCUSSION

In this study, the relationship between the release and clearance of VWF and its multimer size was examined by measuring the level and collagen binding activity of VWF released following stimulation by DDAVP in patients with type 1 VWD and haemophilia A controls. The finding of an increase in the ratio of VWF:CB to VWF:Ag following DDAVP infusion in the majority of subjects is consistent with an increase in circulating HMW multimers of VWF.(Ruggeri, *et al* 1982) This illustrates a structural difference between VWF released following DDAVP and circulating VWF, which may affect the clearance kinetics of VWF as discussed in Chapter 4. The ratio of VWF:CB to VWF:Ag was found to decrease significantly following DDAVP in two type 1 VWD patients from one kindred. Although this is suggestive of removal of HMW multimers from the circulation, the underlying mechanism is not clear and warrants further investigation. The ratio was also found to decrease to a less degree following DDAVP in four further type 1 VWD patients and three controls: the reason for this is not clear, although it is possible that this reflects normal variation.

The increased plasma clearance of VWF:Ag demonstrated in this subgroup of type 1 VWD patients is consistent with the findings for the whole type 1 cohort previously investigated (Chapter 4). In this study the half-life values of VWF:CB following DDAVP were also shown to be significantly shorter in the type 1 VWD patients as compared to the controls. The observation that the half-lives of VWF:Ag and VWF:CB did not generally differ on an individual basis is suggestive of no preferential clearance of released HMW multimers. This contrasts with previously reported findings in similar studies of released VWF,(Casonato 2005, van Genderen P. J. J 1997) as well as some findings in animal studies of infused VWF.(Turecek, *et al* 1997) However, a recent animal model has shown clearance of infused VWF to be independent of multimer size,(Lenting, *et al* 2004) consistent with the findings of released VWF in this study.

In view of the normal multimeric pattern and apparently normal function of the residing circulating plasma VWF in type 1 VWD, it is not clear whether increased clearance of VWF arises subsequent to its increased proteolysis. Having demonstrated no relationship in this study between the plasma clearance of VWF and the size of its multimers by indirect measurement, VWF proteolysis was directly examined in the type 1 VWD patients. The absence of a relationship between ADAMTS-13 activity and the half-lives of both VWF:Ag and VWF:CB suggests that

proteolysis by ADAMTS-13 does not directly relate to the plasma clearance of HMW multimers of VWF in patients with type 1 VWD. The question as to whether increased clearance of VWF could result from increased susceptibility to ADAMTS-13-mediated proteolysis has also been addressed. This does not appear to be the case. Prolonged *in vitro* incubation of purified patient VWF (containing HMW multimers) with ADAMTS-13 results in degradation of the VWF to its LMW multimers and ultimately to dimeric proteolytic fragments. Based on this principle, the susceptibility of VWF to proteolysis was measured in patient samples pre- and post-DDAVP thereby facilitating comparison between circulating and released VWF. Comparison of the multimer patterns before and 3 h after incubation of both pre- and post-DDAVP plasma with ADAMTS-13 revealed that there was no evidence of increased susceptibility of VWF to proteolysis in 25 out of 26 type 1 VWD patients studied. Only the plasma of one patient showed increased proteolysis. Thus the differences in VWF clearance rates of the type 1 VWD patients in this study are not reflected by differences in susceptibility to proteolysis by ADAMTS-13.

The one study patient who did demonstrate increased susceptibility to proteolysis was shown to be heterozygous for the Y1584C mutation. Although this mutation is known for its ability to influence susceptibility to ADAMTS-13 *in vitro*,(Bowen and Collins 2004) the relationship between Y1584C and the *in vivo* plasma clearance of VWF has not been examined previously. It is possible that the increased proteolysis associated with C1584 is due to the proximity of this residue to the ADAMTS-13 cleavage site in the A2 domain of VWF. C1584 is fully solvent exposed and available for new inter- or intramolecular disulphide linkages that could alter the conformation of the A2 domain and enhance access to the cleavage site for ADAMTS-13.(O'Brien, *et al* 2003, Sutherland, *et al* 2004) The VWF half-life in the type 1 VWD patient identified in this study as heterozygous for Y1584C was reduced at 4.8 h. However, the half-lives in the 25 'wild types' were also reduced (median 3.8 h) and therefore the increased clearance observed in these patients cannot be explained by Y1584C. Taken together with the normal plasma VWF:Ag clearance found in the Y1584C haemophilia A control (16 h), these results suggest that the plasma clearance of VWF is unlikely to be significantly affected by the presence of this mutation. The Y1584C mutation is further discussed in Chapter 9.

The absence of a relationship between ADAMTS-13 mediated proteolysis and VWF clearance supports indirect experimental data. Firstly, basal VWF levels have not been shown to be increased in ADAMTS-13 deficient mice when compared to normal mice of the same genetic background. (Motto, *et al* 2005) Secondly, no

difference in VWF clearance rates has been demonstrated between wt-VWF and a variant containing a type 2A mutation (which increases VWF susceptibility to cleavage by ADAMTS-13) in a rat model.(Stoddart, *et al* 1996)

Having found no evidence of a relationship between VWF proteolysis and the rate of VWF clearance, the mechanism by which increased ADAMTS-13 proteolysis may affect circulating VWF levels remains unknown. In contrast to previous reports in normal individuals,(Mannucci, *et al* 2004) type 1 VWD patients of blood group O were not found to have higher activities of ADAMTS-13 than non-O patients in this study. Recent *in-vitro* studies have shown a variation in the susceptibility of VWF to ADAMTS-13 mediated proteolysis according to blood group in the order O = B > A = AB.(Bowen 2003, O'Donnell, *et al* 2005) These observations suggest that the presence of ABH antigens on one or more of the glycosylation moieties that flank the VWF cleavage site for ADAMTS-13 may influence proteolysis and through this mechanism influence plasma VWF level. However this possibility remains to be shown. It is possible that VWF proteolysis and VWF level are not directly related although both may be influenced by ABO blood group.

It is also possible that rather than influencing VWF level, ADAMTS-13-mediated proteolysis affects the function of VWF and it is this that gives rise to symptoms of bleeding. It has been proposed that following proteolysis of the VWF-platelet string, the fragments of VWF that are released into the circulation are no longer subject to shear stress and the subsequent stages of clot formation at the cell surface result from the participation of the remaining portion of the VWF-platelet string.(Dong 2005) Increased rates of proteolysis of the VWF-platelet string could therefore result in defective haemostasis via a variety of mechanisms that are independent of circulating VWF levels. (Bowen and Collins 2006) As well as explaining some of the variability in bleeding phenotype between patients with similar VWF:Ag levels, this theory may account for the compromise in primary haemostasis observed in some individuals with normal standard functional assays of VWF.(Bowen and Collins 2006) It may also, at least partly account for the enrichment of blood group O in type 1 VWD.(Gill, *et al* 1987)

The relationship between plasma levels of VWF-related parameters and ADAMTS-13 activity over a range of concentrations prior to and over 6 h following infusion of DDAVP was examined in this group of type 1 VWD patients. The decrease in ADAMTS-13 activity after infusion of DDAVP in the type 1 VWD patients is consistent with previously reported findings in normal subjects and type 1 VWD

patients.(Mannucci, *et al* 2004, Reiter, *et al* 2003) DDAVP as a direct cause of this reduction in ADAMTS-13 activity has been excluded by studies of both DDAVP and VWF-containing plasma concentrate in patients with type 3 VWD.(Mannucci, *et al* 2004). Inverse correlation was shown between the decrease in ADAMTS-13 activity and simultaneous increase in VWF level and collagen binding activity following DDAVP. However, multimer analysis indicated an increase in the low and intermediate VWF multimers 1 h following DDAVP compared to baseline. Although these findings may appear to contradict the observed overall increase in the ratio of VWF:CB to VWF:Ag following DDAVP, it is possible that the VWF:CB reflects the presence of very HMW forms which are not in sufficient quantity to visualise on multimer analysis or which do not transfer efficiently from the multimer gel. In contrast to previously reported findings, no inverse correlation between absolute values of ADAMTS-13 and VWF:Ag or VWF:CB was found at any time point prior to or following DDAVP in this study. (Mannucci, *et al* 2004, Reiter RA 2003) Taken together, these findings suggest that the relationship between circulating VWF and ADAMTS-13 levels in patients with type 1 VWD is likely to be complex. Although ADAMTS-13 may localise onto UL-VWF released following DDAVP, resulting in an increase in proteolysis and reduction in detectable ADAMTS-13 activity, such a direct regulatory mechanism between ADAMTS-13 and its substrate has yet to be established. Further studies are warranted to elucidate both the mechanism and biological significance of these findings.

Chapter 7

GLYCOSYLATION AND CLEARANCE OF VWF

7.1 INTRODUCTION

VWF is a highly glycosylated and sialylated protein containing 10 sites for O-linked and 12 sites for N-linked glycosylation. The structures of the VWF oligosaccharide side chains are very diverse and although their functional role remains unclear, the nature and extent of glycosylation of plasma VWF may play a significant role in the genetic variation of VWF plasma levels and/or its clearance.

Exposed galactose (Gal) residues are required for the binding of VWF to the hepatic asialoglycoprotein receptor (ASGPR). Therefore, variability in exposure of Gal may influence plasma VWF clearance *in vivo*. A mutation which alters cell type-specific expression of the *Galgt2* glycosyltransferase gene has been shown to result in increased clearance of VWF in a murine model giving rise to a partial quantitative deficiency of VWF.(Mohlke, *et al* 1999b) Another murine model has demonstrated increased clearance of endogenous VWF in the absence of the sialyltransferase ST3Gal-IV, (Ellies, *et al* 2002) and the finding of significant reduction in VWF half-life in rabbits following removal of sialyl-groups further supports the protective effect of sialylation.(Sodetz, *et al* 1977)

The lectin *Ricinus communis* agglutinin I (RCA I) has a high affinity for non-reducing terminal ß-linked Gal residues on VWF,(Matsui, *et al* 1991, Osawa and Tsuji 1987) and an increased RCA-I/VWF ratio, suggestive of increased Gal exposure, has been found in some patients with a partial quantitative deficiency of VWF.(Ellies, *et al* 2002) *Erythina crystagalli* agglutinin (ECA) also has an affinity for exposed Gal on the N-linked structure N-acetyllactosamine (Gal(ß1-4)GlcNAc) and O-linked GalNAc.(Debray, *et al* 1986, Iglesias, *et al* 1982)

The expression of A antigen on VWF released following DDAVP infusion has previously been shown to be increased when compared to plasma VWF.(Brown, *et al* 2002) This suggests that oligosaccharide structures may differ between pools of VWF, which may be relevant to the function and/or clearance of VWF.

7.2 AIMS

The aim of this study was to investigate variability in the exposure of galactose residues as a possible mechanism for increased clearance of VWF resulting in a reduction in VWF levels. Binding of the lectins RCA-I and ECA was examined in a cohort of patients with type 1 VWD and the relationship with VWF levels and the plasma clearance of VWF released following DDAVP was investigated. To determine whether differences in glycosylation profiles between circulating and released VWF could account for the increased VWF clearance observed in some type 1 VWD patients, lectin binding was examined both prior to and following administration of DDAVP.

7.3 MATERIALS AND METHODS

Binding of the lectins RCA-I and ECA to VWF was measured using ELISA assays that were developed and optimised for the purposes of this study, based on a previously described method.(Ellies, *et al* 2002) VWF:Ag levels were measured in parallel and results were expressed as a ratio of RCA-I or ECA binding to VWF per unit of VWF:Ag.

Several technical difficulties were encountered during the development and work-up of these immunoassays: the problem of non-specific binding of the lectin to the coating antibody was largely eliminated by substantially reducing the concentration of the coating antibody and increasing the concentration of bovine serum albumin in the block. Although these measures were successful in the cases of RCA-I and ECA, the non-specific binding observed with Conconavalin A (Con A) was not overcome, and therefore studies using Con A were abandoned.

7.3.1 Patients and controls

A subgroup of type 1 VWD patients (n = 29) was investigated in whom VWF:Ag $t_{1/2}$ had been measured following administration of DDAVP (Chapter 4). Binding of RCA-I and ECA to VWF was analysed in plasma samples at T_0. In addition, RCA-I binding to VWF was measured at T_1 and T_6 in the VWD study group. Reference ranges were derived from the plasma samples of 20 normal controls (n = 20).

7.3.2 *Measurement of Ricinus communis agglutinin-I binding to VWF.*

Principle

The assays were based on the ELISA for measurement of VWF:Ag (3.2.2), using polyclonal rabbit anti-human antibodies to capture VWF and biotinylated lectins as additional detector antibodies to bind to specific VWF oligosaccharide structures.

Chemicals, reagents, controls and equipment

Chemicals, reagents, controls and equipment were as described for the VWF:Ag ELISA (Chapter 3, Section 3.2.2) with the following modifications:

1. Dilution buffer: 150 mM TBS/ 0.1% Tw-20/ 3% BSA (Sigma-Aldrich, Dorset, UK), pH 8.0
2. Block: 3% BSA in 150 mM TBS, pH 8.0

   TBS, 150mM, pH 8.0

a. NaCl	11.68 g
b. TRIZMA-Base	12.12 g
c. Distilled H_2O	2000 mL

3. Capture antibody: Biotinylated *Ricinus Communis* Agglutinin I (RCA-I)) (Vector Laboratories, Peterborough, UK)
4. HRP-conjugated streptavidin (Vector Laboratories, Peterborough, UK)

Method

Plates were coated as per VWF:Ag ELISA with the rabbit anti-human VWF antibody, diluted 1/6000. Following overnight incubation at 4 °C the plate and washing, the plate was blocked with 3% (w/v) BSA/TBS, sealed and incubated on a plate shaker (400-500 oscillations per minute) at room temperature for 90 min before washing a further five times. The standard curve consisted of doubling dilutions of pooled normal plasma from 1/8 to 1/160. The patient plasma samples and reference samples were diluted 1:10 to 1:40 in 3% (w/v) BSA/TBS/0.1% (v/v) Tw-20. Each dilution was dispensed in triplicate, with 100 µL of sample per well. The plate was sealed and incubated on a plate shaker at room temperature for one hour, and then washed five times. RCA-I was diluted 1/5000 in 3% (w/v) BSA/TBS/0.1% (v/v) and 100 µL aliquots were dispensed at a final concentration of 1 µg/ml (w/v) and the plate was incubated for a further hour at room temperature. Following further washing, 100 µL aliquots of HRP-conjugated streptavidin diluted 1/10000 in 3% (w/v) BSA/TBS/0.1% (v/v) were added and the plate was incubated for 7 min. Prior to the end of incubation the substrate solution was prepared and the reaction was incubated and terminated as per the VWF:Ag ELISA method. The plate was read and standard curve derived as per VWF:Ag ELISA. The 1/80 dilution of the

reference plasma was taken as 100%. The RCA-I binding of each dilution of the test samples were extrapolated from the standard curve and the mean values calculated.

Coefficient of variation

Intra-plate: The repeatability was determined by assaying three concentrations of three control samples on six replicates in one assay run. CVs were 7.4, 8.1 and 8.8% respectively.

Inter-plate: The reproducibility was determined by testing three control samples at three dilutions on six replicates over 3 days: CV was consistently found to be less than 10% (8.3, 6.1 and 3.9% respectively).

Negative controls

The specificity for RCA-I was assessed with plasma from a type 3 VWD patient (FVIII:C 2 IU dL^{-1}; VWF:Ag < 1 IU dL^{-1}) diluted ten fold less than normal plasma at serial dilutions (1/1, 1/2, and 1/4). For all dilutions, the response was never above that of buffer. This demonstrates an absence of non-specific binding, although the negative findings could result from the absence of VWF and/or FVIII. To investigate this further, RCA-I binding was measured in plasma from a patient with severe haemophilia A (FVIII:C < 1 IU dL^{-1}, FVIII:Ag < 1 IU dL^{-1}, VWF:Ag 105 IU dL^{-1}): the RCA-I to VWF:Ag ratio was 1.03, indicating normal binding of RCA in the absence of FVIII. This was confirmed by demonstrating no significant alteration in RCA-I binding ($P < 0.01$) in three control samples following the elution of FVIII using $CaCl_2$.

7.3.3 Measurement of Erythina crystagalli agglutinin binding to VWF. Principle, chemicals, reagents, controls and equipment.

As described for the measurement of RCA-I binding to VWF (7.3.2), substituting biotinylated *Erythina Critically* Agglutinin I (ECA) (Vector Laboratories, Peterborough, UK) in place of RCA-I.

Method

As described for the measurement of RCA-I binding to VWF with the following amendments

1. ECA was diluted 1/500 in 3% (w/v) BSA/TBS/0.1% (v/v) and 100 µL aliquots were dispensed at a final concentration of 10 µg/ml and the plate was incubated for 75 min.

2. HRP-conjugated streptavidin was diluted 1/2000 in 3% (w/v) BSA/TBS/0.1% (v/v) and following the addition of 100 μL into each well, the plate was incubated for 10 min.

Coefficient of variation (CV)

Intra-plate: The repeatability was determined by assaying three concentrations of three control samples on six replicates in one assay run. CVs were 6.7, 7.4 and 7.8% respectively.

Inter-plate: The reproducibility was determined by testing three control samples at three dilutions on six replicates over 3 days: CV was consistently less than 10%.

Negative controls

As for RCA-I, the specificity for ECA-I was assessed with plasma from patients with type 3 VWD and severe haemophilia A. There was no binding to plasma from a type 3 VWD control (above) and ECA binding to the FVIII-deficient plasma (above) and plasma eluted of FVIII in three controls (above) was normal.

7.3.4 Data and statistical analysis

Analyses of data were performed using GraphPad Prism (GraphPad Prism version 4.0, GraphPad Software, San Diego, USA).Data was analysed using Spearman's rank correlation and the Mann-Whitney-Wilcoxon and Kruskal-Wallis tests and paired t-test. Data are presented as median values with range.

7.4 RESULTS

7.4.1 Binding of Ricinus communis agglutinin-I to VWF

RCA-I binding to VWF was measured in 29 patients with type 1 VWD at T_0 (Appendix 3) and 20 normal controls at basleine (Appendix 4). The median ratio of RCA-I binding to VWF per unit of VWF:Ag in the type 1 VWD patients was 1.25 at T_0 compared to 1.0 at baseline in the normal controls (Fig 7.1, Table 7.1). This represents a significant increase in RCA-I binding to VWF in the type 1 VWD patients ($P < 0.01$). Inverse correlation was found between the VWF binding of RCA-I and VWF:Ag level in the combined study and control groups ($r = -0.43$, $P < 0.005$, $n = 49$, Fig.7.2). Correlation between RCA-I binding and baseline VWF:Ag was significant in the controls alone ($r = -0.45$, $P < 0.05$, $n = 20$), but not in the VWD patients ($r = -0.18$, $P = 0.4$, $n = 29$)

7.4.2 Binding of Ricinus communis agglutinin-I to VWF released following DDAVP

RCA-I binding to VWF at was measured at T_1 and T_6 in the type 1 VWD patients. There was no significant difference found between RCA-I binding to VWF at T_1 (median 1.19, $P = 0.7$, Fig. 7.3) and T_6 (median 1.16, $P = 0.6$) when compared to T_0 (median 1.25) in the VWD patients (n = 29).

7.4.3 Binding of Erythina Cristagalli Agglutinin to VWF

ECA binding was measured in the same study and control groups. The ratio of ECA binding to VWF per unit of VWF:Ag was significantly higher in the type 1 VWD patients at T_0 (median 1.38, $P < 0.01$, n = 29) than controls at baseline (1.0, n = 20, Fig.7.4, Table 7.1). Inverse correlation was found between ECA binding to VWF and VWF:Ag level in the combined study and control groups (r = -0.53, $P < 0.001$, n = 49, Fig.7.5). This correlation was also found to be significant in the controls alone (r = -0.66, $P < 0.005$, n = 20), but not in the VWD patients (r = -0.16, $P = 0.38$, n = 29).

Table 7.1. Binding of the lectins RCA-I and ECA to VWF in type 1 VWD patients and normal controls.

Data is presented as median with range (#) and 95% confidence intervals (C.I.) where stated.

* $P < 0.01$; NT not tested

	VWF:Ag ($IUdL^{-1}$)	VWF:Ag $t_{1/2}$ (h)	RCA-I binding to VWF	ECA binding to VWF
Type 1 VWD (n = 29)	46 (11 – 84#)	4.1 (1.9 – 9.6#)	1.25* (0.78 – 2.05#) 95% C.I. 1.11 – 1.29	1.38* (0.75 – 2.50#) 95% C.I. 1.34 – 1.67
Normal controls (n = 20)	115 (66 – 194#)	NT	1.0 (0.59 – 1.4#) 95% C.I. 0.92 -1.09	1.0 (0.49 – 1.87#) 95% C.I. 0.88 – 1.28

Figure 7.1. Binding of *Ricinus communis* agglutinin I to VWF in type 1 VWD patients and control subjects. RCA-I binding to VWF in type 1 VWD patients was increased (median 1.25, $P < 0.01$, n = 29) compared to the controls (median 1.0).

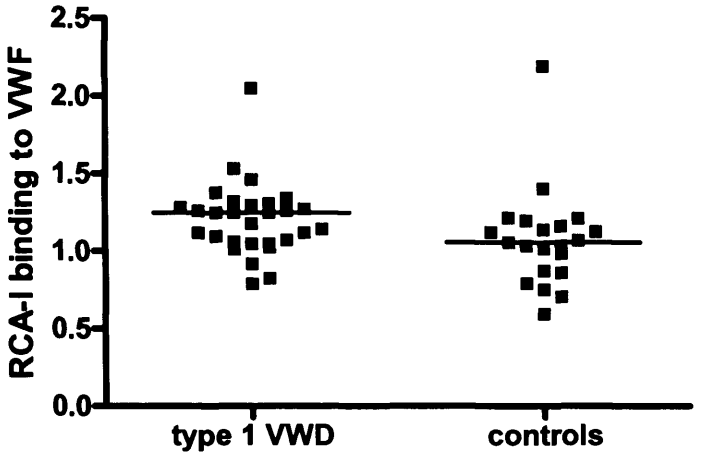


Figure 7.2. Relationship between binding of *Ricinus communis* agglutinin I to VWF in type 1 VWD patients (◆,n = 29) and control subjects (△, n = 20) and circulating VWF:Ag levels. Inverse correlation was found between RCA-I binding to VWF and baseline VWF:Ag in the combined study and control groups (r = -0.43, $P < 0.005$, n = 49) Correlation between RCA-I binding and baseline VWF:Ag was significant in the controls alone (r = -0.45, $P < 0.05$, n = 20), but not in the VWD patients (r = -0.18, $P = 0.4$, n = 29).

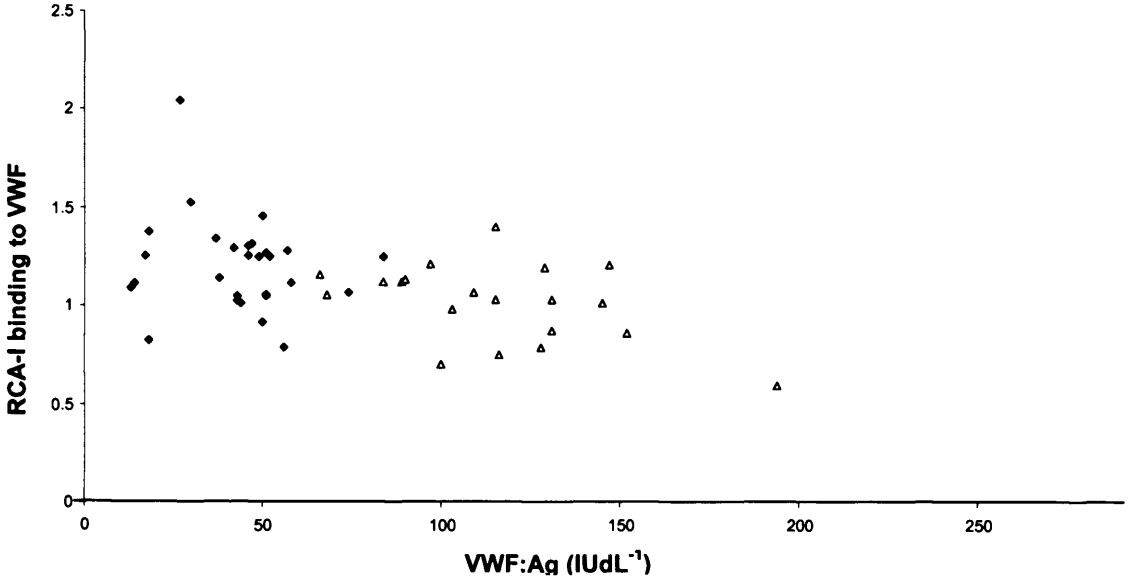


Figure 7.3. RCA-I binding to VWF prior to and 1 h and 6 h following DDAVP in type 1 VWD patients (n = 29). Plasma samples were analysed prior to (T_0) and 1 h and 6 h following DDAVP infusion (T_1, T_6). Statistical analysis showed there to be no significant difference in the median ratio of RCA-I/VWF to VWF:Ag between circulating VWF (median 1.25, $P = 0.8$, n = 29) and released VWF at T_1 (1.13) and T_6 (1.19).

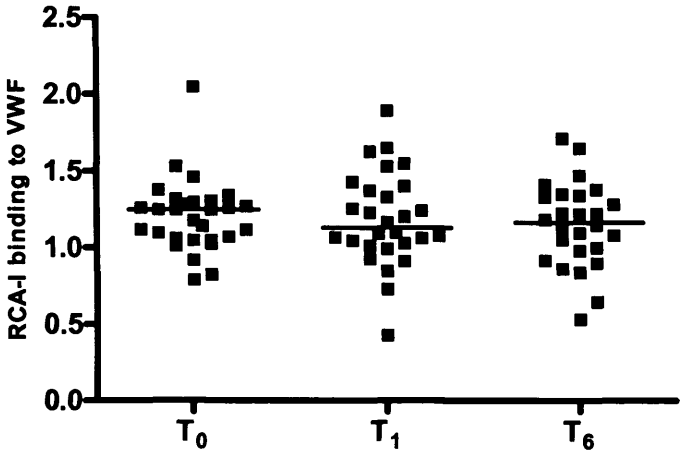


Figure 7.4. Binding of *Erythina crystagalli* agglutinin to VWF in type 1 VWD patients and control subjects. ECA binding to VWF in type 1 VWD patients was increased (median 1.38, $P < 0.01$, n = 29) compared to the controls are shown (median 1.0).

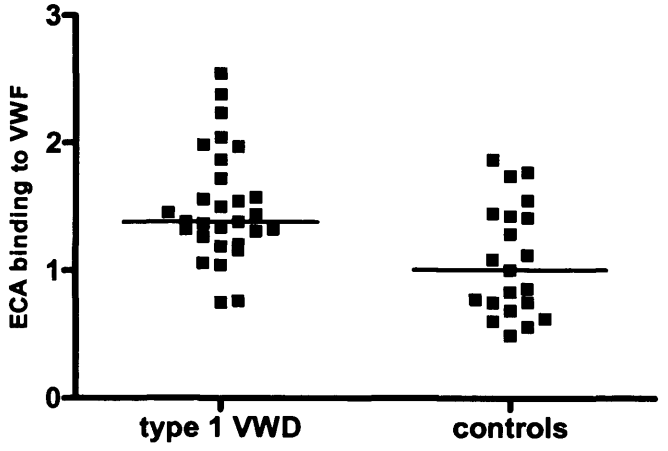
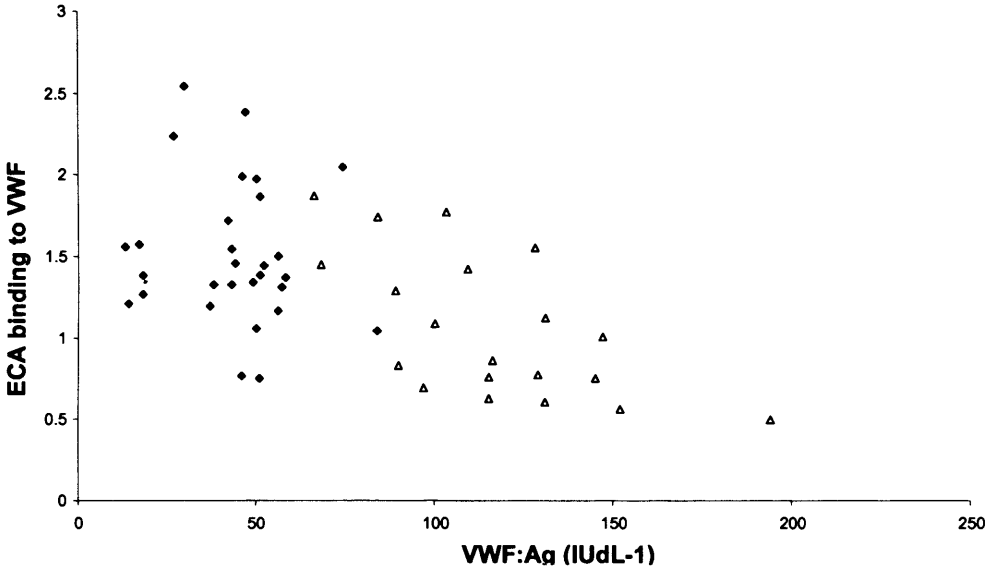


Figure 7.5. Relationship between binding of *Erythina crystagalli* agglutinin to VWF in type 1 VWD patients (◆,n = 29) and normal subjects (△ ,n = 20) and circulating plasma VWF:Ag levels. Inverse correlation was found between ECA binding to VWF and baseline VWF:Ag in the combined study and controls groups (r = -0.52, $P < 0.001$, n = 49).Correlation between ECA binding and baseline VWF:Ag was significant in the controls alone (r = -0.64, $P < 0.005$, n = 20), but not in the VWD patients (r = -0.16, $P = 0.38$, n = 29)



7.4.4 RCA-I and ECA binding and clearance of VWF

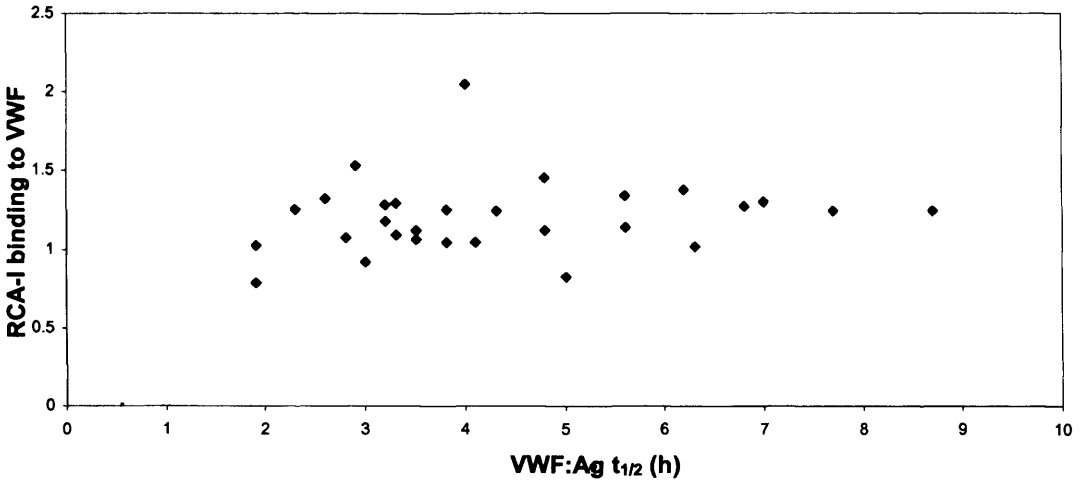
There was no correlation between either RCA-I or ECA binding and VWF:Ag $t_{1/2}$ in the type 1 VWD patients (r = 0.19, $P = 0.3$; r = -0.10, $P = 0.6$, n = 29, Fig.7.6).
To avoid the possibility of a masking effect when analysed as a group, VWF:Ag $t_{1/2}$ values were analysed according to RCA-I binding with increased binding defined as a ratio of RCA-I/VWF to VWF:Ag > 1.10. The median VWF:Ag $t_{1/2}$ value was found to be longer in patients where binding was within the normal range (4.8 h, range 2.3 – 8.7, n = 18, Fig.7.7) compared to patients with increased binding (3.5 h, 0.9 – 9.6, n = 11). However, this difference was not shown to be significant, $P = 0.06$.

7.4.5 Relationship between binding of RCA-I and ECA to VWF

Direct correlation was found between RCA-I and ECA binding in the study and control groups, (r = 0.4, $P < 0.01$, n = 49, data not shown).

Figure 7.6. Relationship between RCA-I (A) and ECA (B) binding to VWF and half-life of VWF in type 1 VWD patients. No correlation was demonstrated between RCA-I or ECA binding to VWF with VWF:Ag $t_{1/2}$ (r = 0.19, *P* = 0.3; r = -0.10, *P* = 0.6, n = 29 respectively).

A



B

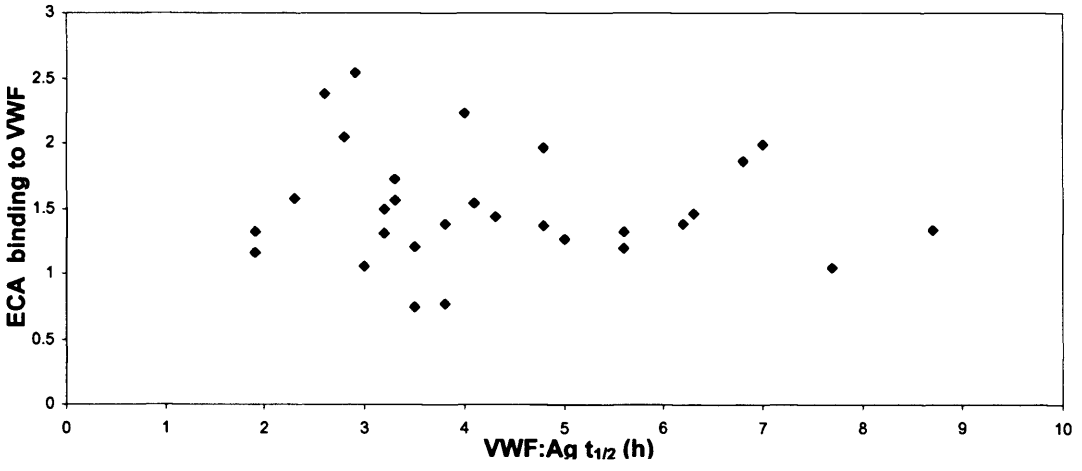
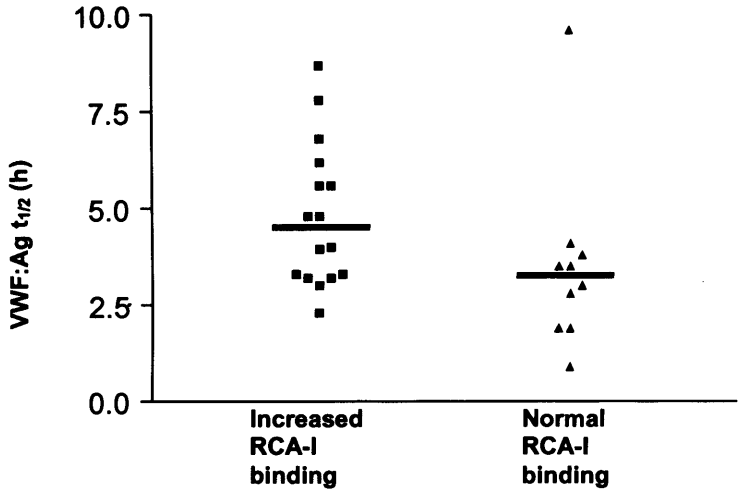


Figure 7.7. Half-life values of VWF:Ag according to binding of RCA-I to VWF in patients with type 1 VWD. Increased RCA-I binding was defined as a ratio of RCA-I/VWF to VWF:Ag > 1.10. Median VWF:Ag $t_{1/2}$ in patients with increased binding is shown as 4.8 h (n = 18) compared to 3.5 h (n = 11) in the patients in whom RCA-I binding was not increased. No significant difference was demonstrated between the groups, $P = 0.06$.



7.4.6 RCA-I and ECA binding and ABO blood group

No difference in RCA-I or ECA binding to VWF was found between subjects according to ABO blood group ($P > 0.2$, n = 50, data not shown).

7.5 DISCUSSION

The word lectin is derived from the Latin, legere, meaning to pick up or choose and was first used in the 1940s following the discovery that certain agglutinins were capable of 'selecting' types of cells based on their blood group activities. The term is now used in a broader sense to describe carbohydrate-binding and cell-agglutinating proteins of non-immune origin. Lectins are ubiquitous, being found in plants, viruses, animals and microorganisms, but in spite of this, their function in nature is not clear. Lectins often demonstrate a preference for oligosaccharides, which they bind with 2 -3 fold greater affinity than monosaccharides.(Sharon 1989) The oligosaccharide-binding specificities of various lectins have been determined using structurally defined glycoconjugates. Therefore lectins are useful tools for the

fractionation and identification of carbohydrate structure of glycoproteins and have been utilised in the determination of oligosaccharide structures of VWF.(Matsui, *et al* 1991, Osawa and Tsuji 1987)

RCA -I is one of two galactose (Gal) binding lectins isolated from *Ricinus communis* (castor bean) seeds and has a high affinity for non-reducing terminal ß Gal residues.(Osawa and Tsuji 1987) *Erythina crystagalli* agglutinin (ECA) is also a legume lectin and has been shown to bind strongly to exposed Gal within N-acetyllactosamine (Gal(ß1-4)GlcNAc) residues (Debray, *et al* 1986, Iglesias, *et al* 1982) Therefore the predominant affinities of RCA-I and ECA towards VWF involve N-linked glycan structures (Chapter 1, Fig.1.5A): In addition, both lectins bind weakly to O-linked GalNAc,(Iglesias, *et al* 1982, Matsui, *et al* 1991) and RCA-I has also been shown to bind to O-linked exposed Gal residues (Fig. 1.5B).

In this study, the relationship between the clearance of released VWF and exposure of Gal residues on VWF has been investigated by developing immunoassays to detect RCA-I and ECA-I binding to VWF. The increased binding of RCA-I to VWF found in type 1 VWD patients, consistent with previously reported findings,(Ellies, *et al* 2002) was shown to relate to lowered circulating plasma concentration of VWF:Ag. Similar findings were obtained for ECA binding. The correlation between the binding of both lectins and VWF:Ag levels was weaker in the type 1 VWD patients than the controls, a finding that may reflect insufficient sample size or the heterogeneity of the pathogenesis of reduced VWF levels in the VWD group.

Increased binding of RCA-I and ECA to VWF were not shown to relate to increased VWF clearance of regulated released VWF in type 1 VWD patients. It is possible that increased Gal exposure results in reduced VWF levels via an alternative mechanism, or may indeed represent normal variability. The absence of a relationship between VWF clearance and exposed Gal may reflect the primary importance of clearance, by mechanisms unrelated to exposed Gal, for determining the steady-state distribution of glycosylation in VWF. Further analysis of the VWD group showed VWF half-life to differ between patients according to whether or not RCA-I binding was increased. Although this was not shown to be statistically significant, the increased RCA-I binding observed in patients with longer half-lives suggests there may be a direct relationship between RCA-I binding and VWF half-life. A possible explanation for this is the increased likelihood of desialylation the longer VWF resides in the circulation. If this were the case, a greater reduction in RCA-I binding between samples pre and 1 h post DDAVP may be expected in

patients with longer VWF half-lives: this was not observed in this study (data not shown). Increased binding of peanut agglutinin, a lectin highly specific for the O-glycan structure Gal(β1-3)GalNAc, has recently been reported as relating to low VWF levels and enhanced VWF clearance.(van Schooten, et al 2007) As the detection of O-linked glycosylation in these patients required prior desialylation of VWF, these findings also suggest that the resultant increased VWF clearance was independent of exposed Gal residues.

As discussed in Chapter 4, structural differences in VWF released following DDAVP and plasma VWF may result in differences in the clearance kinetics between plasma and newly released VWF. Indeed the expression of A antigen on VWF released following DDAVP infusion has previously been shown to be increased when compared to plasma VWF.(Brown, *et al* 2002) This is suggestive of rapid VWF glycosylation in response to DDAVP and that VWF derived from endothelial cell storage pools may contain different oligosaccharide side chain structures than circulating VWF. However, no significant difference between RCA-I binding to circulating VWF and VWF released following DDAVP infusion was demonstrated in this study. This suggests that the increased clearance of VWF demonstrated within this cohort of type 1 VWD patients does not reflect VWF released following DDAVP as having a different Gal exposure from circulating VWF in the steady-state. The absence of variation of RCA-I and ECA binding toVWF observed between ABO blood groups is unexpected given the known variation in the terminal sugar structures of the ABH antigens. Inadequacies in the sample size could account for this finding.

Further analysis of the VWF oligosaccharide structures in individuals with type 1 VWD is warranted. Although lectins may be useful to detect variability in the glycosylation profile, definitive analysis of the carbohydrate composition of VWF by mass spectrometry and other methods is more likely to elucidate any effect of glycosylation on VWF clearance.

Chapter 8

MOLECULAR ANALYSIS OF VWF GENE

8.1 INTRODUCTION

At the outset of this study, it had been proposed that a partial quantitative deficiency of VWF may represent a complex genetic trait.(Sadler 2002) While many molecular defects in the VWF gene (*VWF*) had been identified in cases of types 2 and 3 VWD,(Baronciani, *et al* 2000, Fressinaud, *et al* 2002, Mohlke, *et al* 1999a) molecular analysis of *VWF* in individuals with type 1 VWD had identified the causative mutation in only a minority of patients.(Mohlke, *et al* 1999a) Furthermore, non-linkage between *VWF* and the type 1 phenotype had been described in some families.(Casana, *et al* 2001, Castaman, *et al* 1999) Fourteen *VWF* mutations reported to be associated with type 1 VWD were included on the ISTH VWF mutation database (http://www.vwf.group.shef.ac.uk/index.html) at the time of this study. Of these, thirteen had been demonstrated in exons 28 (10) and 26 (3) with the remaining mutation in exon 19. Three large studies were underway to examine the molecular basis of type 1 VWD in relation to clinical phenotype.

It had been proposed that the A1-A3 and D'-D3 domains of VWF may contain receptor-recognition sites affecting *in vivo* VWF plasma clearance.(Lenting, *et al* 2004) Several dominant VWD mutations within D'-D3 domain have subsequently been shown to result in or in associated with increased VWF clearance,(Haberichter, *et al* 2006a, Lenting, *et al* 2004, Schooten, *et al* 2005). These include the Vicenza variant, resulting from the mutation R1205H, and classified as VWD subtype 2M at the time of this study.(Sadler 1994) It is therefore possible that missense mutations in *VWF* may underlie the decreased half-lives observed in some of the type 1 VWD patients in this study.

VWF is bound, through specific regions within its A1 and A3 domains, to the different types of collagen that constitute the main component of extracellular matrices. VWF has been shown to bind to the fibrillar collagens types I and III via the A3 domain,(Cruz, *et al* 1995, Lankhof, *et al* 1996) and the non-fibrillar collagen type VI via the A1 domain.(Hoylaerts, *et al* 1997) Several mutations in the A3 domain have been reported that result in defective binding of VWF to collagen in the presence of normal multimers.(Ribba, *et al* 2001, Schneppenheim 2001)

8.2 AIMS

The overall aim of this study was to identify *VWF* mutations associated with increased VWF clearance or that may otherwise contribute towards the type 1 phenotype in a cohort of patients in whom plasma clearance of VWF following DDAVP administration had been measured. In addition, patients who had been excluded from the type 1 VWD study cohort following the finding of discordant ratios of VWF:CB to VWF:Ag were further investigated.

8.3 MATERIALS AND METHODS

8.3.1 Patients

Genotypic analysis was performed in 28 of the type 1 VWD patients (25 kindred) in whom clearance of VWF released following DDAVP had been examined (Chapter 4, Appendix 3). Four of the type 1 VWD patients had steady-state VWF:Ag levels reduced to < 20 $IUdL^{-1}$ The three patients excluded from the study following the finding of discordantly low VWF:CB activities (Patients 3, 20 and 42, Appendix 2) and their families were also investigated. Within the type 1 VWD group, two patients displayed low levels of FVIII:C (Patients 5 and 25). These patients and their families were further investigated.

8.3.2 Phenotypic analysis

FVIII:C, VWF:Ag, VWF:RCo, VWF:CB, VWFpp assays and analysis of VWF multimers were performed as described in Chapters 3, 5 and 6. Determination of VWF:Ag $t_{1/2}$ values is described in Chapter 3.

8.3.3 Genotypic analysis

Regions of the *VWF* gene, encoding domains A1, A2, A3 and D'-D3 of the VWF protein were sequenced.

Amplification of Exons

Genomic DNA was extracted from peripheral blood lymphocytes using a standard phenol/chloroform method as described in Chapter 3, Section 3.4. The relevant exons (18 – 20, 24, 26 – 33) of *VWF* and flanking intronic regions were amplified as described in Chapter 3, Section 3.4.3 using the following gene specific primers and PCR conditions shown in Table 8.1. Amplicons were visualised on a 1.5% agarose gel with 0.1% Ethidium bromide as previously described.

Primers

Exon 18:

Forward primer (F) 5'GTCCCCCTGAGTGGGCAACT3'
Reverse primer (R) 5'AACTGAAGGGCAGGCACCAG3'

Exon 19:
F 5'AGGAGGGCTTTAGATCAGTCACT3'
R 5'GTGCGGAAGGTCCTGTGG3'

Exon 20:
F 5'TGTTCCTTCATTGCCTCCAT3'
R 5'TAGGAGGGGTGGGAGCTGTG3'

Exon 24:
F 5'GGGGGTTGTGGGTGGTATGA3'
R 5'AAAAAGCCGAAGTGGTGTCT3'

Exon 26:
F 5'CAACATTATCTCCAGATGGC3'
R 5'GTCCTATCTCTGACCTGCAA3'

Exon 27:
F 5'GTTAAAAATGAGGCTTCCTC3'
R 5'TTTACCCAAAACCTAGTCTCTA3'

Exon 28:
28A
F 5'CAGAAGTGTCCACAGGTTCT3'
R 5'TGTCCGATCCTTCCAGGACCT3'

28B *(as described in Chapter 3, Section 3.4.3)*
F 5'TGGTTCTGGATGTGGCGTTC3'
R 5'TCTTGGCAGATGCATGTAGC3'

Exon 29:
F 5'GTAGGCCTGGTGGCCATTGT3'

Exon 30:
R 5'AGGAGCAAAGTGACTGCTCCG3'

Exon 31:
F 5'ATCCACCGTTAAGACGGGGTGTCG3'
R 5'GGCTGGGGTTACTTTTGGATGTTGT3'

Exon 32:
F 5'TGAACATCTTCCTCATAGGGCTGA3'
R 5'CCATGAACAGAAACTTAAAG3'

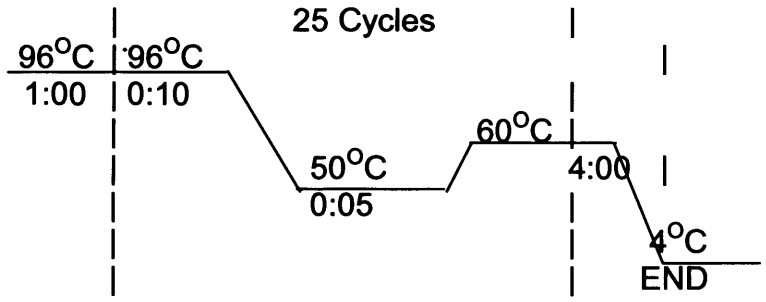
Exon 33:
F 5'CCTCAGCCTCATGTCCCTAT3'
R 5'CCCCAAACACATTCTTAACC3'

Table 8.1. Conditions for PCR amplification of *VWF* exons. Method is described in Chapter 3, Section 3.4.3. dNTPs and $MgCl_2$ were used for all exons except exon 20 (in which KCl was used*), at respective concentrations of 1.25 mM and 50 mM. Temperatures for amplification (amp), replication (rep) and annealing (ann) are given. 40 cycles were performed for each exon.

Exon	**18**	**19**	**20**	**24**	**26**	**27**	**28A**	**28B**	**29**	**30**	**31**	**32**	**33**
NH_4 buffer (μl)	5	5		5	5	5	5	5	5	5	5	5	5
dNTPs (μl)	5	5	5	5	5	5	5	5	5	5	5	5	5
$MgCl_2$ (μl)	1.5	1	5*	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Amp (°C)	94	94	94	94	94	94	94	94	94	94	94	94	94
Rep (°C)	57	60	60	60	51	53	59	58	60	60	61	54	57
Ann (°C)	72	72	72	72	72	72	72	72	72	72	72	72	72

Sequence analysis of VWF

Prior to sequence analysis, the products obtained from PCR amplification of the exons of VWF listed above were purified using a commercial kit (QIAquick PCR Purification kit, Qiagen Ltd., West Sussex, UK). This process removes excess oligonucleotide primers and remaining deoxyribonucleotide triphosphates and salts that were used in the PCR. Both the forward and reverse strands were directly sequenced by automated sequencing technology using a fluorescent detection system (Applied Biosystems 3100 Avant Sequencer). The cycle sequencing reactions were carried out using the recommended protocol. Each sequencing reaction consisted of 1 μL of Big Dye Terminators v1.1© (on ice), I μL of template DNA, 3.2 pmol of sequencing primer, 3.5 μL of Terminator Reaction Buffer©, and 13.5 μL of dH_2O for a final reaction volume of 20 μL. This was then placed in a thermocycler (GeneAmp PCR system) on the following program:



Sequencing reactions were carried out in 96 well microtitre plates. Following amplification the products were purified using the following protocol: 50 μL of 95% ethanol and 2 μL of sodium acetate were added to each sample, mixed and left to stand for 15 min. The plate was then spun in a refrigerated centrifuge at 1650 rpm at 4°C for 45 min. The supernatant was removed by inverting the plate, placing it on a layer of tissues and spun up to 185 rpm. 70 μL of 70% ethanol was then added to each sample, and the plate was again centifuged at 1650 rpm at 4°C for 15 minutes. The supernatant was removed as previously described and spun for 1 min. Finally, 20 μL HiDi Formamide(C) was added to the samples to resuspend the DNA. Automated capillary sequencing was then performed (ABI 3100 Avant Genetic Analyser)

8.3.4 Investigation of FVIII binding

Microtitre plates (Dynatech, Immulon 4) were coated with 100 μL of 1:200 dilution of monoclonal antibody (Monoclonal anti VWF Ab lot RFF-VIII:R1) raised against the GpIba binding site of VWF. After leaving at 4°C overnight, the plates were washed

five times with TBS, pH 8.0 (Chapter 7, Section 7.3.1). Serial dilutions of the standards (1:100 – 1:800) and the patients were performed by doubling dilutions and 100 μL dispensed into the wells. Following incubation at 4°C for 48 h, the plate was washed five times in TBS . Endogenous FVIII was removed by incubating with 100 μL of 350 mM $CaCl_2$ in TBS for 1 h at room temperature. The plate was washed 3 times and further incubated with 100 μL 350 mM $CaCl_2$ for 1 h.

1 U/mL recombinant FVIII (Helixate, Baxter, UK) was mixed with re-incubation buffer (50 mM Tris, 100 mM NaCl pH 8.0, 2% BSA, 1 mL of 1M $CaCl_2$, 0.002% Tween) and added to the wells. This was incubated for a further 2 h at 37 °C. The plate was then washed a further three times. FVIII binding was then measured by chromogenic assay using the Coatest VIII:C/4 Chromogenic kit (Quadratech, UK). Optical densities were read at 405 nm using the Dynex MRX (Dynex Technologies, UK) microtitre plate reader.

Molecular analysis of *VWF* for type 2N mutations was performed by sequencing of exons 18, 19, 20, 24 and 26, as described in Section 8.3.2

8.4 RESULTS

All DNA base changes are heterozygous unless otherwise stated.

Analysis of regions of *VWF* encoding the A1-A3 and D'D3 domains of VWF identified a total of nine mutations in eight of the 28 type 1 VWD patients investigated (n = 8, Appendix 3). These mutations are discussed below and phenotypic and genotypic data are summarised in Table 8.2. Of the four patients with baseline VWF:Ag levels < 20 $IUdL^{-1}$, mutations were identified in three (Patients 7, 14, 34, Appendix 3).

8.4.1 Sequencing of exons 18 – 20

2303 A>G (R768Q)

2303 A>G (R768Q) was identified in exon 18 encoding the D' domain in one patient (Patient 6), a 48 year old female who had presented with a postpartum haemorrhage. Other family members including her three offspring were subsequently shown to be affected.

2771 G>A (R924Q)

2771 G>A (R924Q) was identified in exon 21 of Patients 10 and 25 (kindred 10) and 2561 G>A (R854Q) was identified in Patient 25. Both of these mutations are within the VWF-D'D3 domain. Kindred 10 is discussed in detail in Section 8.4.5.

Table 8.2. Mutations identified by sequence analysis of exons 18 – 20 and 26 – 33 in 28 individuals with type 1 VWD.

Previously unreported mutations denoted by #;

Bleeding times performed at the time of diagnosis are shown.

NT: not tested

* range derived from 20 normal controls (Chapter 5)

Kindred	Patient	ABO Blood Group	Bleeding Time (min)	FVIII:C ($IUdL^{-1}$)	VWF:Ag ($IUdL^{-1}$)	VWF:RCo ($IUdL^{-1}$)	VWF:CB ($IUdL^{-1}$)	VWFpp/ VWF:Ag ratio	VWF:Ag $t_{1/2}$ (h)	Exon	Mutation(s)	Amino acid Substitution
			<8	50 - 150	50 - 150	50 - 150	50 - 150	0.07 – 0.3*				
5	5	O	NT	18	25	26	26	0.15	1.9	27	3613A>C#	R1205S
6	6	A	18	34	42	38	44	0.06	3.5	18	2303A>G#	R768Q
10	25	A	7	13	30	23	34	0.13	5.6	21 20	2771G>A 2561G>A	R924Q R854Q
10	10	A	NT	66	27	23	50	0.10	3.2	21	2771G>A	R924Q
11	12	A	8	62	34	30	48	0.13	4.8	28	4751A>G	Y1584C
12	14	O	25	20	13	17	10	0.45	3.3	27	3613C>T#	R1205C
28	31	O	NT	27	18	16	12	0.55	3.5	28	4247A>T#	I1416N
40	43	A	>20	22	14	11	9	0.49	3	28	4247A>T#	I1416N

8.4.2 Sequencing of exons 26 and 27

Sequencing of exon 27 identified two nucleotide substitutions at 3613 resulting in arginine substitutions at 1205 in the VWF-D3 domain: 3613 C>T (R1205C) in Patient 14 and 3613 A>C (R1205S) in Patient 5. Neither mutation has been reported previously. VWF:Ag and VWF:CB levels prior to and over 6 h following DDAVP infusion are shown in Fig. 8.1. Respective VWF:Ag $t_{1/2}$ values of 3.3 h and 1.9 h were calculated and the VWFpp/VWF:Ag ratio was increased in Patient 14.

3613 C>T (R1205C)

Patient 14, a 41 year old male, presented aged 17 years with prolonged bleeding following dental treatment. He had a lifelong clinical history of bleeding symptoms. He had no siblings and no other family members were known to have a bleeding disorder. VWF multimeric pattern was normal.

3613 A>C (R1205S)

The clinical history and results for Patient 5 and kindred are given in Section 8.4.5.

8.4.3 Sequencing of exon 28

4247 T>A (I1416N)

At the time of the study, 4247 T>A (I1416N), identified in the VWF-A1 domain in Patients 31 and 43, had not been reported as a causative mutation in VWD. Patients 31 and 43 were both male, aged 48 and 54 years respectively, with a moderate to severe clinical phenotype and dominant family history. I1416N was also identified in a type 2M VWD kindred investigated in Chapter 9: VWF levels and functional parameters pre- and post DDAVP for I1416N patients (n = 4) are shown in Table 9.6. Patients demonstrated a functionally restricted release of VWF, with a predominant platelet-binding defect. VWF multimeric analysis was normal pre- and post DDAVP (data not shown). Both the ratio of VWFpp to VWF:Ag and clearance of VWF:Ag was increased in I1416N patients (Table 9.6), with concordance demonstrated between the half-lives of VWF:Ag and VWF:CB (Appendix 3). A detailed discussion of I1416N is presented in Chapter 9.

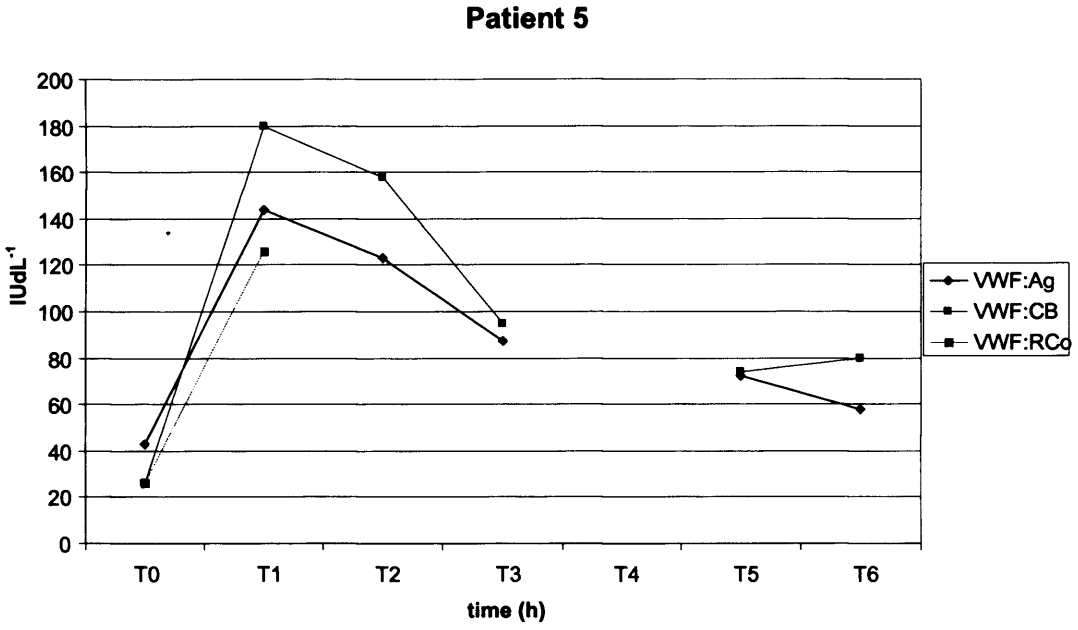
4751 A>G (Y1584C)

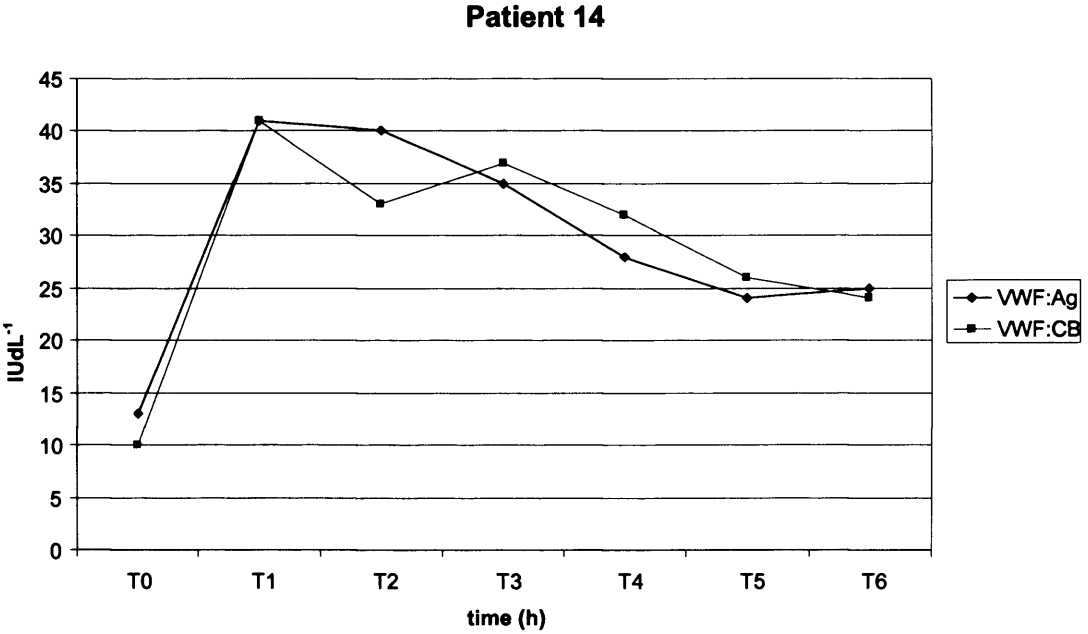
The VWF-A2 domain mutation 4751 A>G (Y1584C) was identified in Patient 12, confirming the findings by PCR restriction analysis reported in Chapter 6. Patient 12 is a 52 year old female who presented with excessive bleeding following thyroidectomy 10 years previously. There was no significant family history of bleeding although her mother was also found to have reduced VWF levels. A detailed discussion of 4751A>G (Y1584C) mutation is presented in Chapter 9.

8.4.4 Sequencing of exons 29 – 33

No mutations were identified in exons 29, 30, 31, 32 or 33 encoding the VWF-A3 domain.

Figure 8.1. VWF:Ag level and VWF:CB activity prior to and over 6 h following DDAVP in Patients 5 and 14 in whom A>C and C>T nucleotide substitutions at 3613 were identified, resulting in R1205S and R1205C respectively. In addition, pre- and 1 h post VWF:RCo activities are shown for Patient 5.





8.4.5 Investigation of patients with low VWF:CB activity: Patients 3, 40 and 42

Patients 3, 20 and 42 were excluded from the type 1 VWD study cohort due to the finding of discordantly low VWF:CB activities (Appendix 2). This section reports the results of further investigation of these patients and their families.

Patient 3

Patient 3 (II:1) is a male from a kindred with a dominant family history of VWD with a severe bleeding phenotype (Fig. 8.2). Many of the affected family members, including his mother (1:1) and more distant relatives are under the care of the Arthur Bloom Haemophilia Centre in Cardiff. Phenotypic data for Patient 3, his sibling (II:2) and mother (1:1) are detailed in Table 8.3. Multimeric analysis was normal on 1.4% gel on plasma samples pre- and post DDAVP in Patient 3 (data not shown). VWF:Ag and VWF:CB values pre- and over 6 h post DDAVP are shown in Fig. 8.3. Despite initial rises in both VWF:Ag and VWF:CB, the rate of clearance was markedly increased with calculated half-lives of 0.92 h and 1.20 h respectively. VWF:RCo activity increased to 25 $IUdL^{-1}$ 1 h following DDAVP. The molar concentration of VWFpp pre-DDAVP was 3.8 nM (NR 4.5 – 17.8) and the molar ratio of VWFpp to VWF:Ag was 1.5 (NR 0.07 – 0.3). Sequence analysis of *VWF* in Patient 3 demonstrated 3614 G>A in exon 27, encoding R1205H in the VWF-D3 domain. Molecular analysis of the *VWF* gene was also performed in another affected branch of this family as part of the UKHCDO type 1 VWD molecular genetics study (Cumming, *et al* 2006): R1205H was confirmed in the maternal grandmother's cousin and shown to segregate with disease phenotype in this branch of the family.

Figure 8.2. Family tree of Patient 3 (II:1)

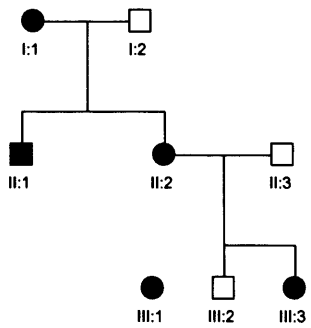
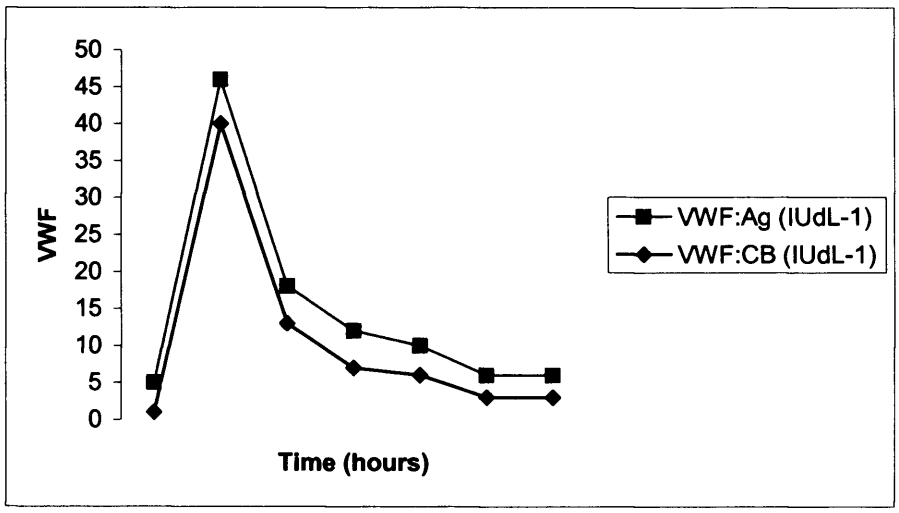


Table 8.3. Phenotypic data for Patient 3 and affected family members.
Multimeric analysis was normal on repeated testing of all family members

Subject	FVIII:C ($IUdL^{-1}$) 50 - 150	VWF:Ag ($IUdL^{-1}$) 50 - 150	VWF:RCo ($IUdL^{-1}$) 50 - 150	VWF:CB ($IUdL^{-1}$) 50 - 175
Patient 3 (II:1)	7	4	<10	1
II:2	30	17	<10	19
I:1	18	10	<10	15

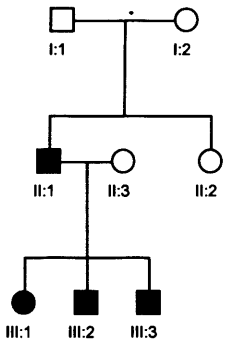
Figure 8.3. VWF:Ag and VWF:CB values prior to and over 6 h following DDAVP in Patient 3. Half-life values of VWF:Ag and VWF:CB were calculated at 0.92 h and 1.2 h respectively.



Patient 20

Patient 20 (II:1, Fig. 8.4) is a male who was diagnosed with VWD at the age of 39 years following investigation of his seven year old daughter for symptoms of easy bruising (proband III:1). Other than mild bruising symptoms in Patient 20, there was no significant clinical history of bleeding in any other family members including following tonsilloadenoidectomy and circumcision. The remainder of the investigations performed at diagnosis in the proband (III:1), her two siblings (III:2 and III:3) and father (Patient 20, II:1) are shown in Table 8.4. On the basis of the clinical and laboratory phenotype, including the finding of normal multimeric pattern, type 1 VWD was diagnosed in all the family members investigated

Figure 8.4. Family tree of Patient 20



Results of 6 h DDAVP study are shown in Fig. 8.5. Calculated half-lives of VWF:Ag and VWF:CB were 5.3 h and 5.1 h respectively. Although steady-state VWF:Ag level and VWF:RCo activity were within normal limits, VWF:CB was reduced with a VWF:CB/VWF:Ag ratio of 0.58, despite normal multimeric analysis (Table 8.5) Neither VWF:RCo nor VWF:CB activities had been measured at the time of diagnosis in any of the offspring of Patient 20, who were subsequently further investigated (Table 8.5). The VWF:Ag level and VWF:RCo activity were both found to be within normal limits in all family members except the proband (III:1). However, there was significant anxiety relating to venepuncture in subjects III:2 and III:3. Unlike Patient 20, a discordant reduction in VWF:CB activity was not demonstrated in any of his offspring, including the proband. Targeted sequence analysis of *VWF*, as described in Section 8.3, identified 5191T>A in exon 30 encoding the VWF-A3 domain mutation S1731T in Patient 20 and his three offspring.

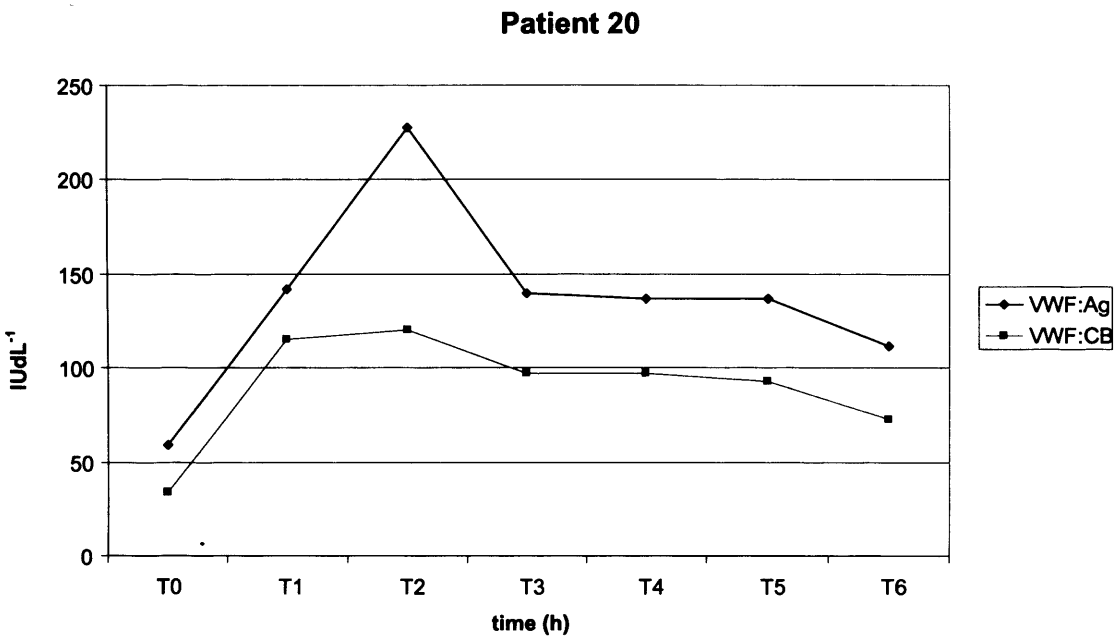
Table 8.4. Investigations performed at time of diagnosis in Patient 20 and offspring (1997). Multimeric analysis was normal in all subjects.
*Functional activity of VWF was measured using an in-house ELISA using monoclonal antibodies.(Murdock, *et al* 1997). Results are shown for tests performed on two separate occasions in subjects II:1 and III:1; in subjects III:2 and III:3, blood tests were performed on one occasion only.

Subject	Age	FVIII:C ($IUdL^{-1}$) 50 -150	VWF:Ag ($IUdL^{-1}$) 50 -150	VWF:Ac* ($IUdL^{-1}$) 50 -150	VWF:Ac/ VWF:Ag
II:1 (Patient 20)	39 years	70, 90	46, 54	46, 51	1, 0.9
III:1	7 years	46, 87	77, 52	84, 47	1.6, 0.9
III:2	5 years	98	72	39	0.5
III:3	12 months	58	54	33	0.6

Table 8.5. Investigations performed in 2005 in Patient 20 and offspring.
Multimeric analysis was normal in all subjects.

Subject	Age (years)	FVIII:C ($IUdL^{-1}$) 50-150	VWF: Ag ($IUdL^{-1}$) 50-150	VWF: RCo ($IUdL^{-1}$) 50-150	VWF: CB ($IUdL^{-1}$) 50-175	VWF:CB/ VWF:Ag ratio	Mutation Analysis
II:1 (Patient 20)	44	72	59	56	34	0.58	5191 T>A (S1731T)
III:1	14	63	43	44	40	0.93	5191 T>A (S1731T)
III:2	12	70	85	68	63	0.74	5191 T>A (S1731T)
III:3	8	90	75	64	63	0.84	5191 T>A (S1731T)

Figure 8.5. VWF:Ag and VWF:CB values in Patient 20 prior to and over 6 h following DDAVP. Calculated half-life values of VWF:Ag and VWF:CB were 5.3 h and 5.1 h respectively.



Patient 42

Patient 42 (II:1, Fig. 8.6) is a 25 year old female with a history of VWD in her mother; both individuals displayed similar laboratory and bleeding phenotypes and there was no other known family history. Patient 42 was recruited into the type 1 VWD study and 6 h data are shown in Fig.8.7. Despite a ratio of VWF:RCo to VWF:Ag of 0.8 at baseline, there was a discordant reduction in VWF:CB activity (VWF:CB to VWF:Ag ratio 0.46 shown on repeated plasma samples) and Patient 42 was consequently excluded from the study. Calculated half-life values of VWF:Ag and VWF:CB were 1.5 h and 1.8 h respectively. The mother of Patient 42 was further investigated and a VWF:CB/VWF:Ag ratio of 0.7 was found. No mutations were demonstrated on sequence analysis of regions of *VWF* encoding the A1-A3 and D'D3 domains. Multimeric analysis was normal in both affected individuals.

Figure 8.6. Family tree of Patient 42

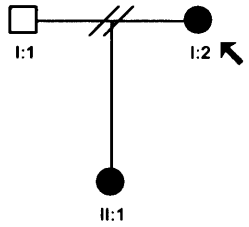
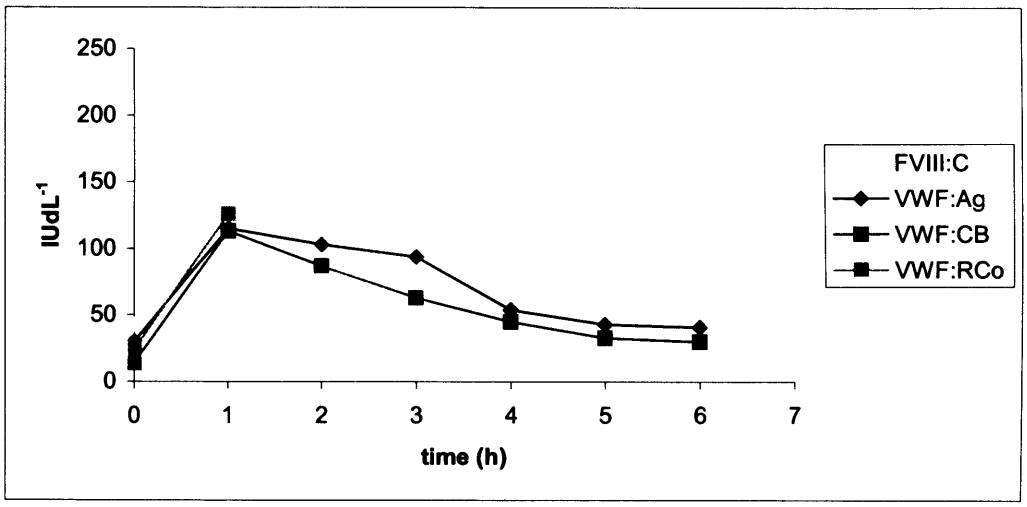


Figure 8.7. VWF:Ag, VWF:CB and FVIII:C values in Patient 42 prior to and over 6 h following DDAVP. Pre- and 1h post VWF:RCo activities are also shown.



8.4.6 Investigation of patients with reduced FVIII:C levels

Two of the type 1 VWD patients investigated in this study also displayed significantly lowered FVIII:C levels (Patients 5 and 25, Appendix 2). The clinical and laboratory phenotype and genotype for these kindred are described below.

Patient 5

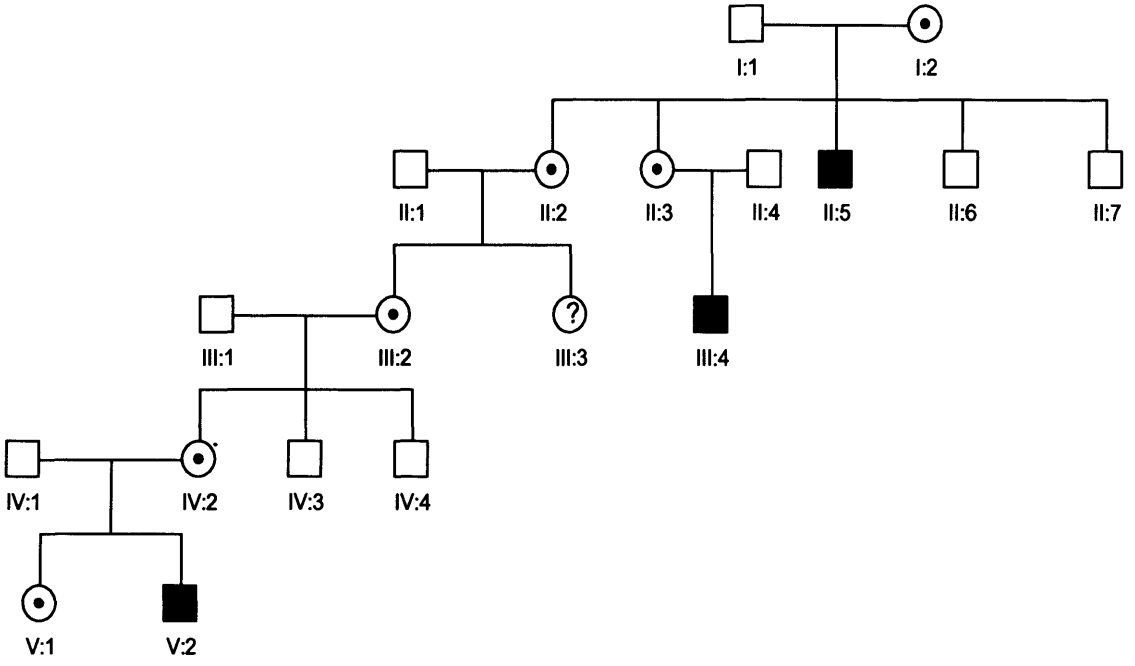
Patient 5 (IV:2, Fig.8.8) was investigated in 1996 following the diagnosis of mild haemophilia A in her 18 month old son. There was a history of haemophilia A in a maternal uncle (II:5) and male cousin (III:4). Patient 5 had a history of menorrhagia but no other significant bleeding history and she had not received DDAVP treatment prior to the study. Phenotypic data for Patient 5 at the time of diagnosis, as well as that of her son (V:2), daughter (V:1) and mother (III:2) are given in Table 8.6.

The 6 h DDAVP data for Patient 5 are shown in Fig.8.1. In addition VWF:RCo activities are shown at T_0 and T_1. Calculated half-life values for VWF:Ag and VWF:CB were 1.9 h and 2.9 h respectively. Sequence analysis of regions of *VWF* encoding the A1-A3 and D'D3 domains identified 3613A>C (R1205S) in the VWF-D3 domain of Patient 5 (IV:2) and her son (V:2) Mutation sequence analysis of the FVIII gene demonstrated 2048A>C (Y664S) mutation in exon 13 of V:2. Mutations at this codon, but not involving this particular base, have previously been associated with haemophilia A. Both Patient 5 (IV:2) and her daughter (V:1) and mother (III:2) were shown to be heterozygous for this mutation

Table 8.6. Investigations performed at time of diagnosis in Patient 5 and family (1996). Multimeric analysis was normal in all subjects. NT: not tested

Subject	**Age**	**FVIII:C ($IUdL^{-1}$) 50 - 150**	**VWF:Ag ($IUdL^{-1}$) 50 - 150**	**VWF:RCo ($IUdL^{-1}$) 50 - 150**	**VWF:CB ($IUdL^{-1}$) 50 - 175**
IV:2 (Patient 5)	34 years	18	25	26	26
V:1	9 years	79	82	83	86
V:2	18 months	4	24	21	25
III:2	61	33	31	45	NT

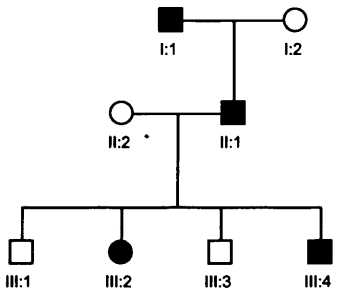
Figure 8.8. Family tree of Patient 5 (IV:2). Symbols are as previously denoted although refer to mutation in FVIII gene. Carrier status of haemophilia A is indicated by dot: this was confirmed in III:2, IV:2 and V:1; the remainder are deemed to be obligate carriers from the family pedigree.



Patient 25 (kindred 10)

Patient 25 (II:1, Fig. 8.9) is a 45 year old male who presented aged 5 years with excessive bleeding following circumcision and dental extractions. There was no significant family history of bleeding. Subsequent testing resulted in the finding of reduced VWF levels in his father (1:1) and two of his children (III:2 and III:4, Fig.8.9, Table 8.8), none whom were clinically affected. Unlike Patient 25, reduction in FVIII levels was not found in other family members (Table 8.8). III:2 was also recruited in the type 1 VWD study (Patient 10, Appendix 2).

Figure 8.9. Family tree of Patient 25 (Kindred 10)



At the outset of the study, Patient 25 (II:1) was known to have reduced FVIII binding activity (Fig.8.10). The mutation 2561 G>A (R854Q) had previously been demonstrated in Patient 25 although had not been shown to be transmitted to the affected offspring, III:2 and III:4.{Jenkins, 1999} The 6 h responses to DDAVP of FVIII:C, VWF:Ag and VWF:CB in Patient 25 are shown in Fig. 8.11. Calculated VWF:Ag $t_{1/2}$ values for Patient 25 and Patient 10 were 5.6 and 3.2 h respectively. Notably, as discussed in Chapter 6 the ratio of VWF:CB to VWF:Ag was shown to decrease significantly following DDAVP in both patients.

Sequence analysis of the targeted regions of *VWF* in Patients 25 and 10 described in Section 8.3 identified 2771G>A (R924Q) in exon 21 of both patients. This kindred was further investigated as part of the UKHCDO type 1 VWD molecular genetics study (Cumming, *et al* 2006): direct DNA sequencing of the essential regions of *VWF* (including exons, splice site boundaries, 5'- and 3'- untranslated regions) was initially performed in Patient 25. Based on these findings, targeted DNA sequencing of the *VWF* gene was subsequently carried out on the other recruited family members (I:1, I:2, II:2, III:1, III:2, III:3, III:4). In addition to Patients 25 and 10, heterozygosity for 2771G>A (R924Q) was demonstrated in the other affected family

members I:1 and III:4. The intron 34 candidate splice site mutation, 5843-8C>G was also identified and shown to segregate with reduced VWF levels. In addition, 2561 G>A (R854Q) was demonstrated in the mother of Patient 25 (I:2), as well as his two unaffected offspring, III:1 and III:3. Segregation of the VWD phenotype with *VWF* was investigated by means of *VWF* haplotype studies. Type 1 VWD was shown to segregate with the haplotype inherited from individual I:1 and the type 2N VWD R854Q allele was shown to be associated with the haplotype inherited from individual I:2.

Table 8.7. Phenotypic data for Kindred 10 (affected members).
Multimeric analysis was normal in all subjects.

Subject	**ABO blood group**	**FVIII:C ($IUdL^{-1}$) 50 - 150**	**VWF:Ag ($IUdL^{-1}$) 50 - 150**	**VWF:RCo ($IUdL^{-1}$) 50 - 150**
II:1 (Patient 25)	A	13	30	34
III:2 (Patient 10)	A	60	41	30
I:1	O	85	45	41
III:4	A	80	40	25

Figure 8.10. Graph showing the FVIII binding of Patient 25 and normal controls. The method is described in Section 8.3.4.

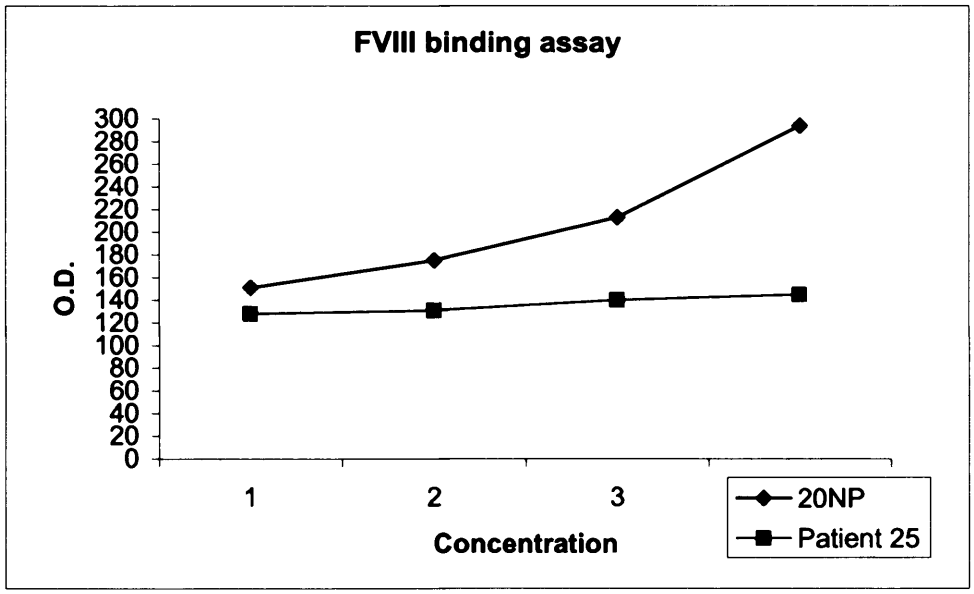
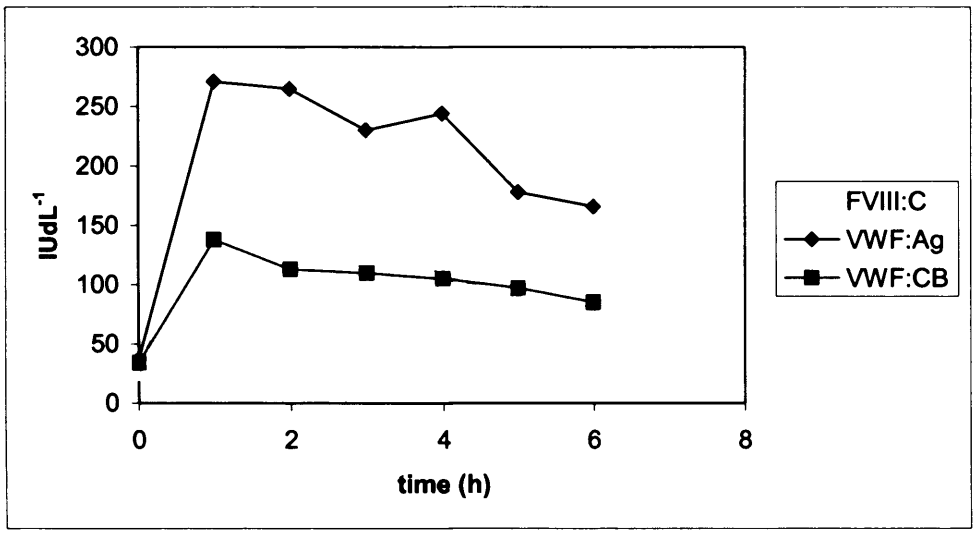


Figure 8.11. FVIII:C, VWF:Ag and VWF:CB values prior to and over 6 h following DDAVP in Patient 25.



8.5 DISCUSSION

In the ISTH-SSC revised classification of 1994, it was assumed that all VWD arose from mutations within the *VWF* gene.(Sadler 1994) However, the recently updated classification recognises that VWD may result from heterogeneous pathophysiologic mechanisms and no longer restricts the diagnosis of VWD to *VWF* mutations.(Sadler, et al 2006) This is pertinent to type 1 VWD, in which mutation analysis has rarely been reported until recently and was generally restricted to the more severe forms of the disease. At the outset of this study, a total of 14 mutations had been reported in association with type 1 VWD. The findings of one international and two national multicentre studies (EU, UKHCDO and Canadian) have recently been reported, providing new insights into the molecular pathogenesis of type 1 VWD.(Cumming, et al 2006, Goodeve, et al 2007, James, et al 2007a) There are now 117 *VWF* mutations included on the ISTH VWF mutation database (www.vwf.group.shef.ac.uk) and the possibility of increased VWF clearance as a molecular mechanism in VWD has led to the proposal of a new and distinct variant of type 1 VWD: type 1C (Sadler, *et al* 2006)

In this study, regions of the *VWF* gene, encoding domains A1,A2,A3 and D'-D3 of the protein were sequenced for possible mutations. These regions have been associated with increased clearance and may contain receptor-recognition sites affecting in vivo clearance(Lenting, et al 2004) Of the 28 type 1 VWD patients

studied, mutations were identified in eight patients from 25 kindred (~30%). Consistent with previous findings,(Eikenboom, et al 2006) a higher likelihood of *VWF* linkage was shown in cases with lower levels of VWF. However, despite a significant proportion of the type 1 VWD patient cohort demonstrating increased VWF clearance following DDAVP, none of the four mutations within the D'-D3 region of VWF reported in association with reduced VWF survival and VWF:Ag levels were identified: 3389G>T (C1130F), 3445T>C (C1149R)(Schooten, et al 2005), 3614 G>A (R1205H)(Lenting, et al 2004) and W1144G.(Haberichter, *et al* 2006a) Significantly increased VWF clearance in association with reduced VWF levels was demonstrated in four of the type 1 VWD patients demonstrating three previously unreported mutations: 4247A>T (I1416N, Patients 31, 43), 3613 A>C (R1205S, Patient 5) and 3613 C>T (R1205C, Patient 14). Three of these patients (Patients 14, 31, 43) demonstrated a more severe phenotype with VWF:Ag levels less than 20IUdL-1 as well as increased ratios of VWFpp to VWF:Ag..

The Vicenza variant, R1205H, resulting from the nucleotide substitution 3614 G>A was the original mutation in which increased VWF clearance was described and subsequently confirmed in a murine model. (Casonato, et al 2002, Lenting, et al 2004). R1205H was identified in one patient who had been excluded from the type 1 VWD study cohort following the finding of a discordant reduction in VWF:CB activity (Patient 3). The significantly reduced VWF level and accelerated plasma VWF clearance observed in this patient together with a moderate to severe bleeding phenotype and dominant penetrance are characteristic of this variant.(Cumming, et al 2006, Lenting, et al 2004) Historically, the Vicenza subtype has been classified as variants of both types 1 and 2 VWD and due to the presence of circulating UL-VWF multimers in some patients, was categorised as type 2M VWD at the time of the study.(Sadler 1994) When the level of VWF is sufficiently high for its precise measurement, concordance with functional activity has been demonstrated, resulting in the re-classification of the Vicenza variant as type 1 VWD in the recent ISTH-SSC recommendations.(Sadler, et al 2006) UL-VWF multimers were not detectable in the R1205H patient in this study, either in circulating plasma or following DDAVP: this finding is consistent with other reports.(Casonato, *et al* 2006, Lester, *et al* 2006) The absence of UL-HMW multimers in Vicenza patients could be misleading if relied upon diagnostically, and the R1205H variant illustrates a case where genetic diagnosis may play an important role. Establishing the diagnosis is particularly relevant to inform the appropriate clinical management given the rapid clearance of VWD released following DDAVP.

It has been suggested that the increased HMW multimers in the Vicenza variant may correlate with the increased turnover of VWF, including increased VWF proteolysis by ADAMTS-13. However, given the rapid plasma clearance of the VWF/R1205H mutant, it is possible that increased proteolysis is not observed in the steady state, resulting in the persistence of circulating UL multimers. In contrast to type 2A VWD, in which the decreased survival mainly concerns the high and intermediate molecular weight multimers,(Dent, *et al* 1990, Gralnick, *et al* 1985) the increased VWF clearance has been shown to involve multimers of all sizes in Vicenza patients. The mechanism by which the R1205H mutation affects VWF clearance is not currently known.

Two further arginine substitutions at 1205 in the D3 domain were identified within the type 1 VWD cohort, R1205C and R1205S, both resulting from nucleotide substitutions at 3613. Increased VWF clearance was demonstrated in both patients. In addition, the R1205C patient (Patient 14) displayed an increase in the VWFpp/VWF:Ag ratio, as well as a similar pattern of VWF release to that seen in the R1205H patient. The VWF response following DDAVP was greater in the R1205S patient (Patient 5) than both R1205H and R1205C patients, although as this patient had a coexistent diagnosis of Haemophilia A, the rise in FVIII was restricted. This co-pathology also leads to difficulties in defining a clear bleeding and laboratory phenotype of R1205S, although this did appear to be milder than for R1205H and R1205C. Functional studies are required to confirm the effect of these mutations on VWF clearance.

Two further patients were excluded from the type 1 VWD cohort due to isolated discrepancies in VWF:CB activity. It is possible that these findings result from the use of type III collagen in this study, which is relatively less sensitive to the presence of HMW multimers than type I collagen. (Penas, et al 2005). However, as well as being reduced in situations where there is a deficiency of VWF or its HMW multimers, a reduction in VWF:CB may also be observed in individuals with mutations in the VWF-A3 domain that interfere with the binding of VWF to collagen.(Goodeve, et al 2007, Ribba, et al 2001, Schneppenheim 2001) Heterozygosity for one of these mutations, 5191 T>A (S1731T) was identified in a patient with a previous diagnosis of type 1 VWD (Patient 20), although the finding of a discordantly low VWF:CB activity had excluded him from the type 1 VWD clearance study cohort. The laboratory phenotype in this patient is similar to that of the two previously reported S1731T cases: all demonstrated borderline low-normal VWF:Ag levels and VWF:RCo activities in the context of normal FVIII:C and

multimeric patterns.(Ribba, et al 2001) No discordant reduction in VWF:CB activity was observed in the offspring of Patient 20, which included the propositus in this kindred, despite all being found to be heterozygous for S1731T. Patient 20 demonstrated a satisfactory response in VWF levels to DDAVP. As expected, the discrepancy in VWF:CB activity persisted following DDAVP despite an increase in HMW multimers. No significant increase in the clearance of VWF:Ag or VWF:CB was found. Within this kindred, only the propositus demonstrated a clinical and laboratory phenotype consistent with type 1 VWD. The phenotypes in her siblings appear normal and the bleeding history in the father, Patient 20, is questionable. Limited molecular analysis of VWF was performed in this kindred and further pathogenic explanation(s), both genetic and non-genetic need to be considered.

In the two related patients previously reported as S1731T heterozygotes, the bleeding phenotype was shown to be mild.(Ribba, et al 2001) However, three other reported VWF-A3 mutations have not been shown to result in any bleeding problems.(Schneppenheim 2001) The relative abundance of VWF-A1 mutations in bleeding patients and lack of bleeding in carriers of VWF-A3 mutations questions the dogma that attributes VWF binding to fibrillar collagen exclusively via the A3 domain.(Romijn, et al 2003) An interaction between the isolated VWF-A1 domain and collagen has recently been demonstrated during platelet recruitment,(Morales, et al 2006) and a reduction in the affinity of VWF for collagen has been found in a type 2B VWD family with a mutation in the A1 domain.(Baronciani, et al 2005) Furthermore, it has recently been shown that the A1 domain is capable of interacting with several types of collagen and is able to assume the function of the A3 domain to trigger platelet recruitment to human collagen fibres.(Bonnefoy, et al 2006) These findings may offer an explanation for the lack of bleeding observed in A3 defective mutants.

The recent inclusion of isolated collagen binding defects in the type 2M subgroup in the 2006 ISTH classification is somewhat controversial.(Sadler, et al 2006) Given the previous VWD classification was strictly focused on platelet-binding defects, at the time of this study, S1731T was unclassified. (Sadler 1994) As discussed above, patients heterozygous for these mutations appear to demonstrate minimal or no bleeding symptoms. The isolated collagen binding defect differs from the plasma VWF in type 1 individuals in whom the binding to collagen is normal,(Favaloro and Koutts 1997) and from 2A and 2B individuals in whom the collagen-binding defect appears to depend only upon the lack of HMW multimers.(Casonato, et al 1999) In type 2M VWD, the loss of binding capacity of VWF to platelet GPIba in the presence

of a full range of VWF multimers retains normal collagen binding capacity.(Hillery, et al 1998) This kindred therefore illustrates the complexity of applying knowledge of VWF mutations to phenotypic data and the lack of clarity that molecular testing may contribute to VWD subtyping.

In contrast to the two patients in whom *VWF* mutations were identified, targeted sequencing of *VWF* did not identify any mutations in the third patient with discordantly low VWF:CB activity in whom accelerated VWF clearance was demonstrated (Patient 42). No discordance in VWF:CB activity was found in the patient's mother despite both individuals displaying near-identical concordant levels of VWF:Ag and VWF:RCo both pre and post DDAVP. These findings are consistent with a type 1 VWD phenotype, which is further supported by the findings of multimeric analysis. It is possible that the findings in this patient result from the use of type III collagen as discussed above.

At the outset of the study, Patient 25 (kindred 10) was known to be heterozygous for 2561G>A (R854Q), although this mutation not been demonstrated in his two affected offspring (including Patient 10). R854Q is the most common type 2N mutation and has been demonstrated in 73% of type 2N VWD patients in homozygous or compound heterozygous form.(Meyer, et al 1997) It has been suggested that R854Q is the only type 2N mutation responsive to DDAVP, as was shown in this patient.(Federici, et al 2004) Further investigation of this kindred in the UKHCDO type 1 VWD molecular genetics study also identified R854Q in the heterozygous state in the unaffected mother and two unaffected offspring of this patient, who are therefore carriers of this recessive type 2 VWD mutation.(Cumming, et al 2006)

2771G>A (R924Q) is also included on the ISTH database as a causative type 2N mutation and in addition to the finding of heterozygosity for R924Q in Patients 25 and 10 in this study, R924Q was identified in all other affected family members in the UKHCDO study.(Cumming, et al 2006) However, such segregation with type 1 VWD phenotype was not found in all families identified with R924Q in the UKHCDO study. The intron 34 candidate splice site mutation, 5843-8C>G was also shown to track with the disease phenotype in this kindred and has been shown to be inherited in phase with R924Q in both family studies and haplotype analysis.(Cumming, et al 2006) Although the frequent finding of R924Q and 5843-C>G in a control population has led to the suggestion that these are common polymorphisms, any possible contribution to the type 1 VWD phenotype is not clear at present. Therefore the

molecular basis of type 1 VWD in this kindred remains uncertain, although it is likely that the low FVIII:C level in Patient 25 results from compound heterozygosity for R854Q and a type 1 VWD allele (carrying R924Q and 5843-8C>G). Notably, Patients 10 and 25 both demonstrated significant reductions in VWF:CB/VWF:Ag ratio following DDAVP, the mechanism for which is not clear and further investigation is warranted.

The common aim of the three recent molecular studies was to examine the genetic basis of type 1 VWD by analysis of *VWF* in clinically affected patients and to relate the findings to the patient's clinical and laboratory phenotype and response to treatment.(Cumming, et al 2006, Goodeve, et al 2007, James, et al 2007a) Despite the finding of numerous novel mutations throughout *VWF*, functional studies are lacking in the majority of these *VWF* gene sequence variants. Therefore, at the present time it is not known whether these newly identified mutations are causative of VWD or if they are merely segregating with the mutant allele. These studies did not identify mutations in the *VWF* gene in up to 45% of patients with type 1 VWD. Taken together with the currently unexplained genetic basis for 40% of the variation in VWF levels,(Levy and Ginsburg 2001) this non-linkage to *VWF* supports a role for other genetic loci in the pathogenesis of type 1 VWD, especially in mild phenotypes. It is well established that *Mvwf1* on chromosome 11 results in an autosomal partial quantitative deficiency of VWF in a murine model due to the aberrant post-translational modification of VWF.(Mohlke, et al 1999b) Further candidate modifier genes have recently been reported in mouse models,(Lemmerhirt, et al 2007, Lemmerhirt, et al 2006, Shavit 2007) and studies of human orthologs are warranted.

In summary, the lower frequency of identified *VWF* mutations in this study (~30%) compared to recently published data can be attributed to the analysis of only selected regions of *VWF*. None of the four previously described mutations in the D'-D3 domain said to be associated with increased VWF clearance were found within this type 1 VWD cohort. This highlights the problem of generalising a study of small numbers of patients from selected type 1 VWD families and challenges the commonly held assumption of a clear link between *VWF* gene mutation and increased clearance of VWF: it is possible that these represent two separate but co-incidental findings in a proportion of type 1 VWD patients. This study did not examine the D4 or CK domains, within which two further mutations have been shown to be associated with increased clearance.(Haberichter, et al 2006, Schooten, et al 2005) Three of the five type 1 VWD patients shown to have increased ratios of VWFpp to VWF:Ag as well as significantly increased clearance of

VWF following DDAVP, were found to have mutations within regions of *VWF* that may affect *in vivo* clearance: R1205C and I1416N. Functional studies are necessary to clarify whether these mutations are causative of increased VWF clearance. One of the D'-D3 mutations previously associated with increased VWF clearance, R1205H (Vicenza), was identified in a patient who had been excluded from the type 1 cohort. This patient demonstrated a significant increase in both VWF clearance following DDAVP and the ratio of VWFpp to VWF:Ag in the context of markedly reduced VWF levels.

A clearer picture is now emerging of the clinical relevance of mutation analysis in type 1 VWD, including awareness of mutations associated with increased VWF clearance. However, the findings in this study suggest that the molecular basis for increased clearance and its classification as a distinct entity (type 1C) may be premature.(Sadler, et al 2006). Sequence analysis of *VWF* is labour-intensive and although it may provide important supplementary diagnostic information in some cases of type 1 VWD as discussed, the frequent finding of non-linkage in type 1 VWD renders it unlikely that genotypic analysis will replace the current measurement of functional activity and multimeric analysis in the diagnostic laboratory setting. Finally, as this study illustrates, the application of knowledge of *VWF* mutations to phenotypic data is not straightforward.

Chapter 9

TYPE 2M VWD

9.1 INTRODUCTION

Type 2M VWD is a variant of VWD in which the decreased binding of VWF to platelets occurs in the presence of normal VWF multimers. The proposed mechanism is a functional loss of binding of VWF to the platelet receptor, glycoprotein Iba (GPIba). This mechanism is supported by data from molecular studies, with 16 of the 18 reported causative mutations being located in the region of the A1 domain of VWF containing the GPIba binding site (http://www.vwf.group.shef.ac.uk/index.html).

The diagnosis of type 2M VWD is made on the basis of a personal and family history of bleeding, a reduction in VWF level with a discordant reduction in functional activity, and a normal multimeric pattern. Although VWF:CB is sensitive to functional variants associated with the loss of HMW multimers, i.e. type 2A and 2B in VWD, the assay has been shown not to reliably discriminate type 2M VWD.(Riddell, *et al* 2002)

Most type 2M VWD patients have not been shown to demonstrate a satisfactory response to DDAVP,(Federici, *et al* 2004, Mannucci, *et al* 1988) and it is recommended practice in the UK to perform a therapeutic trial of DDAVP in all type 2M patients.(Pasi, *et al* 2004) The limitations of judging the effectiveness of DDAVP treatment based solely on the initial rise of VWF in patients with type 1 VWD have been discussed in Chapter 4. As a result, it is recommended practice to measure VWF levels at more than one time point following a test dose of DDAVP, including patients with type 2M patients.(Pasi, *et al* 2004)

Patients with the Vicenza variant were included in the type 2M subtype at the time of this study, as discussed in Chapter 8.(Sadler 1994) Increased VWF clearance had been reported in Vicenza patients, which had been proposed as the pathogenic mechanism of the Vicenza mutation, R1205H.(Casonato, *et al* 2002) The Y1584C mutation has been reported as being a founder haplotype in type 1 VWD,(O'Brien, *et al* 2003) and its prevalence in other bleeding disorders was not known at the time of this study.

In this study, a cohort of 25 patients (15 kindred) were investigated, of whom 14 kindred had been previously investigated at the Royal Free hospital and shown to have phenotypic parameters consistent with type 2M VWD.(Nitu-Whalley IC 2000) Sequencing of a 936 bp fragment of the exon 28 gene had previously identified mutations in the VWF-A1 domain in five kindred, with a further kindred having Del

642.(Nitu-Whalley IC 2000) The phenotypic and genotypic data for this cohort at the outset of this study is summarised in Table 9.1.

9.2 AIMS

The objectives of this study were to determine the antigenic and functional response to DDAVP as well as the plasma clearance of released VWF within a cohort of patients in whom previous investigation at the Royal Free hospital had resulted in a probable or confirmed diagnosis of type 2M VWD.(Nitu-Whalley IC 2000) The possibility that the increased *in vitro* proteolysis of VWF by ADAMTS-13 demonstrated in Y1584C heterozygotes may affect the plasma clearance of VWF was examined in these patients. Genotypic analysis of *VWF* was performed to identify likely causative mutations in exon 28. In addition, regions of the *VWF* gene encoding the D'D3 domains (which have been associated with increased clearance) were sequenced.

9.3 MATERIALS AND METHODS

9.3.1 Patients

A cohort of patients previously diagnosed with type 2M VWD was further investigated for possible causative mutations in targeted regions of the *VWF* gene (n = 25, Table 9.1). The diagnosis of type 2M VWD was based on previous clinical and laboratory findings, (Sadler 1994) with a qualitative defect of VWF defined by a ratio of VWF:RCo to VWF:Ag of less than 0.7. Genotypic analysis had demonstrated causative mutations in the VWF-A1 domain in a proportion of these patients (Table 9.1). The patient cohort was invited to participate in a study investigating the response to DDAVP and clearance of released VWF over a 6 h time period and on this basis, seven patients from five kindred were recruited (3 males, 4 females). Exclusion criteria were as described for type 1 VWD patients and controls in Chapter 3, Section 3.5. Patients are numbered with the prefix 'M'.

9.3.2 Coagulation investigations

VWF:Ag, VWF:RCo and FVIII:C assays were performed as previously described in Chapter 3, Section 3.2. The VWF:CB and VWFpp immunoassays are described in Chapter 6, Section 6.3.2 and Chapter 5, Section 5.3.2 respectively.

9.3.3 Multimeric analysis

The method for the analysis of VWF multimers is described in Chapter 3, Section 3.3. Samples were electrophoresed on 1.4% agarose gels.

9.3.4 Genotypic Analysis

PCR amplification and sequencing of exons 18 – 20, 26, 27 and 28

Genomic DNA extraction is described in Chapter 3, section 3.4. PCR amplification of exons 18 – 20, 26, 27 and 28 and flanking intronic regions was performed as described in Chapter 3, Section 3.4.3 using the gene specific primers given in 8.3.3. The primers used for exon 28 differed from those used in the previous study of these patients. Sequencing of PCR products is described in Chapter 8, Section 8.3.3.

9.3.5 Measurement of clearance of VWF:Ag and VWF:CB following administration of DDAVP

VWF:Ag and VWF:CB were measured prior to (T_0) and over 6 h following DDAVP ($T_1 - T_6$). The method used to calculate VWF:Ag $t_{1/2}$ and VWF:CB $t_{1/2}$ is described in Chapter 3, Section 3.5. Results were analysed and compared to the data obtained for type 1 VWD patients and haemophilia A controls in Chapters 4 and 6.

9.3.6 Data and Statistical Analysis

Analyses of data were performed using GraphPad Prism (GraphPad Prism version 4.0, GraphPad Software, San Diego, USA). Data is presented as median values with range given in brackets. Statistical analyses were performed using the Mann-Whitney Wilcoxon, Kruskal-Wallis and paired t-tests and correlation analysis by Spearman's rank.

Table 9.1: Phenotypic and genotypic data in 15 kindred with previously diagnosed type 2M VWD.

NI: not identified NT: not tested

↓ HMW: multimeric analysis showed slight decrease in high molecular weight multimers (Fig.9.1)

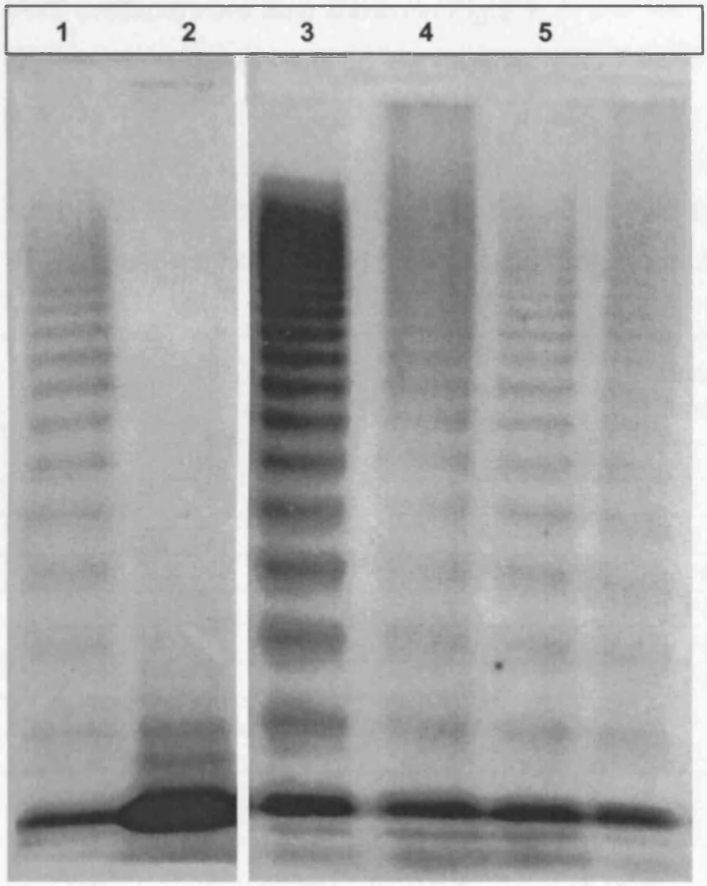
ULHMW: unusually large HMW multimers (Fig.9.1)

*VWF:RCo based on one set of results

Novel candidate mutations.(Nitu-Whalley IC 2000)

Kindred	Patient Number	ABO Blood group	Bleeding Time (min)	FVIII:C ($IUdL^{-1}$)	VWF:Ag ($IUdL^{-1}$)	VWF:RCo ($IUdL^{-1}$)	VWF:RCo/ VWF:Ag	VWF:CB ($IUdL^{-1}$)	Multimers	Mutation	Amino-acid substitution
			<8	50 - 150	50 - 150	50 - 150	1 - 50 - 175				
1	M/1	A	5.5	72	49	16*	0.33	NT	Normal		NI
1	M/2	A	3.5	63	46	30*	0.65	66	Normal		NI
1	M/3	A	3.5	50	49	11*	0.22	61	Normal		NI
2	M/4	O	8	58	43	14*	0.32	50	Normal		NI
3	M/5	O	6	100	45	29	0.64	NT	Normal		NI
4	M/6	A	9.5	68	17	<5	<0.29	NT	Normal		NI
4	M/7	A	20	90	26	5	0.19	64	Normal		NI
5	M/8	O	13	34	17	12	0.7	NT	Normal		NI
6	M/9	O	7.5	22	8	<5	<0.63	6	Normal		NI
6	M/10	O	NT	20	9	<5	<0.56	6	Normal		NI
7	M/11	O	>20	32	18	6	0.33	12	ULHMW		NI
8	M/12	A	NT	29	33	<5	<0.15	55	Normal	4247 G>T	I1416T#
8	M/13	O	NT	50	40	<5	<0.13	54	Normal	4247 G>T	I1416T#
9	M/14	A	NT	73	38	25	0.66	NT	Normal		K1405del
9	M/15	A	NT	52	50	30	0.6	NT	Normal		K1405del
10	M/16	O	20	30	19	<5	<0.26	5	↓ HMW	3961 T>G	Y1321D#
10	M/17	O	20	40	14	<5	<0.36	NT	↓ HMW	3961 T>G	Y1321D#
11	M/18	O	>20	38	23	<5	<0.22	19	Normal	4121 G>T	R1374L
11	M/19	O	NT	22	11	<5	<0.45	21	Normal	4121 G>T	R1374L
11	M/20	O	NT	25	13	<5	<0.38	24	Normal	4121 G>T	R1374L
12	M/21	A	14	32	15	<5	<0.33	19	Normal		NI
13	M/22	A	20	165	76	20	0.26	55	Normal	3835 G>T	V1279F#
14	M/23	B	NT	48	19	<5	<0.26	14	ULHMW	3943 C>T	R1315C
12	M/24	A	20	58	16	<5	<0.31	13	Normal		NI
15	M/25	A	NT	128	55	34	0.62	NT	Normal		NI

Figure 9.1. Autoradiograph of VWF multimers. Multimers are shown for plasma samples from a normal individual (Lane 1), type 2A VWD control (Lane 2), Patient M/23 showing unusually large (UL) multimers (Lane 4), Patient M/16 showing a decrease of HMW multimers (Lane 5) and Patient M/11 showing UL multimers (Lane 6). Samples were electrophoresed on 1.4% agarose gels and the direction of electrophoresis is from top to bottom



9.4 RESULTS

9.4.1 Sequence analysis of VWF

All DNA base changes are heterozygous unless otherwise stated.

Sequence analysis of targeted regions of the *VWF* gene was performed in a cohort of patients previously diagnosed with type 2M VWD (n = 25) and the findings are shown in Table 9.2. Sequencing of exon 28A encoding the VWF-A1 domain identified 4120 C>T (R1374C) in Patients M/9 and M/10 (Kindred 6), 3943 C>T (R1315C) in Patient M/11 (Kindred 7) and 4247 T>A (I1416N) in Patients M/21 and M/24 (Kindred 12). The previously identified A1 mutations were also confirmed. Sequencing of exon 28B of *VWF* encoding the VWF-A2 domain identified 4751 A>G

(Y1584C) in five patients from three kindred (20%; 95% C.I. 4.9 – 38.5%): patients M/1, M/2, M/3 (Kindred 1), M/8 (Kindred 5) and M/16 (Kindred 10). A novel candidate mutation had previously been identified in the A1 domain, 3961 T>G (Y1321D) in Kindred 10, both in the father (Patient M/16) and his daughter (Patient M/17, Table 9.1). Both affected individuals displayed similar bleeding and laboratory phenotypes, with a slight decrease in HMW multimers (Fig.9.1).The multimer patterns for Patients M/11 and M/23 showing the presence of both HMW and UL VWF multimers are also shown in Fig.9.1.

No mutations were identified in exons 18 – 20, 26 and 27 encoding the D'-D3 domain of VWF.

Table 9.2. Mutations identified in exon 28 of *VWF* in cohort of patients with VWD previously classified type 2M (n = 25). No mutation identified (NI)

Kindred	Patient No.	ABO Blood group	Mutation (heterozygous)	Amino-acid substitution
1	M/1	A	4751 A>G	Y1584C
1	M/2	A	4751 A>G	Y1584C
1	M/3	A	4751A>G	Y1584C
2	M/4	O	NI	-
3	M/5	O	NI	-
4	M/6	A	NI	-
4	M/7	A	NI	-
5	M/8	O	4751 A>G	Y1584C
6	M/9	O	4120 C>T	R1374C
6	M/10	O	4120 C>T	R1374C
7	M/11	O	3943 C>T	R1315C
8	M/12	A	***4247 T>C***	***I1416T***
8	M/13	O	4247 T>C	I1416T
9	M/14	A		K1405del
9	M/15	A		K1405del
10	M/16	O	3961 T>G 4751 A>G	Y1321D Y1584C
10	M/17	O	3961 T>G	Y1321D
11	M/18	O	4121 G>T	R1374L
11	M/19	O	4121 G>T	R1374L
11	M/20	O	4121 G>T	R1374L
12	M/21	A	4247 T>A	I1416N
13	M/22	A	3835 G>T	V1279F
14	M/23	B	3943 C>T	R1315C
12	M/24	A	4247 T>A	I1416N
15	M/25	A	NI	-

9.4.2 VWF parameters pre- and 1 h post DDAVP

A subgroup of patients was recruited into the DDAVP study (n = 7). VWF:Ag levels and ratios of VWF:RCo and VWF:CB to VWF:Ag at T_0 and T_1 are shown in Table 9.3. Notably, there was clear discordance between the reductions in VWF:RCo activities and VWF:Ag levels at T_0 in only one patient (M/20). The ratio of VWF:RCo to VWF:Ag at T_0 was found to be greater than 0.7 in three patients (M/2, M/3 and M/4) and was borderline in the remaining three patients from two kindred (M/21, M/24, M/25). It was found that while there was a concordant rise in VWF:Ag and VWF:RCo in Patients M/2, M/3, M/4 and M/25 to satisfactory levels at T_1, the 1 h response, in particular the VWF:RCo activity was restricted in Patients M/20, M/21 and M/24. The ratio of VWF:CB/VWF:Ag was shown to be higher at T_1 than T_0 in four patients (M/2, M/3, M/21, M/24), lower in one patient (M/4) and in two patients remained unchanged (M/20, M/25).

9.4.3 Plasma clearance of VWF:Ag and VWF:CB post DDAVP

VWF:Ag levels and VWF:CB activities were measured at T_0 - T_6 and calculated VWF:Ag $t_{1/2}$ and VWF:CB $t_{1/2}$ values are shown in Table 9.4 (n = 7). No significant difference in VWF:Ag $t_{1/2}$ was demonstrated between the type 2M patients (median 6.4 h, n = 7, Fig.9.2) and either type 1 VWD patients (4.2 h, $P = 0.08$, n = 40, Chapter 4) or haemophilia A controls, (10.1 h, $P = 0.18$, n = 17, Chapter 4). Overall, no difference was found between VWF:Ag $t_{1/2}$ and VWF:CB $t_{1/2}$ values ($P = 0.2$, n = 6, Table 9.4. The notable exception to this is Patient M/3, who is discussed below. Within the two kindred in whom two affected members were studied (Patients M/2, M/3, Kindred 1 and Patients M/21, M/24, Kindred 12), baseline VWF related parameters, DDAVP response and half-life values were comparable (Tables 9.3 and 9.4).

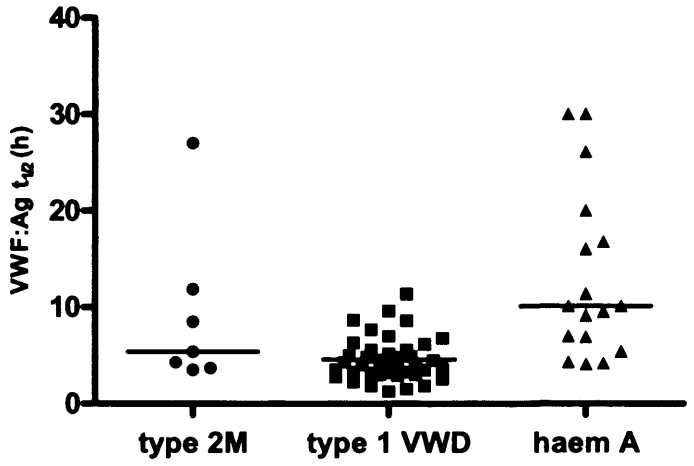
Table 9.3. VWF parameters pre (T^0) and 1 h post DDAVP (T^1) in patients previously diagnosed with type 2M VWD (n = 7).
Multimeric analysis was normal in all patients. *Based on minimum of 3 repeat samples.

Kindred	Patient Number	VWF:Ag T_0 ($IUdL^{-1}$)	VWF:RCo*/ VWF:Ag T_0	VWF:CB/ VWF:Ag T_0	VWF:Ag T_1 ($IUdL^{-1}$)	VWF:RCo/ VWF:Ag T_1	VWF:CB/ VWF:Ag T_1
1	M/2	52	0.81	0.86	173	0.98	1.04
1	M/3	55	0.78	1.09	139	1.04	1.26
2	M/4	42	0.86	1.11	129	0.97	1.0
11	M/20	20	<0.5	0.7	51	<0.20	0.7
12	M/21	15	<0.67	1.00	48	0.56	1.25
12	M/24	16	0.68	0.81	52	0.36	1.25
15	M/25	65	0.66	1.12	126	0.88	1.11

Table 9.4. Half-life values of VWF:Ag and VWF:CB following DDAVP (n = 7). No significant difference was found between VWF:Ag $t_{1/2}$ and VWF:CB $t_{1/2}$ values in each individual, $P = 0.2$. NT not tested

Patient Number	Kindred	VWF:Ag $t_{1/2}$ (h)	VWF:CB $t_{1/2}$ (h)
M/2	1	8.5	NT
M/3	1	11.9	6.2
M/4	2	5.4	6.6
M/20	11	3.7	3.2
M/21	12	4.3	3.5
M/24	12	3.5	3.3
M/25	15	27	11.1

Figure 9.2. Calculated VWF:Ag half-life values for patients previously diagnosed with type 2M VWD. Values are shown against VWF:Ag $t_{1/2}$ values obtained in type 1 VWD patients and haemophilia A controls. No significant difference in median VWF:Ag $t_{1/2}$ values was demonstrated between the type 2M patients (median 6.4 h, n = 7) and either the type 1 VWD patients (4.2 h, $P = 0.08$, n = 40) or haemophilia A controls (10.1 h, $P = 0.18$, n = 17).



9.4.4 Y1584C mutation and VWF clearance

Of the seven patients in whom VWF clearance was measured, 4751A>G (Y1584C) was demonstrated in two (Patients M/2 and M3, Kindred 1) The VWF:Ag $t_{1/2}$ values in these individuals were 8.5 h and 11.9 h respectively (Table 9.4). The third affected member in this kindred was over 60 years of age at the time of the study and was therefore not eligible. The median VWF:Ag $t_{1/2}$ in the five 4751A patients was 4.3 h (range 3.5 – 27 h).

9.4.5 Clinical and laboratory phenotype and genotype.

Quantitative defects of VWF

The laboratory phenotype in Patients M/2, M/3, M/4 and M/25 are consistent with a mild partial quantitative deficiency of VWF: in all cases the VWF:Ag is borderline normal/ reduced and the ratio of VWF:RCo to VWF:Ag = 0.66, with substantial release of functionally intact VWF following DDAVP administration (Table 9.3). Despite the previous finding of a discordant reduction in VWF:RCo activity in these patients (Table 9.1), a minimum of three repeated samples demonstrated concordance between VFW:RCo and VWF:Ag. Within Kindred 1, Patients M/2 and M/3 are brothers, who, along with their mother, Patient M/1, were diagnosed with VWD when Patient M/3 presented with bleeding complications following surgery at the age of 21 years. The clinical history in Patient M/1 at that time included postoperative bleeding and menorrhagia necessitating blood transfusion, however there was no significant clinical history in Patient M/2, including following tonsilloadenoidectomy. Patients M/2 and M/3 were also found to have reduced platelet ADP consistent with a platelet storage pool disorder. As reported above, Patients M/1, M/2 and M/3 (Kindred 1) were found to have the mutation 4751 A>G (Y1584C) in the VWF-A2 domain. Phenotypic data of all the patients in this study in whom Y1584C was identified (n = 5) together with the one the type 1 VWD patient (Patient 12, Appendix 3) and haemophilia A control (Control 11, Appendix 3) reported in Chapter 6, are shown in Table 9.5. All four Y1584C subjects in whom VWF release was examined (Patients M/2, M/3, Patient 12, Control 11) demonstrated an increase in the VWF:CB/VWF:Ag ratio following DDAVP (Table 9.5). All except one (Patient 12) demonstrated normal VWF:Ag $t_{1/2}$ values. Notably, VWF:CB $t_{1/2}$ was shorter than VWF:Ag $t_{1/2}$ in the three subjects in whom clearance of both parameters was measured (Patients M/3, 12, Control 11, Table 9.5).

Patient M/4 is a female who presented at the age of 30 years with haemoptysis and gum bleeding. She had previously undergone tonsilloadenoidectomy with no bleeding complications and there was no significant family history of bleeding.

Patient M/25 is a female who presented aged 21 years with a syncopal episode secondary to severe anaemia requiring blood transfusion. Her clinical history included epistaxis, menorrhagia and easy bruising and there was no significant family history. Targeted molecular sequencing did not identify mutations in *VWF* in either of these patients.

Qualitative defects of VWF

In contrast to the patients described above, Patients M/20 (Kindred 11) and M/21 and M/24 (Kindred 12) demonstrated a variety of qualitative defects of VWF with absent or restricted responses in VWF:RCo activity following DDAVP (Table 9.3). VWF:CB activity was shown to increase following DDAVP in Patients M/21 and M/24 was unchanged in Patient M/20 (Table 9.3). In addition, VWF:Ag $t_{1/2}$ values were found to be shorter than the median $t_{1/2}$ in these patients (Table 9.4). The VWF-A1 domain mutation 4121 G>T (R1374L) had previously been identified in Kindred 11 (Patient M/20) and sequencing of exon 28 in this study identified 4247 T>A (I1416N) in Kindred 12 (Patients M/21 and M/24). Patient M/21 presented at the age of 5 years with gastrointestinal haemorrhage. His mother, Patient M/24, reported a significant bleeding history necessitating blood transfusion. There was also a history of excessive bleeding in the maternal grandmother and a maternal cousin, who had been diagnosed with VWD in another centre. I1416N was also identified in two patients with type 1 VWD (Patients 31 and 43, Chapter 8, Appendix 3) and phenotypic data for all I1416N patients is summarised in Table 9.6. I1416N has recently been reported in one patient with a loss of HMW multimers consistent with type 2A VWD phenotype.(Goodeve, *et al* 2007) Notably, as reflected by the VWF:CB functional activity (Table 9.6), multimeric analysis was normal both pre- and post-DDAVP in all patients identified with I1416N in this study (data not shown). Furthermore, the increase in VWF:CB/VWF:Ag ratio following DDAVP is consistent with release of HMW multimers of VWF (Table 9.6). Patients displayed a moderate to severe clinical and laboratory phenotype, (Table 9.6) with a variable platelet-binding defect in circulating VWF and a predominantly qualitative restrictive defect in VWF release (Patients M/21,M/24,Table 9.3). Both the ratio of VWFpp to VWF:Ag and clearance of VWF:Ag was increased in all I1416N patients (Table 9.6), with concordance between the half-lives of VWF:Ag and VWF:CB, as shown for M21 and M24 (Table 9.4).

Table 9.5. Phenotypic data and half-life values in patients with VWD subtypes 1 and 2M and haemophilia A controls in whom heterozygosity for 4751 A>G (Y1584C) was identified (n = 7).

T_0: pre DDAVP, T_1: 1 h post DDAVP, T_6: 6 h post DDAVP

NT: not tested; NA: not applicable

#3961 T>G (Y1321D) identified;

↓ HMW: slight decrease in high molecular weight multimers

Subject	Previous diagnosis	Bleeding Time (min)	VWF:Ag T_0 ($IUdL^{-1}$)	VWF:CB/ VWF:Ag T_0	VWF:Ag T_1 ($IUdL^{-1}$)	VWF:CB/ VWF:Ag T_1	VWF:CB/ VWF:Ag T_6	FVIII/ VWF:Ag ratio	Multimeric analysis	VWF:Ag $t_{1/2}$ (h)	VWF:CB $t_{1/2}$ (h)
			<8	**50 - 150**							
Patient 12	type 1 VWD	8	34	0.97	217	1.22	1.06	1.8	Normal	4.8	3.5
Control 11	Haem A	NT	50	1.0	165	1.2	1.2	NA	Normal	6.0	11
M/1	type 2M VWD	5.5	54	0.95	NT	NT	NT	1.3	Normal	NT	NT
M/2	type 2M VWD	3.5	50	0.86	173	1.04	NT	1.1	Normal	8.5	NT
M/3	type 2M VWD	3.5	47	1.09	139	1.26	1.12	1.2	Normal	11.9	6.2
M/8	type 2M VWD	13	17	NT	NT	NT	NT	2.0	Normal	NT	NT
M/16#	type 2M VWD	10	19	0.26	NT	NT	NT	1.6	↓ HMW	NT	NT

Table 9.6. Phenotypic data and half-life values in patients with VWD subtypes 1 and 2M and in whom heterozygosity for 4247 T>C (I1416N) was identified (n = 4). VWF multimeric analysis was normal in all subjects.

Patient	Previous diagnosis	Bleeding Time (min)	VWF: Ag T_0 ($IUdL^{-1}$)	VWF:CB/ VWF:Ag (T_0)	VWF: Ag T_1 ($IUdL^{-1}$)	VWF:CB/ VWF:Ag (T_1)	FVIII:C/VWF:Ag ratio (T_0)	VWFpp/Ag ratio (T_0)	VWF:Ag $t_{1/2}$ (h)
31	type 1 VWD	NT	18	0.67	92	1.10	1.5	0.55	3.5
43	type 1 VWD	>20	11	0.81	53	1.19	2.7	0.49	3.0
M/21	type 2M VWD	14	15	1.0	48	1.25	2.1	0.56	3.5
M/24	type 2M VWD	20	16	0.81	52	1.25	3.6	0.57	3.3

9.5 DISCUSSION

The recently updated classification of VWD defines the type 2M subtype as including 'qualitative variants with decreased VWF-dependent platelet adhesion without a selective deficiency of HMW VWF multimers. The assembly and secretion of large molecular weight multimers is approximately normal and the functional defect is caused by mutations that disrupt binding of VWF to platelets or subendothelium.'(Sadler, *et al* 2006)

In this study, targeted analysis of the *VWF* gene was performed in a cohort of patients with a historical diagnosis of type 2M VWD.(Nitu-Whalley IC 2000) This included sequence analysis of the region of *VWF* that encodes the GPIba binding site within the A1 domain of VWF. Previous mutation analysis had identified six mutations in this region in 11 of the 25 patients examined (six of 15 kindred).(Nitu-Whalley IC 2000) In this study, three VWF-A1 mutations were identified in a further five patients (three kindred). It is possible that these mutations were not identified in the original study because of overlap between the primer used at that time and a polymorphic site and the primers used in this study had been redesigned to take account of this. One VWF-A2 domain mutation (Y1584C) was identified in five patients in this study (three kindred). One of these patients had previously been identified as having a VWF-A1 mutation. Combining the findings in both studies, a total of eight A1 domain mutations and one A2 domain mutation were identified in 20 of these 25 patients (11 kindred).

The majority of patients registered with a diagnosis of type 2M VWD at the Royal Free Hospital had not received DDAVP at the outset of this study. One of the objectives of this study was to determine the antigenic and functional response to DDAVP as well as the plasma clearance of VWF released following DDAVP. Seven patients recruited from the cohort described above were investigated. In contrast to previous findings, concordance between circulating VWF levels and functional activity was demonstrated in four patients from three kindred in this study (Patients M/2 and M/3 (Kindred 1), Patient M/4, Patient M/25), consistent with a type 1 VWD phenotype. This was supported by the finding of a good functional VWF response following DDAVP in these patients as well as an absence of mutations in the VWF-A1 domain. As the VWF:CB assay is sensitive to loss of HMW multimers and not to platelet-binding defects, VWF function was determined by VWF:RCo activity in both studies. The cause of the discrepancy in VWF:RCo activities between studies found in some patients is not clear; it is known that the VWF:RCo inter- and intra-assay variation is considerable and coefficient of variation (CV) values up to 80% have

been demonstrated in the most recent United Kingdom National External Quality Assessment Scheme (UKNEQAS). Furthermore, the EU type 1 VWD study has demonstrated considerable overlap in the ratio of VWF:RCo to VWF:Ag between subjects with normal and abnormal multimeric patterns.(Goodeve, *et al* 2007)

In the remaining patients investigated (Patient M/20 (Kindred 11) and Patients M/21 and M/24 (Kindred 12)), the predominant functional defect related to VWF-platelet binding, consistent with the type 2M variant. Mutations in the VWF-A1 domain have been demonstrated in these patients. The finding of a restricted VWF response to DDAVP in this subgroup of patients, especially in VWF:RCo activity is consistent with the poor responses to DDAVP reported in the majority of patients with type 2M VWD.(Federici, *et al* 2004) These data reinforce the importance of laboratory monitoring of VWF:RCo activity following DDAVP in these patients.

Within both subgroups of patients studied (type 1 and type 2M phenotype), the ratio of VWF:CB to VWF:Ag was found to increase following DDAVP in the majority of patients, consistent with release of HMW multimers. This was similar to the findings in type 1 VWD patients and haemophilia A controls as discussed in Chapter 6. A wide range in the half-life values of VWF:Ag released following DDAVP was found, accounting for the lack of significant difference between the half-lives in this group of patients and both type 1 VWD and haemophilia A patients, in whom VWF clearance has previously been shown to be increased and normal respectively (Chapter 4). Although patient numbers in this study cohort are insufficient for formal analysis, the half-lives of VWF in the subgroup of patients with type 2M phenotype appeared to be shortened, the significance of which is not clear. In contrast, VWF clearance rates were within normal limits in the subgroup of patients with VWF parameters consistent with type 1 VWD. No mutations were identified on sequence analysis of regions of *VWF* encoding the D'-D3 domains of VWF, which have previously been reported in association with increased VWF clearance.(Haberichter, *et al* 2006a, Lenting, *et al* 2004, Schooten, *et al* 2005) Although this study provides only limited data on VWF clearance in type 2M VWD patients, further studies are warranted.

Genotypic analysis of the type 1 VWD subgroup demonstrated the VWF-A2 domain mutation 4751 A>G (Y1584C) in Patients M/2 and M/3 (Kindred 1). A detailed discussion of Y1584C is presented below. Investigation of the patients with type 2M phenotype had previously demonstrated 4121C>T (R1374L) in Kindred 11 (Patient M/20), and in this study 4247A>T (I1416N) was identified in Kindred 12 (Patients M/21 and M/24). I1416N was also identified in two patients with type 1 VWD

(Chapter 8). The clinical and laboratory phenotype of all I1416N patients was shown to be similar: a dominant and penetrant pattern of inheritance was demonstrated, together with a poor functional response in VWF:RCo activity as compared to a much better (but still restricted) response in VWF:Ag and VWF:CB and normal multimeric pattern. In addition, all patients were shown to have a shortened VWF half-life as well as an increased ratio of FVIII to VWF:Ag. These findings suggest defective VWF synthesis/secretion, release of functionally deficient VWF (predominant platelet-binding defect) and increased VWF clearance as possible pathogenic mechanisms of I1416N. A further mutation affecting I1416 has previously been identified in a kindred in this study, 4247 T>C (I1416T, Kindred 8), and reported as a novel candidate VWD type 2M mutation.(Nitu-Whalley IC 2000)

The effect of R1374 mutations on VWF function is not clear, which has led to the classification of these mutations as both type 2A and type 2M VWD variants.(Casonato, *et al* 2001b, Goodeve, *et al* 2007, Hilbert, *et al* 1995) Multimeric analysis had previously been shown to be normal in the two kindred within this cohort in whom R1374 mutations were identified (Kindred 6 and 11). The laboratory phenotype in Kindred 6 (Patients M/9 and M/10) in whom 4120 C>T (R1374C) was demonstrated in this study is suggestive of a disproportionate reduction in VWF:RCo activity compared to both VWF:Ag and VWF:CB, although it is possible that the levels are too low to meaningfully differentiate. Discordance between functional activities of VWF:RCo and VWF:CB was more clearly defined in Kindred 11 in whom 4121 G>T (R1374L) had previously been identified.(Nitu-Whalley IC 2000) Furthermore, the VWF:RCo/VWF:Ag ratio decreased following DDAVP in Patient M/20 (Kindred 11) with no change in the VWF:CB/VWF:Ag ratio. These findings are consistent with a predominant platelet-binding effect of R1374 mutations, with a relatively minor effect on VWF multimer size, consistent with the functional abnormality that defines type 2M VWD.

Controversy also surrounds the functional defect that results from the nucleotide substitution 3943 C>T (R1315C), identified in Patient M/11 in this study and previously identified in Patient M/23.(Nitu-Whalley IC 2000) Both patients demonstrated circulating UL-HMW multimers. As well as being reported as a type 2M mutation(Casana, *et al* 1998) and a type 2A mutation,(Goodeve, *et al* 2007) R1315C has also been described in cases of type 1 VWD (James, *et al* 2007a) While it is possible that these discrepancies may reflect variation in the sensitivity of multimeric analysis, this mutation illustrates the lack of clinically informative data that can result from the classification of VWD on a molecular basis.

The prevalence of 4751 A>G (Y1584C) was determined in the cohort of patients with a historical diagnosis of type 2M VWD. Y1584C was demonstrated in five of the 25 patients investigated (Kindred 1, 5, 10). Sequence analysis of the A1, A2 and D'-D3 domains identified no other mutations in four of these patients. A candidate mutation in the A1 domain, 3961 T>G (Y1321D) had previously been identified in the kindred of the remaining Y1584C patient (Patient M/16, Kindred 10); both affected members of this kindred displayed a similar clinical and laboratory phenotype. As previously described, the phenotype in Kindred 1 was found to be consistent with type 1 VWD. Only targeted areas of the *VWF* gene were sequenced in these patients, and therefore the significance of Y1584C as causative or contributory towards the disease phenotype is not clear. Furthermore, although heterozygosity for Y1584C was demonstrated in one fifth of the patients and kindred in this study, the phenotypes of these patients were found to be heterogeneous: three of the five Y1584C and two Y1584Y patients demonstrated phenotypic parameters consistent with a partial quantitative deficiency of VWF and a lack of HMW multimers was found in the patient heterozygous for both Y1321D and Y1584C. This results in the demonstration of heterozygosity for Y1584C in only one of the 19 patients previously reported as having type 2M VWD.

Y1584C is the most frequently occurring *VWF* gene mutation in type 1 VWD with reported frequencies of 11% and 15% in type 1 VWD index cases in the EU and Canadian studies respectively,(Goodeve, *et al* 2007, James, *et al* 2007a) and 19% of affected kindred members in the UK study.(Cumming, *et al* 2006) These data contrast with reported frequencies in the normal population of 0% (O'Brien, *et al* 2003) and 1%.(Bowen, *et al* 2005) As reported in Chapter 6, Y1584C was demonstrated in one of the 27 patients (24 kindred) investigated with type 1 VWD (~4%). Taken together with the finding of Y1584C in Kindred 1 in this study results in an overall prevalence of Y1584C in type 1 VWD kindred of ~8 % in and ~15% in affected kindred members.

While Y1584C has been shown to be associated with a reduction in VWF levels,(Bowen, et al 2005) the mechanism is not known. When expressed in its homozygous form, C1584 has been shown to result in increased intracellular retention of VWF.(O'Brien, *et al* 2003) Analysis of the Y1584C patients in this cohort together with the Y1584C type 1 VWD patient and haemophilia A control (Chapter 6) showed the ratio of FVIII to VWF:Ag to be increased in some patients, suggestive of a defect in VWF synthesis or secretion. However, it is possible that this could be accounted for by confounding factors including other (as yet unidentified) *VWF*

mutations. All Y1584C patients who had been stimulated with DDAVP were shown to demonstrate satisfactory responses in both the level and functional activity of VWF, with increased ratios of VWF:CB to VWF:Ag consistent with release of HMW multimers. The half-lives of VWF:Ag released following DDAVP were not found to be significantly shortened in these patients. This contrasts with the increased VWF clearance found in type 1 VWD patients, irrespective of the presence of the Y1584C mutation (Chapter 6). However, the VWF:CB half-life was found to be shorter than VWF:Ag half-life in the three Y1584C patients in whom both parameters were measured. This is consistent with *in vitro* finding of increased ADAMTS-13 mediated VWF proteolysis resulting from C1584. (Bowen and Collins 2004, Keeney, *et al* 2007) Although the findings in this study do not define a clear mechanism of Y1584C, these data do suggest that increased *in vitro* ADAMTS-13 proteolysis does not significantly affect the plasma clearance of VWF.

The absence of consistent co-segregation of C1584 with either low VWF levels or bleeding phenotype in affected type 1 VWD kindred (Bowen, *et al* 2005, James, *et al* 2006, Lanke, *et al* 2005) suggests that on its own, rather than being causative, C1584 may be a risk factor for type 1 VWD. Y1584C has been shown to be associated with blood group O in 95% of type 1 VWD patients,(Bowen, *et al* 2005, O'Brien, *et al* 2003) an even higher proportion than the reported prevalence of blood group O in type 1 VWD patients of 77%.(Gill, *et al* 1987) A recent study of a large population of blood donors has shown C1584 to exert a greater influence on VWF levels than blood group O. (Davies, *et al* 2006) Moreover, the combined effect of blood group O and C1584 was shown to be similar to their product, suggestive of a synergistic interaction between blood group O and Y1584C that results in reduction in VWF level.

An alternative explanation for the apparent enrichment of Y1584C in type 1 VWD has been proposed, which suggests that C1584 may be more prevalent in patients in whom primary haemostasis is defective, irrespective of where the defect lies in the platelet-VWF-vessell wall axis.(Bowen and Collins 2006) Therefore, rather than being causative of VWD, C1584 may increase the probability that an individual will present with bleeding symptoms of sufficient severity to warrant investigation.

This study highlights the lack of clear distinction between VWD types 1, 2M and 2A, with overlap demonstrated at both phenotypic and genotypic level. The findings in this study are consistent with previous data demonstrating the limited value of DDAVP in patients with type 2M VWD,(Federici, *et al* 2004) and thus re-enforce the

clinical importance of distinguishing type 2M from type 1 VWD. It has recently been proposed that types 2A and 2M VWD should no longer be differentiated, thereby all variants with a decreased platelet-dependent function would be categorised together, regardless of the multimeric distribution.(Batlle 2007) Although this would simplify the classification and clinically important information would not be missed (such as the limited value of DDAVP in both subtypes), it does not address the main diagnostic difficulty in classifying VWD subtypes 1 and 2M.

Finally, this study illustrates the need for a better functional measurement of VWF activity. Some of the limitations of the VWF:RCo assay have been highlighted: this is currently the primary assay used to distinguish type 2M from type 1 VWD. Although a ratio of VWF:RCo to VWF:Ag of less than 0.7 is generally used to distinguish qualitative VWF defects,(Federici, *et al* 2002) a significantly higher likelihood of identifying A1 domain mutations has recently been shown in patients with VWF:RCo/VWF:Ag ratios of <0.4.(James, *et al* 2007b) The use of a ratio of 0.7 to differentiate qualitative from quantitative defects of VWF may account for the apparent previous over-diagnosis of type 2M VWD in some of the patients in this study. It has recently been proposed that the structural normality of plasma VWF could be assessed by the multimer pattern alone.(Goodeve, *et al* 2007) The findings in this study support the use of diagnostic genotypic analysis in the differentiation of VWD types 1 and 2M. Forthcoming recommendations from the ISTH/SSC working party on laboratory assays for VWD will hopefully address these issues.

Chapter 10

CONCLUSIONS

Type 1 VWD is the most common variant of VWD and yet until recently was the least understood of the VWD subtypes. The work presented in this thesis coincided with the onset of three large studies of type 1 VWD, which have since provided new insights into this disorder and resulted in considerable improvement in the understanding of its molecular basis. (James *et al*, 2007, Goodeve *et al* 2007, Cumming *et al* 2006) Despite the finding of a large number of *VWF* mutations in these studies, functional data are largely lacking. It is now well established that lowered VWF levels do not appear to be linked to the *VWF* gene in a significant proportion of type 1 VWD patients, especially those with milder phenotypes. (James, *et al* 2006, Eikenboom *et al*, 2006)

In this thesis, the release and clearance of VWF released following DDAVP has been examined in a group of patients with a partial quantitative deficiency of VWF. In keeping with previous reports, increased clearance of VWF has been demonstrated in a significant proportion of patients, supporting the view that decreased survival of VWF in plasma may be a mechanism underlying low VWF levels in some patients with type 1 VWD. (Brown, *et al* 2003, Michiels, *et al* 2002, van Genderen, *et al* 1997) A variety of parameters have been investigated in an attempt to identify *VWF* gene linked and non-linked variables that may affect VWF clearance. No single underlying common characteristic or variable was shown to predominate within the study group, despite the majority of these patients demonstrating increased rates of VWF clearance. Such absence of a common mechanism is however not surprising, given that type 1 VWD is a classification that can include patients with a ten-fold difference in absolute VWF levels.

The method used to calculate the half-life of VWF released following DDAVP in this thesis is well established and validated by the findings in the control group.(Brown, *et al* 2003, van Genderen P, 1997, Michiels *et al,* 2002). The demonstration of increased clearance of VWF released following DDAVP may be of therapeutic relevance in type 1 VWD patients. However, the findings in this thesis raise the possibility that this approach may provide limited information about the clearance of constitutively released VWF, which accounts for the majority of circulating VWF in plasma. This could account for the lack of consistency shown between the plasma clearance of VWF released following DDAVP and the relative circulating concentrations of VWFpp and VWF:Ag. It has been proposed that measurement of VWFpp may identify patients with shortened VWF:Ag half-lives.(Haberichter, *et al* 2006) Although this thesis demonstrates increased VWF clearance following DDAVP in all patients with increased VWFpp/VWF:Ag ratios (a suggested index of

accelerated VWF clearance), increased VWF clearance was also commonly found in the context of normal VWFpp/VWF:Ag ratios. While the significance of these data is not clear, it can be concluded that the VWFpp/VWF:Ag ratio appears to be of limited value in predicting increased clearance of VWF released following DDAVP. This is particularly relevant given the current commercial development of the VWFpp assay.

Despite being the largest study of its kind to date, the patient and control numbers in this study may render it under-powered in its ability to demonstrate findings that are statistically significant. Pertinent to this is the study of ABO blood group effect on the clearance of VWF: while no significant difference in VWF clearance rates was observed between individuals of different ABO blood groups following DDAVP, ratios of VWFpp/VWF:Ag ratio were shown to be significantly increased in individuals of blood group O compared to A. This discrepancy further illustrates the potential limitations of the approach used to assess VWF clearance in this thesis as discussed above. As well as being limited by patient numbers, heterogeneity of the pathogenic mechanisms within the VWD study group was also found to complicate the investigation of determinants of VWF clearance in this thesis. The study group included patients with VWF:Ag levels ranging from 5 – 50 $IUdL^{-1}$. Consistent with previously reported findings, the likelihood of defining the underlying molecular mechanism in the *VWF* gene was found to be higher in patients of more severe phenotype, defined by a reduction in VWF:Ag levels to < 20 $IUdL^{-1}$. (Eikenboom *et al,* 2006) Less clear-cut and more common was the finding of patients with circulating VWF:Ag values approaching normal levels who demonstrated increased rates of VWF clearance, yet normal ratios of VWFpp to VWF:Ag, and in whom no mutations were found within the regions of the *VWF* gene analysed. The genetic determinants of milder type 1 VWD phenotypes are known to be more complex and are more likely to involve contributions from other factors, including ABO blood group and increased VWF clearance, as the findings in this thesis suggest. Strict criteria in the selection of patients are necessary in future studies to overcome the problems encountered in association with patient heterogeneity in this thesis. However, the recruitment of patients with milder phenotypes is often difficult as these patients are likely to be less motivated than patients with more severe bleeding problems.

The *VWF* gene mutations identified in this thesis include the VWF-D3 mutation R1205H, (Vicenza) which has previously been demonstrated to be causative of increased VWF clearance (Lenting *et al*, 2004) and recently re-classified as a

variant of type 1 VWD. (Sadler, 2006) Two novel candidate mutations were also identified that result from arginine substitutions at 1205, R1205C and R1205S, as well as a novel candidate mutation in the VWF-A1 domain, I1416N. These mutations were all associated with significantly accelerated VWF clearance and were in general identified in patients with steady-state VWF levels reduced to < 20 $IUdL^{-1}$. Patients demonstrated both increased rates of clearance of released VWF and increased steady-state ratios VWFpp to VWF:Ag. In addition, all patients identified with the mutation I1416N demonstrated restricted functional release of VWF in the context of normal VWF multimeric analysis, consistent with a type 2M phenotype. Expression of these mutants is essential to confirm the significance of these findings.

The findings in this thesis suggest that increased plasma clearance of VWF does not consistently reflect steady-state VWF levels in type 1 VWD. Firstly, there was only a weak correlation between plasma VWF:Ag levels and the half-life values for VWF:Ag, and secondly, no difference was observed for the VWFpp/VWF:Ag ratio between the type 1 VWD patients and controls. These findings suggest that reduced steady state VWF levels are either not, or only in part, due to increased clearance of VWF. A defect in intracellular transport and secretion by endothelial cells may also contribute towards the deficiency of VWF in these patients. It is also possible that because of structural differences between VWF released following DDAVP administration and plasma VWF, clearance kinetics of plasma and newly released VWF may differ. Indeed, VWF released following DDAVP infusion has previously been shown to differ from circulating VWF in terms of multimer composition and expression of A antigen. (Ruggeri, *et al* 1982, Brown, *et al* 2002) In this thesis, an increased HMW multimeric content of VWF, reflected by the relative increase in VWF:CB activity compared to VWF level, was observed following DDAVP in the majority of type 1 VWD patients and controls. While differential glycosylation of DDAVP-released VWF may be important in determining its half-life, this thesis did not demonstrate a significant difference in the exposure of galactose residues by the binding of the lectin RCA-I between circulating and released VWF. Furthermore, consistent with previously reported findings, inverse correlation was found between the binding of RCA-I to VWF and the steady-state VWF level. (Ellies *et al*, 2002) However, this relationship appeared to be independent of VWF clearance and together with recent reports, these findings suggest that VWF clearance is independent of the exposure of N- and O-linked galactose residues.(van Schooten *et al,* 2007)

Although the ratio of VWF:CB to VWF:Ag was shown to increase following DDAVP in the majority of type 1 VWD patients and controls, two type 1 VWD patients from the same kindred demonstrated significant reductions in the ratio of VWF:CB to VWF:Ag. In both patients the VWF-D3 mutation R924Q mutation was identified, although the pathological significance of this mutation is not currently established. Neither patient was shown to demonstrate increased proteolysis of VWF by ADAMTS-13. While these findings are suggestive of the removal of HMW multimers, which may not be related to VWF proteolysis, the underlying mechanism is not clear and further studies are warranted.

It is not known how VWF proteolysis, plasma multimer distribution and VWF clearance are integrated *in vivo*. In this thesis, the size of VWF multimers in relation to the release and clearance following DDAVP has been examined. Clearance of VWF was not shown to be associated with either the absolute levels of ADAMTS-13 or susceptibility of VWF to cleavage by ADAMTS-13. These findings are supported by recent indirect experimental data in which plasma VWF levels in wild-type and ADAMTS-13 deficient mice were not shown to differ.(Motto *et al*, 2005) After ABO blood group, C1584 has been shown to be the second most influential genetic modulator of VWF level in humans,(Davies *et al*, 2006) and is known to influence the susceptibility of VWF to ADAMTS-13 mediated proteolysis.(Bowen and Collins, 2004) Despite the demonstration of increased rates of proteolysis in Y1584C heterozygotes, the findings in this thesis suggest that the Y1584C mutation does not have a major effect on VWF clearance.

Some of the complexities of VWD subtyping have been illustrated throughout this thesis and the relevance of developing a classification system that is both clinically and scientific informative has been discussed. The recently revised ISTH-SSC recommendations have attempted to address some of the limitations of the previous 1994 VWD classification.(Sadler, *et al* 2006) These include the correlation of VWD phenotype with the likely response to DDAVP treatment. In addition, it has been proposed that that type 1 VWD patients demonstrating an increased VWF clearance phenotype should be classified as a distinct entity (type 1C). The findings in this thesis suggest that such a distinction may be premature at this stage and results of ongoing clinical studies comparing the biological response following DDAVP with clinical efficacy may provide a more clinically relevant basis for distinguishing patients with increased VWF clearance.

To date, the asialoglycoprotein receptor is the only identified endocytic receptor that has been shown to mediate the removal of VWF from the circulation and its reactivity appears to be limited to hyposialylated VWF or VWF with an altered glycosylation profile.(Ellies, *et al* 2002, Mohlke, *et al* 1999) Preliminary data suggests that macrophage-like cells present in both the liver and the spleen may contribute towards the clearance of VWF (unpublished). An improved knowledge of the mechanisms of VWF clearance, including the identification of VWF clearance receptors, may help clarify the pathogenic role of altered VWF clearance in patients with type 1 VWD. As discussed, future studies of VWF clearance in type 1 VWD would benefit from the improved stratification of patients, with the focus being on patients with milder phenotypes. An approach of studying the clearance of VWF that has been constitutively released from the endothelial pool may provide a more accurate measurement of VWF clearance than the DDAVP-induced release studied in this thesis. Unlike regulated secretion, VWF secreted via the constitutive pathway is largely composed of dimers and small multimers. Methods such as plasma fractionation or chromatography could be used to separate VWF multimers by size and the plasma clearance of these multimers could be studied in a suitable murine model, notwithstanding the possible limitations of different receptor and proteolysis mechanisms.

In conclusion, the work presented in this thesis contributes novel observations on different aspects of VWF clearance. Although increased clearance of VWF released following DDAVP was a prevalent finding in the type 1 VWD cohort studied in this thesis, no unifying underlying mechanism, either *VWF* gene linked or non-linked, was shown to predominate in these patients. The findings in this thesis support the hypothesis that the phenotype of type 1 VWD is not due to a single causative effect but is likely to be due to a composite effect of a number of factors under separate genetic control.

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Appendices

Appendix 1

Nucleotide sequence of VWF cDNA and amino acid residues of pre-pro-VWF. The numbering of the nucleotides and amino acid residues are according to the ISTH subcommittee on VWF recommendations. (Goodeve and Peake, 2001) Numbering of the nucleotides commences with the initiator ATG and the numbering of the amino acid residues commences from the initiator methionine.

The nucleotide triplets (comprising A = adenine, T = thymine, G = guanine and C = cytosine) are in the upper line and the corresponding amino acid residues (see end of sequence for abbreviations) are given below the nucleotide triplets. The numbering shows the number of the nucleotide and then the amino acid residue at the start of each line, e.g. 73/25. The amino acid residues to which the N-linked and O-linked oligosaccharide side chains are attached have been highlighted in red (N) and blue (S or T) respectively.

1/1
ATG ATT CCT GCC AGA TTT GCC GGG GTG CTG CTT GCT CTG GCC CTC ATT TTG CCA GGG ACC CTT TGT GCA GAA
M I P A R F A G V L L A L A L I L P G T L C A E

73/25
GGA ACT CGC GGC AGG TCA TCC ACG GCC CGA TGC AGC CTT TTC GGA AGT GAC TTC GTC AAC ACC TTT GAT GGG
G T R G R S S T A R C S L F G S D F V N T F D G

145/49
AGC ATG TAC AGC TTT GCG GGA TAC TGC AGT TAC CTC CTG GCA GGG GGC TGC CAG AAA CGC TCC TTC TCG ATT
S M Y S F A G Y C S Y L L A G G C Q K R S F S I

217/73
ATT GGG GAC TTC CAG AAT GGC AAG AGA GTG AGC CTC TCC GTG TAT CTT GGG GAA TTT TTT GAC ATC CAT TTG
I G D F Q N G K R V S L S V Y L G E F F D I H L

289/97
TTT GTC AAT GGT ACC GTG ACA CAG GGG GAC CAA AGA GTC TCC ATG CCC TAT GCC TCC AAA GGG CTG TAT CTA
F V N G T V T Q G D Q R V S M P Y A S K G L Y L

361/121
GAA ACT GAG GCT GGG TAC TAC AAG CTG TCC GGT GAG GCC TAT GGC TTT GTG GCC AGG ATC GAT GGC AGC GGC
E T E A G Y Y K L S G E A Y G F V A R I D G S G

433/145
AAC TTT CAA GTC CTG CTG TCA GAC AGA TAC TTC AAC AAG ACC TGC GGG CTG TGT GGC AAC TTT AAC ATC TTT
N F Q V L L S D R Y F N K T C G L C G N F N I F

505/169
GCT GAA GAT GAC TTT ATG ACC CAA GAA GGG ACC TTG ACC TCG GAC CCT TAT GAC TTT GCC AAC TCA TGG GCT
A E D D F M T Q E G T L T S D P Y D F A N S W A

577/193
CTG AGC AGT GGA GAA CAG TGG TGT GAA CGG GCA TCT CCT CCC AGC AGC TCA TGC AAC ATC TCC TCT GGG GAA
L S S G E Q W C E R A S P P S S S C N I S S G E

649/217
ATG CAG AAG GGC CTG TGG GAG CAG TGC CAG CTT CTG AAG AGC ACC TCG GTG TTT GCC CGC TGC CAC CCT CTG
M Q K G L W E Q C Q L L K S T S V F A R C H P L

721/241
GTG GAC CCC GAG CCT TTT GTG GCC CTG TGT GAG AAG ACT TTG TGT GAG TGT GCT GGG GGG CTG GAG TGC GCC
V D P E P F V A L C E K T L C E C A G G L E C A

793/265
TGC CCT GCC CTC CTG GAG TAC GCC CGG ACC TGT GCC CAG GAG GGA ATG GTG CTG TAC GGC TGG ACC GAC CAC
C P A L L E Y A R T C A Q E G M V L Y G W T D H

865/289
AGC GCG TGC AGC CCA GTG TGC CCT GCT GGT ATG GAG TAT AGG CAG TGT GTG TCC CCT TGC GCC AGG ACC TGC
S A C S P V C P A G M E Y R Q C V S P C A R T C

937/313
CAG AGC CTG CAC ATC AAT GAA ATG TGT CAG GAG CGA TGC GTG GAT GGC TGC AGC TGC CCT GAG GGA CAG CTC
Q S L H I N E M C Q E R C V D G C S C P E G Q L

1009/337
CTG GAT GAA GGC CTC TGC GTG GAG AGC ACC GAG TGT CCC TGC GTG CAT TCC GGA AAG CGC TAC CCT CCC GGC
L D E G L C V E S T E C P C V H S G K R Y P P G

1081/361
ACC TCC CTC TCT CGA GAC TGC AAC ACC TGC ATT TGC CGA AAC AGC CAG TGG ATC TGC AGC AAT GAA GAA TGT
T S L S R D C N T C I C R N S Q W I C S N E E C

1153/385
CCA GGG GAG TGC CTT GTC ACA GGT CAA TCA CAC TTC AAG AGC TTT GAC AAC AGA TAC TTC ACC TTC AGT GGG
P G E C L V T G Q S H F K S F D N R Y F T F S G

1225/409
ATC TGC CAG TAC CTG CTG GCC CGG GAT TGC CAG GAC CAC TCC TTC TCC ATT GTC ATT GAG ACT GTC CAG TGT
I C Q Y L L A R D C Q D H S F S I V I E T V Q C

1297/433
GCT GAT GAC CGC GAC GCT GTG TGC ACC CGC TCC GTC ACC GTC CGG CTG CCT GGC CTG CAC AAC AGC CTT GTG
A D D R D A V C T R S V T V R L P G L H N S L V

1369/457
AAA CTG AAG CAT GGG GCA GGA GTT GCC ATG GAT GGC CAG GAC GTC CAG CTC CCC CTC CTG AAA GGT GAC CTC
K L K H G A G V A M D G Q D V Q L P L L K G D L

1441/481
CGC ATC CAG CAT ACA GTG ACG GCC TCC GTG CGC CTC AGC TAC GGG GAG GAC CTG CAG ATG GAC TGG GAT GGC
R I Q H T V T A S V R L S Y G E D L Q M D W D G

1513/505
CGC GGG AGG CTG CTG GTG AAG CTG TCC CCC GTC TAT GCC GGG AAG ACC TGC GGC CTG TGT GGG AAT TAC AAT
R G R L L V K L S P V Y A G K T C G L C G N Y N

1585/529
GGC AAC CAG GGC GAC GAC TTC CTT ACC CCC TCT GGG CTG GCG GAG CCC CGG GTG GAG GAC TTC GGG AAC GCC
G N Q G D D F L T P S G L A E P R V E D F G N A

1657/553
TGG AAG CTG CAC GGG GAC TGC CAG GAC CTG CAG AAG CAG CAC AGC GAT CCC TGC GCC CTC AAC CCG CGC ATG
W K L H G D C Q D L Q K Q H S D P C A L N P R M

1729/577
ACC AGG TTC TCC GAG GAG GCG TGC GCG GTC CTG ACG TCC CCC ACA TTC GAG GCC TGC CAT CGT GCC GTC AGC
T R F S E E A C A V L T S P T F E A C H R A V S

1801/601
CCG CTG CCC TAC CTG CGG AAC TGC CGC TAC GAC GTG TGC TCC TGC TCG GAC GGC CGC GAG TGC CTG TGC GGC
P L P Y L R N C R Y D V C S C S D G R E C L C G

1873/625
GCC CTG GCC AGC TAT GCC GCG GCC TGC GCG GGG AGA GGC GTG CGC GTC GCG TGG CGC GAG CCA GGC CGC TGT
A L A S Y A A A C A G R G V R V A W R E P G R C

1945/649
GAG CTG AAC TGC CCG AAA GGC CAG GTG TAC CTG CAG TGC GGG ACC CCC TGC AAC CTG ACC TGC CGC TCT CTC
E L N C P K G Q V Y L Q C G T P C N L T C R S L

2017/673
TCT TAC CCG GAT GAG GAA TGC AAT GAG GCC TGC CTG GAG GGC TGC TTC TGC CCC CCA GGG CTC TAC ATG GAT
S Y P D E E C N E A C L E G C F C P P G L Y M D

2089/697
GAG AGG GGG GAC TGC GTG CCC AAG GCC CAG TGC CCC TGT TAC TAT GAC GGT GAG ATC TTC CAG CCA GAA GAC
E R G D C V P K A Q C P C Y Y D G E I F Q P E D

2161/721
ATC TTC TCA GAC CAT CAC ACC ATG TGC TAC TGT GAG GAT GGC TTC ATG CAC TGT ACC ATG AGT GGA GTC CCC
I F S D H H T M C Y C E D G F M H C T M S G V P

2233/745
GGA AGC TTG CTG CCT GAC GCT GTC CTC AGC AGT CCC CTG TCT CAT CGC AGC AAA AGG AGC CTA TCC TGT CGG
G S L L P D A V L S S P L S H R S K R ***Ser*** L S C R

2305/769
CCC CCC ATG GTC AAG CTG GTG TGT CCC GCT GAC AAC CTG CGG GCT GAA GGG CTC GAG TGT ACC AAA ACG TGC
P P M V K L V C P A D N L R A E G L E C T K T C

2377/793
CAG AAC TAT GAC CTG GAG TGC ATG AGC ATG GGC TGT GTC TCT GGC TGC CTC TGC CCC CCG GGC ATG GTC CGG
Q N Y D L E C M S M G C V S G C L C P P G M V R

2449/817
CAT GAG AAC AGA TGT GTG GCC CTG GAA AGG TGT CCC TGC TTC CAT CAG GGC AAG GAG TAT GCC CCT GGA GAA
H E N R C V A L E R C P C F H Q G K E Y A P G E

2521/841
ACA GTG AAG ATT GGC TGC AAC ACT TGT GTC TGT CGG GAC CGG AAG TGG AAC TGC ACA GAC CAT GTG TGT GAT
T V K I G C N T C V C R D R K W N C T D H V C D

2593/865
GCC ACG TGC TCC ACG ATC GGC ATG GCC CAC TAC CTC ACC TTC GAC GGG CTC AAA TAC CTG TTC CCC GGG GAG
A T C S T I G M A H Y L T F D G L K Y L F P G E

2665/889
TGC CAG TAC GTT CTG GTG CAG GAT TAC TGC GGC AGT AAC CCT GGG ACC TTT CGG ATC CTA GTG GGG AAT AAG
C Q Y V L V Q D Y C G S N P G T F R I L V G N K

2737/913
GGA TGC AGC CAC CCC TCA GTG AAA TGC AAG AAA CGG GTC ACC ATC CTG GTG GAG GGA GGA GAG ATT GAG CTG
G C S H P S V K C K K R V T I L V E G G E I E L

2809/937
TTT GAC GGG GAG GTG AAT GTG AAG AGG CCC ATG AAG GAT GAG ACT CAC TTT GAG GTG GTG GAG TCT GGC CGG
F D G E V N V K R P M K D E T H F E V V E S G R

2881/961
TAC ATC ATT CTG CTG CTG GGC AAA GCC CTC TCC GTG GTC TGG GAC CGC CAC CTG AGC ATC TCC GTG GTC CTG
Y I I L L L G K A L S V V W D R H L S I S V V L

2953/985
AAG CAG ACA TAC CAG GAG AAA GTG TGT GGC CTG TGT GGG AAT TTT GAT GGC ATC CAG AAC AAT GAC CTC ACC
K Q T Y Q E K V C G L C G N F D G I Q N N D L T

3025/1009
AGC AGC AAC CTC CAA GTG GAG GAA GAC CCT GTG GAC TTT GGG AAC TCC TGG AAA GTG AGC TCG CAG TGT GCT
S S N L Q V E E D P V D F G N S W K V S S Q C A

3097/1033
GAC ACC AGA AAA GTG CCT CTG GAC TCA TCC CCT GCC ACC TGC CAT AAC AAC ATC ATG AAG CAG ACG ATG GTG
D T R K V P L D S S P A T C H N N I M K Q T M V

3169/1057
GAT TCC TCC TGT AGA ATC CTT ACC AGT GAC GTC TTC CAG GAC TGC AAC AAG CTG GTG GAC CCC GAG CCA TAT
D S S C R I L T S D V F Q D C N K L V D P E P Y

3241/1081
CTG GAT GTC TGC ATT TAC GAC ACC TGC TCC TGT GAG TCC ATT GGG GAC TGC GCC TGC TTC TGC GAC ACC ATT
L D V C I Y D T C S C E S I G D C A C F C D T I

3313/1105
GCT GCC TAT GCC CAC GTG TGT GCC CAG CAT GGC AAG GTG GTG ACC TGG AGG ACG GCC ACA TTG TGC CCC CAG
A A Y A H V C A Q H G K V V T W R T A T L C P Q

3385/1129
AGC TGC GAG GAG AGG AAT CTC CGG GAG AAC GGG TAT GAG TGT GAG TGG CGC TAT AAC AGC TGT GCA CCT GCC
S C E E R N L R E N G Y E C E W R Y N S C A P A

3457/1153
TGT CAA GTC ACG TGT CAG CAC CCT GAG CCA CTG GCC TGC CCT GTG CAG TGT GTG GAG GGC TGC CAT GCC CAC
C Q V T C Q H P E P L A C P V Q C V E G C H A H

3529/1177
TGC CCT CCA GGG AAA ATC CTG GAT GAG CTT TTG CAG ACC TGC GTT GAC CCT GAA GAC TGT CCA GTG TGT GAG
C P P G K I L D E L L Q T C V D P E D C P V C E

3601/1201
GTG GCT GGC CGG CGT TTT GCC TCA GGA AAG AAA GTC ACC TTG AAT CCC AGT GAC CCT GAG CAC TGC CAG ATT
V A G R R F A S G K K V T L N P S D P E H C Q I

3673/1225
TGC CAC TGT GAT GTT GTC AAC CTC ACC TGT GAA GCC TGC CAG GAG CCG GGA GGC CTG GTG GTG CCT CCC ACA
C H C D V V N L T C E A C Q E P G G L V V P P T

3745/1249
GAT GCC CCG GTG AGC CCC ACC ACT CTG TAT GTG GAG GAC ATC TCG GAA CCG CCG TTG CAC GAT TTC TAC TGC
D A P V S P T T L Y V E D I S E P P L H D F Y C

3817/1273
AGC AGG CTA CTG GAC CTG GTC TTC CTG CTG GAT GGC TCC TCC AGG CTG TCC GAG GCT GAG TTT GAA GTG CTG
S R L L D L V F L L D G S S R L S E A E F E V L

3889/1297
AAG GCC TTT GTG GTG GAC ATG ATG GAG CGG CTG CGC ATC TCC CAG AAG TGG GTC CGC GTG GCC GTG GTG GAG
K A F V V D M M E R L R I S Q K W V R V A V V E

3961/1321
TAC CAC GAC GGC TCC CAC GCC TAC ATC GGG CTC AAG GAC CGG AAG CGA CCG TCA GAG CTG CGG CGC ATT GCC
Y H D G S H A Y I G L K D R K R P S E L R R I A

4033/1345
AGC CAG GTG AAG TAT GCG GGC AGC CAG GTG GCC TCC ACC AGC GAG GTC TTG AAA TAC ACA CTG TTC CAA ATC
S Q V K Y A G S Q V A S T S E V L K Y T L F Q I

4105/1369
TTC AGC AAG ATC GAC CGC CCT GAA GCC TCC CGC ATC GCC CTG CTC CTG ATG GCC AGC CAG GAG CCC CAA CGG
F S K I D R P E A S R I A L L L M A S Q E P Q R

4177/1393
ATG TCC CGG AAC TTT GTC CGC TAC GTC CAG GGC CTG AAG AAG AAG AAG GTC ATT GTG ATC CCG GTG GGC ATT
M S R N F V R Y V Q G L K K K K V I V I P V G I

4249/1417
GGG CCC CAT GCC AAC CTC AAG CAG ATC CGC CTC ATC GAG AAG CAG GCC CCT GAG AAC AAG GCC TTC GTG CTG
G P H A N L K Q I R L I E K Q A P E N K A F V L

4321/1441
AGC AGT GTG GAT GAG CTG GAG CAG CAA AGG GAC GAG ATC GTT AGC TAC CTC TGT GAC CTT GCC CCT GAA GCC
S S V D E L E Q Q R D E I V S Y L C D L A P E A

4393/1465
CCT CCT CCT ACT CTG CCC CCC CAC ATG GCA CAA GTC ACT GTG GGC CCG GGG CTC TTG GGG GTT TCG ACC CTG
P P P T L P P H M A Q V T V G P G L L G V S T L

4465/1489
GGG CCC AAG AGG AAC TCC ATG GTT CTG GAT GTG GCG TTC GTC CTG GAA GGA TCG GAC AAA ATT GGT GAA GCC
G P K R N S M V L D V A F V L E G S D K I G E A

4537/1513
GAC TTC AAC AGG AGC AAG GAG TTC ATG GAG GAG GTG ATT CAG CGG ATG GAT GTG GGC CAG GAC AGC ATC CAC
D F N R S K E F M E E V I Q R M D V G Q D S I H

4609/1537
GTC ACG GTG CTG CAG TAC TCC TAC ATG GTG ACC GTG GAG TAC CCC TTC AGC GAG GCA CAG TCC AAA GGG GAC
V T V L Q Y S Y M V T V E Y P F S E A Q S K G D

4681/1561
ATC CTG CAG CGG GTG CGA GAG ATC CGC TAC CAG GGC GGC AAC AGG ACC AAC ACT GGG CTG GCC CTG CGG TAC
I L Q R V R E I R Y Q G G N R T N T G L A L R Y

4753/1585
CTC TCT GAC CAC AGC TTC TTG GTC AGC CAG GGT GAC CGG GAG CAG GCG CCC AAC CTG GTC TAC ATG GTC ACC
L S D H S F L V S Q G D R E Q A P N L V Y M V T

4825/1609
GGA AAT CCT GCC TCT GAT GAG ATC AAG AGG CTG CCT GGA GAC ATC CAG GTG GTG CCC ATT GGA GTG GGC CCT
G N P A S D E I K R L P G D I Q V V P I G V G P

4897/1633
AAT GCC AAC GTG CAG GAG CTG GAG AGG ATT GGC TGG CCC AAT GCC CCT ATC CTC ATC CAG GAC TTT GAG ACG
N A N V Q E L E R I G W P N A P I L I Q D F E T

4969/1657
CTC CCC CGA GAG GCT CCT GAC CTG GTG CTG CAG AGG TGC TGC TCC GGA GAG GGG CTG CAG ATC CCC ACC CTC
L P R E A P D L V L Q R C C S G E G L Q I P T L

5041/1681
TCC CCT GCA CCT GAC TGC AGC CAG CCC CTG GAC GTG ATC CTT CTC CTG GAT GGC TCC TCC AGT TTC CCA GCT
S P A P D C S Q P L D V I L L L D G S S S F P A

5113/1705
TCT TAT TTT GAT GAA ATG AAG AGT TTC GCC AAG GCT TTC ATT TCA AAA GCC AAT ATA GGG CCT CGT CTC ACT
S Y F D E M K S F A K A F I S K A N I G P R L T

5185/1729
CAG GTG TCA GTG CTG CAG TAT GGA AGC ATC ACC ACC ATT GAC GTG CCA TGG AAC GTG GTC CCG GAG AAA GCC
Q V S V L Q Y G S I T T I D V P W N V V P E K A

5257/1753
CAT TTG CTG AGC CTT GTG GAC GTC ATG CAG CGG GAG GGA GGC CCC AGC CAA ATC GGG GAT GCC TTG GGC TTT
H L L S L V D V M Q R E G G P S Q I G D A L G F

5329/1777
GCT GTG CGA TAC TTG ACT TCA GAA ATG CAT GGT GCC AGG CCG GGA GCC TCA AAG GCG GTG GTC ATC CTG GTC
A V R Y L T S E M H G A R P G A S K A V V I L V

5401/1801
ACG GAC GTC TCT GTG GAT TCA GTG GAT GCA GCA GCT GAT GCC GCC AGG TCC AAC AGA GTG ACA GTG TTC CCT
T D V S V D S V D A A A D A A R S N R V T V F P

5473/1825
ATT GGA ATT GGA GAT CGC TAC GAT GCA GCC CAG CTA CGG ATC TTG GCA GGC CCA GCA GGC GAC TCC AAC GTG
I G I G D R Y D A A Q L R I L A G P A G D S N V

5545/1849
GTG AAG CTC CAG CGA ATC GAA GAC CTC CCT ACC ATG GTC ACC TTG GGC AAT TCC TTC CTC CAC AAA CTG TGC
V K L Q R I E D L P T M V T L G N S F L H K L C

5617/1873
TCT GGA TTT GTT AGG ATT TGC ATG GAT GAG GAT GGG AAT GAG AAG AGG CCC GGG GAC GTC TGG ACC TTG CCA
S G F V R I C M D E D G N E K R P G D V W T L P

5689/1897
GAC CAG TGC CAC ACC GTG ACT TGC CAG CCA GAT GGC CAG ACC TTG CTG AAG AGT CAT CGG GTC AAC TGT GAC
D Q C H T V T C Q P D G Q T L L K S H R V N C D

5761/1921
CGG GGG CTG AGG CCT TCG TGC CCT AAC AGC CAG TCC CCT GTT AAA GTG GAA GAG ACC TGT GGC TGC CGC TGG
R G L R P S C P N S Q S P V K V E E T C G C R W

5833/1945
ACC TGC CCC TGC GTG TGC ACA GGC AGC TCC ACT CGG CAC ATC GTG ACC TTT GAT GGG CAG AAT TTC AAG CTG
T C P C V C T G S S T R H I V T F D G Q N F K L

5905/1969
ACT GGC AGC TGT TCT TAT GTC CTA TTT CAA AAC AAG GAG CAG GAC CTG GAG GTG ATT CTC CAT AAT GGT GCC
T G S C S Y V L F Q N K E Q D L E V I L H N G A

5977/1993
TGC AGC CCT GGA GCA AGG CAG GGC TGC ATG AAA TCC ATC GAG GTG AAG CAC AGT GCC CTC TCC GTC GAG CTG
C S P G A R Q G C M K S I E V K H S A L S V E L

6049/2017
CAC AGT GAC ATG GAG GTG ACG GTG AAT GGG AGA CTG GTC TCT GTT CCT TAC GTG GGT GGG AAC ATG GAA GTC
H S D M E V T V N G R L V S V P Y V G G N M E V

6121/2041
AAC GTT TAT GGT GCC ATC ATG CAT GAG GTC AGA TTC AAT CAC CTT GGT CAC ATC TTC ACA TTC ACT CCA CAA
N V Y G A I M H E V R F N H L G H I F T F T P Q

6193/2065
AAC AAT GAG TTC CAA CTG CAG CTC AGC CCC AAG ACT TTT GCT TCA AAG ACG TAT GGT CTG TGT GGG ATC TGT
N N E F Q L Q L S P K T F A S K T Y G L C G I C

6265/2089
GAT GAG AAC GGA GCC AAT GAC TTC ATG CTG AGG GAT GGC ACA GTC ACC ACA GAC TGG AAA ACA CTT GTT CAG
D E N G A N D F M L R D G T V T T D W K T L V Q

6337/2113
GAA TGG ACT GTG CAG CGG CCA GGG CAG ACG TGC CAG CCC ATC CTG GAG GAG CAG TGT CTT GTC CCC GAC AGC
E W T V Q R P G Q T C Q P I L E E Q C L V P D S

6409/2137
TCC CAC TGC CAG GTC CTC CTC TTA CCA CTG TTT GCT GAA TGC CAC AAG GTC CTG GCT CCA GCC ACA TTC TAT
S H C Q V L L L P L F A E C H K V L A P A T F Y

6481/2161
GCC ATC TGC CAG CAG GAC AGT TGC CAC CAG GAG CAA GTG TGT GAG GTG ATC GCC TCT TAT GCC CAC CTC TGT
A I C Q Q D S C H Q E Q V C E V I A S Y A H L C

6553/2185
CGG ACC AAC GGG GTC TGC GTT GAC TGG AGG ACA CCT GAT TTC TGT GCT ATG TCA TGC CCA CCA TCT CTG GTC
R T N G V C V D W R T P D F C A M S C P P S L V

6625/2209
TAC AAC CAC TGT GAG CAT GGC TGT CCC CGG CAC TGT GAT GGC AAC GTG AGC TCC TGT GGG GAC CAT CCC TCC
Y N H C E H G C P R H C D G N V S S C G D H P S

6697/2233
GAA GGC TGT TTC TGC CCT CCA GAT AAA GTC ATG TTG GAA GGC AGC TGT GTC CCT GAA GAG GCC TGC ACT CAG
E G C F C P P D K V M L E G S C V P E E A C T Q

6769/2257
TGC ATT GGT GAG GAT GGA GTC CAG CAC CAG TTC CTG GAA GCC TGG GTC CCG GAC CAC CAG CCC TGT CAG ATC
C I G E D G V Q H Q F L E A W V P D H Q P C Q I

6841/2281
TGC ACA TGC CTC AGC GGG CGG AAG GTC AAC TGC ACA ACG CAG CCC TGC CCC ACG GCC AAA GCT CCC ACG TGT
C T C L S G R K V N C T T Q P C P T A K A P T C

6913/2305
GGC CTG TGT GAA GTA GCC CGC CTC CGC CAG AAT GCA GAC CAG TGC TGC CCC GAG TAT GAG TGT GTG TGT GAC
G L C E V A R L R Q N A D Q C C P E Y E C V C D

6985/2329
CCA GTG AGC TGT GAC CTG CCC CCA GTG CCT CAC TGT GAA CGT GGC CTC CAG CCC ACA CTG ACC AAC CCT GGC
P V S C D L P P V P H C E R G L Q P T L T N P G

7057/2353
GAG TGC AGA CCC AAC TTC ACC TGC GCC TGC AGG AAG GAG GAG TGC AAA AGA GTG TCC CCA CCC TCC TGC CCC
E C R P N F T C A C R K E E C K R V S P P S C P

7129/2377
CCG CAC CGT TTG CCC ACC CTT CGG AAG ACC CAG TGC TGT GAT GAG TAT GAG TGT GCC TGC AAC TGT GTC AAC
P H R L P T L R K T Q C C D E Y E C A C N C V N

7201/2401
TCC ACA GTG AGC TGT CCC CTT GGG TAC TTG GCC TCA ACC GCC ACC AAT GAC TGT GGC TGT ACC ACA ACC ACC
S T V S C P L G Y L A S T A T N D C G C T T T T

7273/2425
TGC CTT CCC GAC AAG GTG TGT GTC CAC CGA AGC ACC ATC TAC CCT GTG GGC CAG TTC TGG GAG GAG GGC TGC
C L P D K V C V H R S T I Y P V G Q F W E E G C
7345/2449
GAT GTG TGC ACC TGC ACC GAC ATG GAG GAT GCC GTG ATG GGC CTC CGC GTG GCC CAG TGC TCC CAG AAG CCC
D V C T C T D M E D A V M G L R V A Q C S Q K P

7417/2473
TGT GAG GAC AGC TGT CGG TCG GGC TTC ACT TAC GTT CTG CAT GAA GGC GAG TGC TGT GGA AGG TGC CTG CCA
C E D S C R S G F T Y V L H E G E C C G R C L P

7489/2497
TCT GCC TGT GAG GTG GTG ACT GGC TCA CCG CGG GGG GAC TCC CAG TCT TCC TGG AAG AGT GTC GGC TCC CAG
S A C E V V T G S P R G D S Q S S W K S V G S Q

7561/2521
TGG GCC TCC CCG GAG AAC CCC TGC CTC ATC AAT GAG TGT GTC CGA GTG AAG GAG GAG GTC TTT ATA CAA CAA
W A S P E N P C L I N E C V R V K E E V F I Q Q

7633/2545
AGG AAC GTC TCC TGC CCC CAG CTG GAG GTC CCT GTC TGC CCC TCG GGC TTT CAG CTG AGC TGT AAG ACC TCA
R N V S C P Q L E V P V C P S G F Q L S C K T S

7705/2569
GCG TGC TGC CCA ACG TGT CGC TGT GAG CGC ATG GAG GCC TGC ATG CTC AAT GGC ACT GTC ATT GGG CCC GGG
A C C P T C R C E R M E A C M L N G T V I G P G

7777/2593
AAG ACT GTG ATG ATC GAT GTG TGC ACG ACC TGC CGC TGC ATG GTG CAG GTG GGG GTC ATC TCT GGA TTC AAG
K T V M I D V C T T C R C M V Q V G V I S G F K

7849/5617
CTG GAG TGC AGG AAG ACC ACC TGC AAC CCC TGC CCC CTG GGT TAC AAG GAA GAA AAT AAC ACA GGT GAA TGT
L E C R K T T C N P C P L G Y K E E N N T G E C

7921/2641
TGT GGG AGA TGT TTG CCT ACG GCT TGC ACC ATT CAG CTA AGA GGA GGA CAG ATC ATG ACA CTG AAG CGT GAT
C G R C L P T A C T I Q L R G G Q I M T L K R D

7993/2665
GAG ACG CTC CAG GAT GGC TGT GAT ACT CAC TTC TGC AAG GTC AAT GAG AGA GGA GAG TAC TTC TGG GAG AAG
E T L Q D G C D T H F C K V N E R G E Y F W E K

8065/2689
AGG GTC ACA GGC TGC CCA CCC TTT GAT GAA CAC AAG TGT CTG GCT GAG GGA GGT AAA ATT ATG AAA ATT CCA
R V T G C P P F D E H K C L A E G G K I M K I P

8137/2713
GGC ACC TGC TGT GAC ACA TGT GAG GAG CCT GAG TGC AAC GAC ATC ACT GCC AGG CTG CAG TAT GTC AAG GTG
G T C C D T C E E P E C N D I T A R L Q Y V K V

8209/2737
GGA AGC TGT AAG TCT GAA GTA GAG GTG GAT ATC CAC TAC TGC CAG GGC AAA TGT GCC AGC AAA GCC ATG TAC
G S C K S E V E V D I H Y C Q G K C A S K A M Y

8281/2761
TCC ATT GAC ATC AAC GAT GTG CAG GAC CAG TGC TCC TGC TGC TCT CCG ACA CGG ACG GAG CCC ATG CAG GTG
S I D I N D V Q D Q C S C C S P T R T E P M Q V

8353/2785
GCC CTG CAC TGC ACC AAT GGC TCT GTT GTG TAC CAT GAG GTT CTC AAT GCC ATG GAG TGC AAA TGC TCC CCC
A L H C T N G S V V Y H E V L N A M E C K C S P

8425/2809
AGG AAG TGC AGC AAG TGAGGCTGCTGCAGCTGCATGGGTGCCTGCT
R K C S K

8471
GCTGCCTGCCTTGGCCTGATGGCCAGGCCAGAGTGCTGCCAGTCCTCTGCATGTTCTGCTCTTGTGCCCT

8541
TCTGAGCCCACAATAAAGGCTGAGCTCTTATCTTGCAAAAA

Abbreviations for amino acid residues.

A	Ala	Alanine	K	Lys	Lysine
R	Arg	Arginine	M	Met	Methionine
N	Asn	Asparagine	F	Phe	Phenylalanine
D	Asp	Aspartic acid	P	Pro	Proline
C	Cys	Cysteine	S	Ser	Serine
Q	Gln	Glutamine	T	Thr	Threonine
E	Glu	Glutamic acid	W	Trp	Tryptophan
G	Gly	Glycine	Y	Tyr	Tyrosine
H	His	Histidine	V	Val	Valine
I	Ile	Isoleucine			
L	Leu	Leucine			

1. Goodeve A, Peake I. A standard nomenclature for von Willebrand factor gene mutations and polymorphisms. Best Pract Res Clin Haematol. 2001;14:235-240

Appendix 2, Table 1. Phenotypic data for type 1 VWD patients at time of diagnosis

Patient Number	Kindred	sex	ABO Blood group	age (years)	FVIII:C ($IUdL^{-1}$)	VWF:Ag ($IUdL^{-1}$)	VWF:RCo ($IUdL^{-1}$)
1	1	M	O	35	56	45	44
2	2	M	A	52	60	34	46
3	3	M	O	46	7	4	5
4	4	F	O	47	52	36	35
5	5	F	O	40	18*	25	26
6	6	F	A	48	34	42	38
7	7	M	A	27	36	14	11
8	8	M	O	36	62	29	34
9	9	F	O	27	80	35	35
10	10	F	A	21	66	27	23
11	8	F	O	60	67	36	41
12	11	F	A	52	62	34	30
13	8	M	O	31	68	36	41
14	12	M	O	41	20	13	17
15	13	M	O	34	64	50	52
16	14	M	O	25	52	36	36
17	15	M	O	32	60	50	36
18	16	M	O	22	70	38	37
19	17	F	O	43	57	39	46
20	18	M	A	44	70	46	56
21	19	F	O	57	52	42	38
22	20	F	A	40	69	22	17
23	21	M	O	32	58	37	30
24	22	F	O	32	64	37	37
25	10	M	A	45	13**	30	23
26	23	F	A	28	68	44	52
27	24	M	A	57	28	10	9
28	25	M	O	31	35	17	14
29	26	M	O	39	70	47	46
30	27	M	A	38	48	30	23
31	28	M	O	48	27	18	16
32	29	F	NT	21	21	9	10
33	30	F	NT	30	70	43	39
34	31	M	NT	45	131	45	47
35	32	M	A	35	76	33	43
36	33	F	A	51	104	40	42
37	34	F	A	44	95	49	41
38	35	F	O	33	27	13	10
39	36	F	O	19	78	45	36
40	37	M	A	52	18	14	32
41	38	F	O	49	61	44	41
42	39	F	A	25	28	25	24
43	40	M	A	54	22	14	11

VWF multimeric analysis was normal in all patients.
Patients 3, 20 and 42 were subsequently found to have discordant ratios of VWF:CB to VWF:Ag and excluded from the study
NT-not tested
* Patient 5 known haemophilia A carrier ** Patient 25 known to have factor VIII binding defect

Appendix 2, Table 2. Phenotypic data for haemophilia A controls

Patient Number	Kindred	sex	ABO Blood group	age (years)	FVIII:C ($IUdL^{-1}$)	VWF:Ag ($IUdL^{-1}$)	VWF:RCo ($IUdL^{-1}$)
1	1	M	A	57	19	238	240
2	2	M	O	31	33	93	99
3	3	M	B	35	38	168	300
4	4	M	B	54	10	192	178
5	5	M	O	56	4	61	48
6	6	M	O	44	5	131	196
7	7	M	O	26	17	82	82
8	8	M	O	19	17	65	NT
9	9	M	O	36	6	45	50
10	10	M	O	44	20	63	84
11	11	M	O	47	5	50	67
12	12	M	A	58	26	193	124
13	13	M	O	41	7	162	128
14	14	M	O	40	26	83	100
15	15	M	O	41	30	159	112
16	16	M	A	41	8	105	92
17	17	M	A	25	38	126	118

NT - not tested

Appendix 3, Table 1. Type 1 VWD patient data (n = 40)

Patient Number	Kindred	sex	ABO Blood group	VWF:Ag T_0 ($IUdL^{-1}$)	VWFpp T_0 (nM)	VWF:CB T_0 ($IUdL^{-1}$)	ADAMTS-13 T_0 (%)	VWFpp/ VWF:Ag ratio	Increase in VWF:Ag from T_0 to T_1 ($IUdL^{-1}$)	VWF:Ag T_1 ($IUdL^{-1}$)	VWF:Ag $t_{1/2}$ (h)	VWFpp $t_{1/2}$ (h)	VWF:CB $t_{1/2}$ (h)	Y1584C	Amino acid Sub-stitution	RCA-I/ VWF:Ag T_0	ECA/ VWF:Ag T_0
1	1	M	O	46	2.847	47	61.792592	0.12	94	140	7	2.9	5.53	NT	NT	1.31	1.99
2	2	M	A	84	2.9	102	82.9	0.06	109	193	7.7	2.6	10.28	tyr/tyr	NI	1.25	1.04
4	4	F	O	27	1.9	37	60.74448	0.14	70	97	4	2.1	4.93	tyr/tyr	NI	2.05	2.24
5	5	F	O	43	3.324	26	76.799224	0.15	101	144	1.9	2.5	2.97	tyr/tyr	R1205S	1.02	1.32
6	6	F	A	51	1.594	44	126.63004	0.06	92	143	3.5	8	9.88	tyr/tyr	R768Q	1.05	0.75
7	7	M	A	17	2.864	14	92.625214	0.34	100	117	2.3	2.6	1.72	tyr/tyr	NI	1.26	1.57
8	8	M	O	50	4.372	44	71.7	0.17	136	186	3	2.3	3.06	tyr/tyr	NI	0.92	1.06
9	9	F	O	49	2.671	49	50.852605	0.11	89	138	8.7	2.6	4.22	NT	NT	1.25	1.34
10	10	F	A	57	2.8	50	99.906122	0.1	115	172	3.2	2.5	7.85	tyr/tyr	R924Q	1.28	1.31
11	8	F	O	51	3.355	60	89.281019	0.13	105	156	6.8	3.5	6.76	tyr/tyr	NI	1.27	1.87
12	11	F	A	58	3.809	56	116.13377	0.13	159	217	4.8	2.4	3.49	cys/tyr	Y1584C	1.12	1.37
13	8	M	O	56	10.891	50	83.836846	0.39	154	210	3.2	1.7	3.76	tyr/tyr	NI	1.18	1.5
14	12	M	O	13	2.944	10	59.305591	0.45	28	41	3.3	3.1	4.65	tyr/tyr	R1205C	1.1	1.56
15	13	M	O	52	3.5	43	94.54418	0.13	128	180	4.3	2.9	4.62	tyr/tyr	NI	1.25	1.44
16	14	M	O	46	3.724	43	75.940924	0.16	153	199	3.8	2.6	3.13	tyr/tyr	NI	1.26	0.76
17	15	M	O	56	3.6	56	88.533646	0.13	177	233	1.9	2.8	3.31	NT	NT	0.79	1.16
18	16	M	O	38	3.3	30	95.856564	0.17	127	165	5.6	1.8	4.98	tyr/tyr	NI	1.14	1.32
19	17	F	O	43	2.497	43	93.643472	0.12	181	224	4.1	2.5	4.21	tyr/tyr	NI	1.05	1.54
21	19	F	O	42	2.4	38	73.124677	0.11	137	179	3.3	2.1	3.43	tyr/tyr	NI	1.29	1.72
22	20	F	A	18	1.4	16	80.166905	0.16	36	54	6.2	2.3	6.51	NT	NI	1.38	1.38
23	21	M	O	51	3	49	95.594954	0.12	111	162	3.8	2.1	3.13	tyr/tyr	NI	1.05	1.38

NI: not identified NT: not tested

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Appendix 3, Table 1. Type 1 VWD patient data (n = 40)

Patient Number	Kindred	sex	ABO Blood group	VWF:Ag T_0 ($IUdL^{-1}$)	VWFpp T_0 (nM)	VWF:CB T_0 ($IUdL^{-1}$)	ADAMTS-13 T_0 (%)	VWFpp/ VWF:Ag ratio	Increase in VWF:Ag from T_0 to T_1 ($IUdL^{-1}$)	VWF:Ag T_1 ($IUdL^{-1}$)	VWF:Ag $t_{1/2}$ (h)	VWFpp $t_{1/2}$ (h)	VWF:CB $t_{1/2}$ (h)	Y1584C	Amino acid Sub-stitution	RCA-I/ VWF:Ag T_0	ECA/ VWF:Ag T_0
25	22	F	O	50	2.7	42	90.6515	0.11	161	211	4.8	2.9	6.31	tyr/tyr	NI	1.46	1.97
25	10	M	A	37	2.4	34	98.537787	0.13	234	271	5.6	1.9	5.68	tyr/tyr	R854Q; R924Q	1.34	1.19
26	23	F	A	74	4.1	74	100.7	0.11	141	215	2.8	2.4	3.37	tyr/tyr	NI	1.07	2.04
27	24	M	A	10	NT	NT	NT	NT	14	24	2.6	NT	NT	tyr/tyr	NI	NT	NT
28	25	M	O	17	NT	NT	NT	NT	29	46	2.9	NT	NT	tyr/tyr	NI	NT	NT
29	26	M	O	47	NT	NT	NT	NT	188	235	5	NT	NT	tyr/tyr	NI	1.32	2.38
30	27	M	A	30	NT	NT	NT	NT	157	187	6.3	NT	NT	tyr/tyr	NI	1.53	2.54
31	28	M	O	18	4.7	NT	NT	0.52	75	93	3.5	2.6	NT	tyr/tyr	I1416N	0.82	1.26
32	29	F	NT	9	NT	NT	NT	NT	104	113	1.5	NT	NT	NT	NT	NT	NT
33	30	F	NT	43	NT	NT	NT	NT	102	145	4.6	NT	NT	NT	NT	NT	NT
34	31	M	NT	45	NT	NT	NT	NT	65	110	8.6	NT	NT	NT	NT	NT	NT
35	32	M	A	33	NT	NT	NT	NT	57	90	5.2	NT	NT	NT	NT	NT	NT
36	33	F	A	40	NT	NT	NT	NT	101	141	11.4	NT	NT	NT	NT	NT	NT
37	34	F	A	49	NT	NT	NT	NT	65	114	4.9	NT	NT	NT	NT	NT	NT
38	35	F	O	13	NT	NT	NT	NT	35	48	3.6	NT	NT	NT	NT	NT	NT
39	36	F	O	45	NT	NT	NT	NT	127	172	4.5	NT	NT	NT	NT	NT	NT
40	37	M	A	14	NT	NT	NT	NT	71	85	1.3	NT	NT	NT	NT	NT	NT
41	38	F	O	44	3.7	52	NT	0.17	83	127	9.6	2.2	5	tyr/tyr	NI	1.01	1.46
43	40	M	A	14	2.7	9	NT	0.39	42	56	3	2	2.4	tyr/tyr	I1416N	1.11	1.21

NI: not identified NT: not tested

Appendix 3, Table 2. Haemophilia A controls data (n = 17)

Control Number	sex	ABO Bloodgroup	VWF:Ag T_0 ($IUdL^{-1}$)	VWFpp T_0 (nM)	VWF:CB T_0 ($IUdL^{-1}$)	ADAMTS-13 T_0 (%)	VWFpp/ VWF:Ag ratio	Increase in VWF:Ag from T_0 to T_1 (IU/dL)	VWF:Ag T_1 ($IUdL^{-1}$)	VWF:Ag $t_{1/2}$ (h)	VWFpp $t_{1/2}$ (h)	VWF:CB $t_{1/2}$ (h)	Y1584C
1	M	A	238	NT	NT	NT	NT	166	404	26.1	NT	NT	NT
2	M	O	93	NT	NT	NT	NT	122	215	10.1	NT	NT	NT
3	M	B	168	NT	NT	NT	NT	135	303	10.1	NT	NT	NT
4	M	B	192	NT	NT	NT	NT	192	384	9.5	NT	NT	NT
5	M	O	61	NT	NT	NT	NT	138	199	9.1	NT	NT	NT
6	M	O	131	NT	NT	NT	NT	200	331	6.9	NT	NT	NT
7	M	O	82	NT	NT	NT	NT	162	244	4.2	NT	NT	NT
8	M	O	65	8.9	55	NT	0.27	144	209	4.3	1.4	3.4	tyr/tyr
9	M	O	45	4.3	49	NT	0.19	154	199	11.4	2.3	16	tyr/tyr
10	M	O	63	22	70	NT	0.7	121	184	5.4	0.8	5.1	tyr/tyr
11	M	O	50	4.2	50	NT	0.17	115	165	16	2.3	11.5	cys/tyr
12	M	A	193	8.4	186	NT	0.09	30	223	>30*	6	35.9	tyr/tyr
13	M	O	162	11	150	NT	0.14	102	264	20	6.1	7.9	tyr/tyr
14	M	O	83	8	112	NT	0.19	185	268	7	2	8.6	tyr/tyr
15	M	O	159	14	92	NT	0.18	43	202	>30*	1.1	26.5	tyr/tyr
16	M	A	105	3.3	98	NT	0.06	202	307	4.1	3.3	4.4	tyr/tyr
17	M	A	126	4.8	145	NT	0.08	123	249	16.8	8.5	5.3	tyr/tyr

NI: not identified
NT: not tested
* In two of the control subjects, the 1 h rise in VWF:Ag concentration was sustained over the 6 h time course and a k value was not obtainable. The VWF:Ag $t_{1/2}$ values for these subjects is shown as being greater than 30 h.

Appendix 4, Normal control group (n = 20)

Control	ABO blood group	VWF:Ag ($IUdL^{-1}$)	VWFpp/ VWF:Ag ratio	RCA-I/ VWF:Ag	ECA/ VWF:Ag
1	O	68	0.22	1.05	1.45
2	AB	145	0.1	1.01	0.75
3	B	129	0.11	0.19	0.78
4	O	128	0.11	0.79	1.55
5	O	100	0.12	0.71	1.09
6	AB	147	0.16	1.21	1
7	O	89	0.11	1.12	1.29
8	O	131	0.17	1.03	1.12
9	AB	152	0.12	0.86	0.56
10	AB	116	0.09	0.75	0.86
11	O	103	0.1	0.98	1.77
12	AB	194	0.11	0.59	0.49
13	O	66	0.07	1.16	1.87
14	AB	90	0.14	1.14	0.83
15	AB	115	0.14	1.03	0.63
16	AB	131	0.13	0.87	0.61
17	AB	109	0.08	1.07	1.42
18	AB	115	0.14	1.4	0.75
19	AB	97	0.3	1.21	0.69
20	O	84	0.09	1.12	1.74