INVESTIGATING THE GENETIC BASIS OF TYPE 3 OF VON WILLEBRAND DISEASE (VWD)

by

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von Willebrand Disease (VWD) is the most common inherited bleeding disorder in humans, resulting from quantitative or qualitative deficiencies of von Willebrand factor (VWF). Type 3 VWD is the rarest and most severe form of the disease. This thesis characterizes the phenotype-genotype correlations of a cohort of Canadian type 3 VWD patients and their family members. Three main findings are highlighted: 1) 50% of families showed evidence of co-dominant inheritance as opposed to recessive, 2) 42% of mutations identified were located in the VWF propeptide region (VWFpp), 3) index cases (IC) with mutations in the VWFpp had a more severe bleeding diatheses than IC with mutations elsewhere.

We investigated two of the identified VWFpp mutations (ex4-5del and Cys633Arg) to elucidate their molecular mechanisms using two cellular models. Patient-derived blood outgrowth endothelial cells (BOEC) are ideal for studying the underlying molecular mechanism of VWF mutations as they represent the native vascular endothelium. BOEC were isolated from type 3 VWD IC and family members with the mutations of interest. A heterologous cellular system was also used to study the VWF mutations in vitro. The VWFpp mutations caused impaired VWF secretion, defective multimerization, qualitative and quantitative defects in Weibel-Palade body (WPB) formation, and resulted in VWF retention within the endoplasmic reticulum. We attempted to restore secretion and multimerization by co-transfecting each mutant with the wild-type VWF propeptide (VWFpp), which was unsuccessful.

Additionally, we investigated a third mutation, c.8419_8422dupTCCC, which is unique to the Canadian VWD population and is found at a high frequency in a specific geographic population. While we hypothesized that this mutation would disrupt dimerization due to its location in the C-terminal cysteine knot (CK) domain of VWF we did not find this to be true.

The results presented within this thesis provide new insight into the genetics and pathobiology of type 3 VWD, the functional contribution of the VWFpp to type 3 VWD and
highlight the utility of BOEC as a cellular model for evaluating the pathogenic mechanisms of VWF mutations.
Co-Authorship

Dr. Paula James supervised research and assisted with manuscript writing and editing.

Dr. David Lillicrap supervised research.

The research technologists Angie Tuttle, Colleen Notley, Christine Brown, Shawn Tinlin, Jayne Leggo, and Meghan Deforest performed sequencing as part of Canadian type 3 VWD study.

Lisa Morrison created the pCIneohuVWFC633R mutant, and performed the transient transfections and corresponding ELISAs as part of her fourth year thesis project, under my supervision.

Lara Casey performed the immunostaining and confocal experiments using the patient BOEC with Cys633Arg mutation. She also performed the PMA experiments on the BOEC and transient transfections. This work constituted her fourth year thesis project, under my supervision.

Carol Hegadorn, Barbara Vidal and Angie Tuttle performed the multimer analyses.

Julie Grabell and Angie Tuttle measured VWF:RCo.

Hemophilia clinic nurses Sherry Purcell and Lisa Thibeault performed phlebotomy on the patients.

Matt Gordon and Jeff Mewburn provided technical assistance with acquiring confocal images and flow cytometry data.

All other work is my own.
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My husband Robb always joked that I was a lifelong student, but not once did he discourage me from doing this. I thank him for his constant love and support and for never giving up on the vision that this would someday be finished! And last but certainly not least to my two
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# Table of Contents

Abstract ................................................................................................................................. ii
Co-Authorship ....................................................................................................................... iv
Acknowledgements ............................................................................................................... v
List of Figures ....................................................................................................................... xi
List of Tables ......................................................................................................................... xiii
List of Abbreviations ........................................................................................................... xiv

Chapter 1 Introduction to von Willebrand Factor (VWF) and von Willebrand Disease (VWD) .... 1
  1.1 Von Willebrand Factor (VWF) .................................................................................... 1
  1.2 VWF structure ............................................................................................................ 1
  1.3 Biosynthesis of VWF .................................................................................................. 3
  1.4 Intracellular storage .................................................................................................... 7
  1.5 WPB exocytosis ......................................................................................................... 8
  1.6 Clearance of VWF .................................................................................................... 10
  1.7 Catabolism of VWF ................................................................................................. 11
  1.8 Biological interactions of VWF ................................................................................ 12
    1.8.1 VWF and FVIII .................................................................................................. 12
    1.8.2 VWF and subendothelial matrix (collagen) ....................................................... 13
    1.8.3 VWF and platelets ............................................................................................ 14
    1.8.4 Additional VWF interactions ........................................................................... 14
  1.9 VWF gene .................................................................................................................. 16
  1.10 Von Willebrand Disease (VWD) .............................................................................. 16
    1.10.1 Type 1 VWD .................................................................................................. 17
    1.10.2 Type 2 VWD .................................................................................................. 18
    1.10.3 Type 3 VWD .................................................................................................. 19
  1.11 Bleeding assessment tools (BAT) for the diagnosis of VWD ................................. 20
  1.12 Diagnostic assays for VWD .................................................................................... 21
  1.13 Treatment of VWD .................................................................................................. 22
  1.14 Cellular models to study VWD .............................................................................. 23
    1.14.1 Heterologous cellular systems ....................................................................... 23
    1.14.2 Endothelial cellular systems .......................................................................... 23
  1.15 Thesis hypothesis and objectives .......................................................................... 24
    1.15.1 The genetics of Canadian type 3 VWD ........................................................... 25
1.15.2 The contribution of VWF propeptide (VWFpp) mutations to type 3 VWD ........... 25
1.15.3 The pathogenic mechanism of the VWF mutation c.8419_8422dupTCCC .......... 26

Chapter 2 The Genetics of Canadian Type 3 von Willebrand Disease (VWD): Further Evidence for Co-dominant Inheritance of Mutant Alleles ......................................................... 27

2.1 Summary .................................................................................................................. 28
2.2 Introduction .............................................................................................................. 29
2.3 Patients, materials and methods ............................................................................... 31
  2.3.1 Patients ............................................................................................................... 31
  2.3.2 Bleeding questionnaire ......................................................................................... 32
  2.3.3 Coagulation studies ............................................................................................ 32
  2.3.4 Alloantibodies to VWF ...................................................................................... 32
  2.3.5 DNA sequencing ................................................................................................ 33
2.4 Results ...................................................................................................................... 34
  2.4.1 Patients ............................................................................................................... 34
  2.4.2 Bleeding questionnaire ......................................................................................... 36
  2.4.3 Phenotypic analyses ............................................................................................ 37
  2.4.4 Alloantibodies to VWF ...................................................................................... 37
  2.4.5 Genotypic analyses ............................................................................................. 37
    2.4.5.1 Missense mutations ....................................................................................... 42
    2.4.5.2 Partial/total gene deletions .......................................................................... 43
    2.4.5.3 Gene conversion ............................................................................................ 44
  2.4.6 Phenotype-Genotype Correlations ..................................................................... 44
2.5 Discussion ................................................................................................................ 45
2.6 Acknowledgements ................................................................................................. 47

Chapter 3 Investigation of the Contribution of von Willebrand Factor (VWF) Propeptide Mutations to Type 3 von Willebrand Disease (VWD) ....................................................... 48

3.1 Summary .................................................................................................................. 49
3.2 Introduction .............................................................................................................. 50
3.3 Patients, materials and methods ............................................................................. 52
  3.3.1 Patients ............................................................................................................... 52
  3.3.2 BOEC isolation and culture ............................................................................... 52
  3.3.3 Confirmation of endothelial cell phenotype ......................................................... 54
  3.3.4 VWF expression ................................................................................................ 55
  3.3.5 BOEC confocal immunofluorescence microscopy ............................................. 55
Chapter 4 Investigation of the Pathogenic Mechanisms of the von Willebrand Factor (VWF)

Mutation, c.8419_8422dupTCCC

4.1 Summary .................................................................................................................. 94
4.2 Introduction ............................................................................................................... 95
4.3 Patients, materials and methods ............................................................................. 97
  4.3.1 Patients .................................................................................................................. 97
  4.3.2 BOEC isolation .................................................................................................... 97
  4.3.3 Confirmation of endothelial cell phenotype .......................................................... 99
  4.3.4 VWF expression in patient-derived BOEC .......................................................... 100
  4.3.5 Confocal immunofluorescence microscopy of BOEC ......................................... 100
  4.3.6 Regulated secretion of VWF from BOEC ............................................................ 101
  4.3.7 Plasmid construct ................................................................................................ 101
  4.3.8 Cell culture and calcium phosphate transfections .............................................. 102
  4.3.9 Transient transfection confocal immunofluorescence microscopy .................... 102
  4.3.10 Regulated secretion of VWF ................................................................. 103
  4.3.11 Graphing and statistical analysis .................................................................... 103
4.4 Results ....................................................................................................................... 104
List of Figures

Figure 1.1 The historically recognized annotated domains of VWF. ................................................. 2
Figure 1.2 The current annotated domains of VWF. .......................................................... 4
Figure 1.3 The dimeric bouquet assembly of VWF under acidic pH. ............................................. 4
Figure 1.4 A schematic of the biosynthesis of VWF. .............................................................. 5
Figure 2.1 The location of the 31 mutations identified. .......................................................... 41
Figure 3.1 Pedigree and laboratory data for study families. ..................................................... 53
Figure 3.2 VWF expression of ex4-5del BOEC media, lysates, and multimer analysis. .......... 63
Figure 3.3 Qualitative and quantitative defects in Weibel-Palade body (WPB) formation in ex4-5del patient BOEC. .......................................................... 64
Figure 3.4 Lack of co-localization of ex4-5del VWF with P-selectin in patient BOEC............. 65
Figure 3.5 Diffuse ex4-5del VWF co-localizes with the endoplasmic reticulum (ER) marker calnexin. .................................................................................................................. 66
Figure 3.6 Stimulated secretion of ex4-5del patient-derived BOEC. ........................................ 68
Figure 3.7 VWF expression of Cys633Arg BOEC media, lysates, and multimer analysis. ....... 70
Figure 3.8 Qualitative and quantitative defects in Weibel-Palade body (WPB) formation in Cys633Arg BOEC. ........................................................................................................ 71
Figure 3.9 Lack of co-localization of VWF and P-selectin in Cys633Arg BOEC. ..................... 72
Figure 3.10 ER retention of VWF in Cys633Arg patient BOEC.................................................. 73
Figure 3.11 Stimulated secretion of Cys633Arg patient-derived BOEC.................................... 74
Figure 3.12 VWF expression from transient transfections of ex4-5del. .................................. 76
Figure 3.13 Confocal immunofluorescence microscopy of ex4-5del transient transfections. ...... 77
Figure 3.14 Stimulated release of VWF from transient transfections of ex4-5del. ................. 79
Figure 3.15 Proteasomal inhibition of ex4-5del transfected cells............................................. 80
Figure 3.16 VWF expression from transient transfections of Cys633Arg................................. 82
Figure 3.17 Confocal immunofluorescence microscopy of Cys633Arg transient transfections... 83
Figure 3.18 Stimulated release of VWF from transient transfections of Cys633Arg.............. 85
Figure 3.19 Proteasomal inhibition of Cys633Arg transfected cells ........................................ 86
Figure 3.20 Co-transfections of ex4-5del and Cys633Arg with the VWF propeptide. ............. 88
Figure 4.1 The sequence changes as a result of VWF c.8419_8422dupTCCC................................. 96
Figure 4.2 Pedigree and laboratory data for the two families with c.8419_8422dupTCCC........ 98
Figure 4.3 VWF expression in the BOEC media and lysates. .................................................... 107
Figure 4.4 Confocal immunofluorescence microscopy of BOEC from type 3 VWD patients with c.8419_8422dupTCCC. ........................................................................................................................................ 108
Figure 4.5 Partial co-localization of VWF and P-Selectin in compound heterozygous c.8419_8422dupTCCC. ........................................................................................................................................ 109
Figure 4.6 ER retention of VWF c.8419_8422dupTCCC. ........................................................................................................ 110
Figure 4.7 Stimulated release of VWF from patient-derived BOEC. ....................................................................................... 112
Figure 4.8 VWF expression from transient transfections of c.8419_8422dupTCCC. ................................................. 113
Figure 4.9 Confocal immunofluorescence microscopy images from transient transfections of c.8419_8422dupTCCC. ........................................................................................................................................ 115
Figure 4.10 Stimulated release of VWF from transient transfections of c.8419_8422dupTCCC. ................................................. 116
List of Tables

Table 2.1 Characteristics and phenotypic data for the 100 study subjects................................. 35
Table 2.2 Phenotypic and genotypic data for the 34 index cases................................................. 39
Table 2.3 In silico analysis of six missense mutations with PolyPhen-2 and SIFT comparison scores. ................................................................................................................................. 42
Table 3.1 Endothelial markers on patient-derived BOEC ................................................................. 61
Table 4.1 Endothelial markers on patient-derived BOEC. ................................................................. 105
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tr>
<td>ADAMTS13</td>
<td>a disintegrin-like and metalloprotease with thrombospondin type 1 motif, 13th member</td>
</tr>
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<td>ANG-2</td>
<td>angiopoietin-2</td>
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<td>BAT</td>
<td>bleeding assessment tools</td>
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<td>BOEC</td>
<td>blood outgrowth endothelial cells</td>
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<td>BS</td>
<td>bleeding score</td>
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<tr>
<td>DDAVP</td>
<td>1-deamino-8-D-arginine vasopressin</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FVIII</td>
<td>coagulation factor VIII</td>
</tr>
<tr>
<td>GP Ibα</td>
<td>platelet glycoprotein Ibα receptor</td>
</tr>
<tr>
<td>HEK293</td>
<td>human embryonic kidney cells</td>
</tr>
<tr>
<td>HMW</td>
<td>high molecular weight</td>
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<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cells</td>
</tr>
<tr>
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<td>index case</td>
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<td>IF</td>
<td>immunofluorescence</td>
</tr>
<tr>
<td>MNC</td>
<td>mononuclear cell</td>
</tr>
<tr>
<td>MLPA</td>
<td>multiplex ligation-dependent probe amplification</td>
</tr>
<tr>
<td>OC</td>
<td>obligate carrier</td>
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<tr>
<td>OPG</td>
<td>osteoprotegerin</td>
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<tr>
<td>proVWF</td>
<td>unprocessed VWF containing both VWF propeptide and mature VWF protein</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
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<tr>
<td>TGN</td>
<td>trans Golgi network</td>
</tr>
<tr>
<td>TSP-1</td>
<td>thrombospondin-1</td>
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<td>UFM</td>
<td>unaffected family member</td>
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<td>ULVWF</td>
<td>ultralarge von Willebrand factor multimers</td>
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<td>VWF</td>
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<td>VWF</td>
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<td>von Willebrand factor propeptide</td>
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<td>VWFpp/VWF:Ag</td>
<td>ratio of VWF propeptide levels to VWF antigen levels</td>
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<td>von Willebrand factor ristocetin cofactor activity</td>
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<td>WPB</td>
<td>Weibel-Palade bodies</td>
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Chapter 1

Introduction to von Willebrand Factor (VWF) and von Willebrand Disease (VWD)

1.1 Von Willebrand Factor (VWF)

von Willebrand Factor (VWF) is a large, multimeric glycoprotein that plays critical roles in both primary and secondary hemostasis. VWF is responsible for promoting platelet adhesion and aggregation at sites of vascular injury. Additionally, VWF acts as a chaperone protein for the coagulation factor, factor VIII (FVIII).\(^1\) Deficiency or dysfunction in VWF can lead to the inherited bleeding disorder, von Willebrand disease (VWD). This chapter will provide an introduction and overview of the structure and function of VWF, as well as an introduction to VWD. Additionally an overview of the cellular models used to study this disease will be discussed.

1.2 VWF structure

The pre-pro-VWF molecule is 2813 amino acids comprised of a 22 amino signal peptide, a 741 amino acid VWF propeptide (VWFpp), previously called VWF antigen II, and a 2050 amino acid mature subunit.\(^2\) The VWF protein is rich in cysteine, comprising approximately 8% of the amino acid content.\(^1\) All cysteine residues of secreted VWF appear to be paired in disulfide bonds.\(^3\) The domain structure of VWD that is historically recognized in the literature consists of four homologous domains (A-D) of the pro-VWF arranged in the order: D1-D2-D’-D3-A1-A2-
A3-D4-B1-B2-B3-C1-C2-CK (Figure 1.1). These domains provide binding sites for a number of different proteins including FVIII, platelet glycoprotein Ibα (GP Ibα), and platelet integrin αIIbβ3. These highly conserved domains also provide the basis for the secondary and tertiary structure of the mature protein.

**Figure 1.1** The historically recognized annotated domains of VWF.

The historically recognized domains of VWF are illustrated, as well as the locations of the binding sites for other ligands. The figure has been adapted from that which can be found on the International Society for Hemostasis and Thrombosis Scientific and Standardization Committee (ISTH SSC) VWF database (http://www.vwf.group.shef.ac.uk/).
More recently, evidence from electron microscopy studies has shown that the sequence and structure of VWF is more likely comprised of assemblies of many smaller domains (Figure 1.2). In this new model, the historical D domains are assemblies of smaller modules which include von Willebrand D (VWD), 8-cysteine (C8), trypsin inhibitor-like (TIL), fibronectin type 1-like domains (E), and a unique D4N module in D4. Additionally, the previous B and C domains have been re-annotated as six tandem von Willebrand C (VWC) and VWC-like domains. Zhou et al. (2011) showed that under acidic pH levels, VWF assembles into a dimeric “bouquet-like” structure. The revised VWF dimeric bouquet structure is shown in Figure 1.3 from Zhou et al. (2012), with the A2, A3 and D4 assembly resembling the “flowers” and the small tandem C domains resembling the “stem”. These recent changes in our understanding of the VWF structure will propel further studies, providing greater insight into the biosynthesis and secretion of VWF.

1.3 Biosynthesis of VWF

VWF is synthesized in two cell types: endothelial cells and the platelet precursor, the megakaryocyte. The 2813 amino acid pre-pro-VWF is translated in the rough endoplasmic reticulum (RER) and is then translocated to the endoplasmic reticulum (ER) where the 22 amino acid signal peptide is proteolytically cleaved by signal peptidases. A highly regulated series of post-translational modifications subsequently takes place. Figure 1.4 illustrates the main steps of the biosynthesis of VWF, including the cellular compartment at each stage.

N-linked glycosylation through the addition of oligosaccharide chains at 12 sites in the mature VWF and at four additional sites in the VWFpp takes place. N-linked glycosylation is important for the protein structure and function, including facilitating proper folding, disulfide...
Figure 1.2 The current annotated domains of VWF.

The re-annotated domains of VWF are shown in relation to the historical D domain assemblies. The figure has been adapted from Zhou et al. (2012).

Figure 1.3 The dimeric bouquet assembly of VWF under acidic pH.

The revised dimeric bouquet assembly of VWF under acidic conditions from Zhou et al. 2012.
Figure 1.4 A schematic of the biosynthesis of VWF.

Adapted from Romani de Wit and van Mourik (2001).
bond formation, and the prevention of protein aggregation. The N-linked oligosaccharides of VWF are unique because they contain ABO blood group oligosaccharides.\textsuperscript{10}

Dimerization of VWF in a “tail-to-tail” fashion through the formation of inter-chain disulfide bonds at the carboxyl-terminal end of proVWF monomers occurs next. The cysteine knot (CK) domain is located at the carboxyl-terminal end of VWF, containing 90 residues, of which eleven are cysteine residues.\textsuperscript{11} It is these cysteine residues within the CK domain that are responsible for the “tail-to-tail” dimerization of the proVWF subunits in the ER. The dimers are formed through the creation of inter-chain disulfide bonds between paired cysteine residues.\textsuperscript{1} The exit of proVWF dimers from the ER appears to be tightly regulated, by both glycosylation and dimerization. Studies have shown that the inhibition of N-linked glycosylation by the antibiotic tunicamycin prevents dimerization and results in retention of monomeric proVWF molecules in the ER.\textsuperscript{12}

ProVWF dimers are subsequently transported to the Golgi apparatus where O-linked glycosylation at 10 sites,\textsuperscript{2} and high-mannose oligosaccharide processing take place.\textsuperscript{7} Sulfation occurs at either one or both of the asparagine-linked carbohydrate chains of the mature subunit, Asn 384 and/or Asn468 in the \textit{trans}-Golgi network (TGN).\textsuperscript{13}

Multimerization in the acidic environment of the TGN occurs in a “head-to-head” fashion through the formation of inter-chain disulfide bonds of the dimers near the amino-terminus of the subunits.\textsuperscript{1} This process yields multimers that are greater than 20,000 kDa in size. Multimerization is facilitated by the VWFpp, which acts as a pH-dependent endogenous chaperone, facilitating the inter-chain disulfide bond formation by catalyzing protein disulfide interchange.\textsuperscript{14} \textit{In vitro} studies have shown that when the VWFpp is deleted, multimerization does not occur;\textsuperscript{15} however, expression of the VWFpp in \textit{trans} is sufficient for multimer assembly.\textsuperscript{16} A
possible explanation for this can be found by considering the structure of the VWFpp, which contains two CXXC sequences (Cys-Gly-Leu-Cys), the signature sequence for disulfide oxidoreductases. Disulfide oxidoreductases are essential for disulfide bond formation under the acidic conditions of the Golgi apparatus. A final modification in the biosynthetic pathway of VWF is the removal of the large VWFpp which is proteolytically cleaved after the dibasic amino acids Lysine-Arginine, at amino acid 763, most likely by furin, a member of the paired basic amino acid cleaving enzyme (PACE) family. After cleavage, the VWFpp remains in non-covalent association with the VWF multimers.

1.4 Intracellular storage

The majority of VWF appears to be secreted into the circulation in a constitutive fashion, with the remainder stored in the Weibel-Palade bodies (WPB) of endothelial cells or α-granules of platelets and then released upon stimulation. The converse, however, has also been shown, with the majority of VWF being trafficked to WPB prior to basal release.

WPB are rod-shaped, membrane enclosed organelles, 0.1–0.2 μm wide and up to 4 μm long composed of longitudinal striations. When observed in cross-section, the striations are tightly packed tubules that appear to be composed of VWF multimers. Similar tubules have also been observed in platelet α-granules. Tubulation allows a 100-fold compaction of VWF, which is required for proper intracellular storage of VWF and for the orderly secretion of VWF.

The biogenesis of WPB is driven by VWF itself. Evidence for this is seen in VWF-deficient pigs, mice, and dogs which all lack WPB. Targeting of VWF to WPB requires both the VWFpp and the mature protein, with the VWFpp acting as an intra-molecular chaperone,
through non-covalent associations.\textsuperscript{32} \textit{In vitro} studies have shown that the VWFpp will sort to storage organelles when expressed alone, however if the mature VWF is expressed in the absence of the VWFpp it will not.\textsuperscript{33} Additional studies have shown that the VWFpp in \textit{cis} or \textit{trans} is sufficient to target mature VWF to storage organelles.\textsuperscript{34} The critical areas of interaction between the VWFpp and mature protein have been localized to amino acid Arg416 of the VWFpp and amino acid Thr869 of the VWD3 domain, part of the D'D3 assembly (historical D3 domain) of mature VWF.\textsuperscript{35} Michaux \textit{et al.} (2006) demonstrated that the minimum required sequence of VWF to form elongated WPB is the D1-A1 domain which contains the VWFpp.\textsuperscript{28}

While WPB are composed primarily of VWF,\textsuperscript{25,27,36} there are a number of other proteins that have been identified as being stored within WPB that are essential to hemostasis and inflammation, as well as in modulating vascular tone and angiogenesis such as P-selectin, CD63, interleukin-8 (IL-8), osteoprotegerin (OPG), angiopoietin-2 (ANG-2) etc.\textsuperscript{27,37}

\subsection*{1.5 WPB exocytosis}

\textit{In vivo}, VWF plasma levels rise due to \textbeta-\textalpha-adrenergic receptor activation such as that from stress or exercise,\textsuperscript{38,39} as well as thrombin generation\textsuperscript{40} and treatment with the vasopressin analog 1-desamino-8-D-arginine vasopressin (DDAVP).\textsuperscript{41} DDAVP is a widely used treatment option for type 1 VWD and mild/moderate hemophilia,\textsuperscript{41} as it produces a rapid release of VWF from the WPB into the blood stream through the activation of the vasopressin V2 receptor.\textsuperscript{42,43} Cultured endothelial cells do not contain the V2 receptor as this response is not observed \textit{in vitro} unless the cells are transduced to express the V2 receptor.\textsuperscript{44}

\textit{In vitro}, endothelial cells can be stimulated by a number of secretagogues to release
VWF\textsuperscript{45} through a number of different mechanisms. Stimulation of cells with divalent cationic calcium secretagogues such as calcium ionophore, and physiologically relevant agents such as histamine and thrombin, increase intracellular free Ca\textsuperscript{2+} through a phospholipase C-dependent mechanism, which causes a rapid release of VWF from WPB.\textsuperscript{42,46,47} Epinephrine, forskolin, and vasopressin induce VWF release due to a cyclic-amp (c-AMP) mediated response.\textsuperscript{48,49} Finally, phorbol myristate acetate (PMA) is thought to activate protein kinase C, which then mediates phosphorylation of exocytotic proteins.\textsuperscript{50}

Different types of WPB exocytosis have been observed which may be dependent upon the physiological conditions present \textit{in vivo} at the time. Single WPB exocytosis has been confirmed, whereby a single WPB will fuse with the plasma membrane.\textsuperscript{51} A second mode, termed the “lingering kiss” has been observed whereby a WPB fuses transiently with the plasma membrane, creating a small pore through which small molecules, such as IL-8 and CD63, are selectively released and larger molecules, such as VWF and P-selectin, are retained.\textsuperscript{52} A third type of exocytosis, “multigranular exocytosis” has been proposed to be a significant mode of exocytosis by which several WPB merge into a “secretory pod” before fusing with the plasma membrane.\textsuperscript{53}

Upon release from the cell, the VWF multimers and VWFpp dissociate under physiologic pH and are secreted together in 1:1 stoichiometric amounts.\textsuperscript{54} The shift in pH from 5.5 within the WPB\textsuperscript{55} to neutral pH causes the VWFpp to be released from the mature protein, allowing the chain of VWF multimers in the tubules to unfold in an orderly manner, generating VWF strings.\textsuperscript{28} Recent studies have shown that a fraction of VWF and VWFpp actually remain associated in the circulation, limiting access of the VWF A1 domain for GP Iba binding.\textsuperscript{56} The VWFpp circulates in the plasma for a short time, with a half-life of about 2 hours and plasma levels of about 1
μg/mL whereas multimeric VWF circulates with a half-life of approximately 8-12 hours and plasma levels of about 10 μg/mL.\textsuperscript{48,57,58}

1.6 Clearance of VWF

For some time it has been known that ABO blood group affects VWF levels, with blood group O individuals having an average of a 25% reduction in VWF levels compared with non-O blood groups.\textsuperscript{59} Recent studies have shown that H antigen expression mediates the ABO effect.\textsuperscript{60} ABO groups are added to the N-linked oligosaccharide chains of VWF prior to secretion and it is likely that glycosylation of VWF has influences on plasma VWF clearance.\textsuperscript{61}

Until recently there had been limited information on the cells and/or receptors involved in the clearance of VWF. Evidence indicating cells in the liver take up the majority of VWF, with the spleen also contributing to the clearance of VWF existed.\textsuperscript{62} As well, more recently macrophages have been identified as being efficient in the uptake of VWF both \textit{in vitro} and \textit{in vivo}.\textsuperscript{63}

Currently, there is emerging evidence on VWF clearance mechanisms due to information from a genome-wide association study (GWAS) meta-analysis which linked VWF levels to the genes \textit{STXBP5, SCARA5, STAB2, STX2, TC2N, and CLEC4M}.\textsuperscript{64} Studies are underway to investigate the encoded proteins and their involvement in VWF clearance. Van Loon \textit{et al.} (2012) investigated the role of genetic variations in syntaxin binding protein 5 (\textit{STXBP5}) and its substrate syntaxin 2 (\textit{STX2}) on VWF levels, because of the potential involvement of these proteins in WPB exocytosis.\textsuperscript{65} The genetic variation in \textit{STX2} was found to be associated with VWF levels and the genetic variation in \textit{STXBP5} was associated with the bleeding phenotype in
female patients.\textsuperscript{65} Recent studies on C-type lectin receptor, CLEC4M, showed its ability to bind, internalize, and clear VWF.\textsuperscript{66} Additionally, this study showed the contribution of \textit{CLEC4M} polymorphisms to variations in plasma VWF levels.\textsuperscript{66}

Other recent work has shown that macrophage low-density lipoprotein receptor–related protein-1 (LRP1) modulates VWF clearance possibly via shear stress induced interactions or as part of an LRP1-β2-integrin complex.\textsuperscript{67}

\section*{1.7 Catabolism of VWF}

VWF multimers secreted in the regulatory fashion from WPB are ultra large molecular weight multimers (ULMWM), which are more adhesive.\textsuperscript{21} ULMWM bind to P-selectin, causing a conformational change leading to the partial unfolding of VWF which exposes exosites in the A2 domain of VWF (cleavage site Tyrosine (Y)1605-Methionine (M)1606) through which proteolytic cleavage by \textbf{A} disintegrin-like and \textbf{m}etalloprotease with \textbf{t}hrombospondin type \textbf{1} motifs \textbf{13} (ADAMTS13) occurs.\textsuperscript{68}

Under normal conditions, VWF circulates in the plasma in a globular form. This globular conformation keeps the A2 domain hidden and prevents cleavage by ADAMTS13.\textsuperscript{69,70} ADAMTS13, however, is still able to bind globular VWF, through interactions between the C-terminal distal domains of ADAMTS13 and the C-terminal region of VWF (D4-CK domains; amino acids 1874-2813).\textsuperscript{70,71} This initial interaction may be the first step in a multi-step process, which ultimately results in VWF cleavage by ADAMTS13.
1.8 Biological interactions of VWF

VWF has a number of key biological functions which include binding to other ligands involved in the hemostatic process such as FVIII, components of the subendothelial matrix, specifically collagen, and platelet surface glycoproteins. These interactions are critical for the proper functioning of primary and secondary hemostasis. Additionally, VWF interacts with other ligands such as thrombospondin-1 (TSP-1), OPG, heparin, and ANG-2.

1.8.1 VWF and FVIII

Coagulation factor FVIII is a blood clotting protein which functions as a cofactor in the intrinsic pathway of the coagulation cascade, which ultimately produces a fibrin clot.\(^72\) Deficiencies of FVIII result in the X-linked bleeding disorder Hemophilia A.\(^72\) Once FVIII is released into the circulation it immediately binds to its carrier protein VWF in a non-covalent complex, upon which it is protected from premature proteolytic cleavage by activated protein C (APC) and premature clearance.\(^73\) Each monomer of multimeric VWF is able to bind one FVIII molecule with high affinity. This stoichiometry appears to be limited though \textit{in vivo} by the number of FVIII molecules present (0.2 μg/mL in plasma), resulting in an approximately 1:50 (FVIII:VWF) ratio.\(^74\) The FVIII binding site on VWF has been localized to the N-terminus region of VWF, within the D’D3 domain.\(^75,76\) Mutations within this region prevent the VWF-FVIII complex from forming. The acidic region of the light chain and the C2 domain of FVIII have been shown to form the high affinity binding site for VWF.\(^77\) Studies have also shown that if the VWFpp is not cleaved then the binding of FVIII to VWF will not occur \(^78,79\) or at least there will be a reduced affinity binding between the two\(^80\) most likely because the uncleaved VWFpp
sterically hinders FVIII from binding to the D'D3 region. The VWF-FVIII complex becomes dissociated once thrombin cleaves FVIII and then activated FVIII (FVIIIa) is released and able to perform its cofactor role in the coagulation cascade.81

1.8.2 VWF and subendothelial matrix (collagen)

VWF is required for the maintenance of vascular integrity and does so by binding to collagen in the exposed subendothelium and helping to recruit platelets at sites of injury. When the endothelium is disturbed due to vessel injury, subendothelial matrix proteins, which are not normally in contact with flowing blood, such as collagens, are exposed. VWF acts as a molecular bridge between collagen and circulating platelets to enable VWF to bind platelets with affinity sufficient to snare platelets from the rapidly flowing blood and retain them at the site of injury. Several collagens are found in the vessel wall, however types I, III and VI appear to be the most important with respect to the interactions with VWF. The VWF and collagen type VI interaction is believed to occur in the superficial subendothelium, initiating the early VWF-dependent platelet adhesion under low shear conditions.82 Fibrillar collagens type I and III are present deeper in the subendothelium and their interaction with VWF enhances platelet adhesion and collagen-induced platelet aggregation.83 VWF binds to type VI collagen through the A1 domain and collagens type I and III through sites in the A1 and A3 domains.84-86
1.8.3 VWF and platelets

At sites of vascular injury, VWF mediates adhesive interactions of platelets exposed to flowing blood through two distinct platelet receptors for VWF, the GP Ibα (GP Ib–IX–V complex) and the integrin αIIbβ3 (GP IIb–IIIa complex) receptors. The VWF A1 domain undergoes a conformational change in response to shear stress conditions or to collagen binding, resulting in a shift from low to high binding affinity for GP Ibα. After initial binding to VWF, platelets will roll across the surface causing more interactions with platelet receptors and resulting in platelet tethering. Once platelets become tethered they are reversibly activated and adhere to the surface and subsequently bind more VWF and fibrinogen to the integrin αIIbβ3 which mediates additional platelet recruitment and aggregation. The GP IB-IX-V complex binds to the A1 domain of VWF while the GP IIb-IIIa complex binds to the RGD (Arg-Gly-Asp) sequence in the previously annotated C1 domain, now annotated as the VWC4 domain.

1.8.4 Additional VWF interactions

VWF has interactions with a number of other proteins; these specific interactions are less well known presently. TSP-1 is an adhesive glycoprotein which modulates cell adhesion and is found in platelet α-granules along with VWF. Although the physiological relevance of the VWF-TSP-1 interaction is unclear, TSP-1 has been shown to act as a VWF reductase, playing a role in regulating VWF size by uncoupling multimers through the reduction of the disulfide bonds linking each subunit. Pimanda et al. (2002) showed that TSP-1 binds to the A3 domain of VWF, which is also a docking site for ADAMTS13 under flow conditions. However the
relationship between TSP-1 and ADAMTS13 remained unclear. A recent study suggests that TSP-1 may act in a competitive inhibitory manner against ADAMTS13 binding and cleaving of VWF and this interaction may occur in the A2 and A3 domains. This study however was performed under static conditions and further studies under flow conditions need to be carried out.

OPG is a member of the tumor necrosis-factor receptor superfamily, and plays an important role in bone remodeling and has been implicated in endothelial cell survival. Zannettino et al. (2005) had previously shown association of VWF and OPG in WPB and more recently the two proteins were shown to associate together in platelet α-granules. The VWF-OPG interaction occurs through the A1 domain of VWF with the complex most likely forming in the TGN, and then co-storage of the proteins in WPB. The two proteins remain associated in the plasma. OPG also binds to TSP-1 and may prove a link between TSP-1 and the regulation of VWF size.

Heparin is another molecule which also binds to VWF through the A1 domain. Heparin can inhibit the interaction between VWF and GP Ibα which may affect platelet adhesion for individuals who are on heparin therapy.

ANG-2, an angiogenic regulator, is known to co-localize with VWF in WPB. More recently, it has been shown that ANG-2 binds to VWF through the A1 domain, regardless of VWF conformation, and these two proteins remain associated upon exocytosis from WPB. The continued association of VWF and ANG-2 in the circulation may facilitate each protein’s role in both hemostatic and angiogenic processes.
1.9 VWF gene

The VWF gene is located on the short arm of chromosome 12 at 12p13.3 and spans 178 kB. Characterization of VWF occurred simultaneously by four groups in the USA and Europe in 1985. The VWF gene is comprised of 52 exons that vary in length from 40 bp (exon 50) to 1.3 kb (exon 28), with introns ranging from 97 bp (intron 29) to 19.9 kb (intron 6). VWF transcribes a 9 kb mRNA which encodes the 2813 amino acid pre-pro-VWF molecule. Sequence analysis of genetic defects in VWF is complicated by the presence of a partial, unprocessed pseudogene which is located on chromosome 22 (22q.11-13). The VWF pseudogene corresponds to 12 exons (exons 23-34) of VWF with ~ 97% sequence homology. Mutations in VWF can lead to the inherited bleeding disorder von Willebrand Disease (VWD).

1.10 Von Willebrand Disease (VWD)

In 1926 Swedish physician, Dr. Erik von Willebrand, first described a severe inherited bleeding disorder in a family from the Åland islands archipelago. Originally called “pseudo-hemophilia” because it was distinct from hemophilia but displayed similarities, this bleeding disorder was later renamed VWD. Presently, VWD is recognized as the most common inherited bleeding disorder in humans with the prevalence of symptomatic VWD estimated to be approximately 1 in 1,000 (0.1%). Patients with VWD present clinically with excessive and prolonged mucocutaneous bleeding resulting from either quantitative or qualitative deficiencies of VWF.
VWD is currently classified into three types, as recognized by the International Society on Thrombosis and Hemostasis (ISTH).\textsuperscript{112} Types 1 and 3 VWD result from quantitative deficiencies of VWF whereas Type 2 VWD is characterized by qualitatively abnormal VWF.\textsuperscript{112}

\subsection*{1.10.1 Type 1 VWD}

Type 1 VWD is the most common type of VWD, representing approximately 75-80\% of all VWD cases, resulting from a partial quantitative deficiency of VWF.\textsuperscript{113} VWF levels (VWF antigen (VWF:Ag) and/or VWF ristocetin cofactor (VWF:RCo)) are generally reduced to between 0.05 and 0.50 IU/mL, however there is no international agreement on this and some centers consider an upper end cut-off of 0.30 IU/mL for the diagnosis of type 1 VWD (see below, section 1.12 for VWF assay details).\textsuperscript{114} Symptoms include mild to moderate mucocutaneous bleeding such as easy bruising, epistaxis, bleeding from minor wounds and oral cavity bleeding. Type 1 VWD is challenging to diagnose, as there is great variability in normal VWF levels, as well as incomplete penetrance, and variable expressivity within families. Additionally, VWF levels are affected by environmental influences such as age, stress, estrogens,\textsuperscript{115} pregnancy,\textsuperscript{116} ethnicity,\textsuperscript{117} and exercise,\textsuperscript{118,119} and genetic factors such as ABO blood group.\textsuperscript{59}

Type 1 VWD is mostly inherited in an autosomal dominant fashion but, as mentioned above, in some cases incomplete penetrance and variable expressivity are observed. Additionally, cases with recessive type 1 VWD have been reported.\textsuperscript{120} Recently, three cohort studies on the mutational spectrum of type 1 VWD have been published.\textsuperscript{121-123} In all three, mutations were identified in approximately 65\% of index cases (IC), leaving the genotype of 35\% of cases undetermined. Thus it is likely that there are additional genetic modifiers of VWF levels that may

17
lead to misdiagnoses of VWD. The influence of other potential genetic loci that modulate VWF levels is currently being investigated.\textsuperscript{64-66} Missense mutations account for approximately 70% of mutation types, with only about 10-15% of the total identified mutations resulting in null alleles.\textsuperscript{121-123} Mutations causing type 1 VWD generally lead to inefficient protein synthesis, storage, or secretion, intracellular retention or degradation, or faster clearance.\textsuperscript{124}

\subsection*{1.10.2 Type 2 VWD}

Type 2 VWD is characterized by functionally abnormal VWF and is further divided into the following four subtypes: Types 2A, 2B, 2M and 2N. Type 2A is characterized by a decrease in VWF-dependent platelet adhesion due to a loss of high molecular weight (HMW) VWF multimers. VWF:Ag and FVIII levels may be normal or only slightly decreased whereas the VWF:RCo will be markedly reduced.\textsuperscript{125} Type 2B also results in the loss of HMW multimers in most cases but there is an increased affinity for platelet GP Ibα, and often patients have thrombocytopenia.\textsuperscript{125,126} Type 2M is characterized by defective VWF-platelet interactions in spite of a normal size distribution of VWF multimers with a modest reduction of VWF:Ag levels, and disproportionately reduced VWF:RCo.\textsuperscript{127} Finally, Type 2N results in significantly decreased binding affinity of VWF to FVIII and mimics mild hemophilia A. In these patients, VWF levels can be normal but the FVIII levels are reduced to 0.05 to 0.40 IU/mL.\textsuperscript{128}

The inheritance pattern of type 2 VWD varies depending on the subtypes: types 2A, 2M and 2B are generally inherited in an autosomal dominant fashion whereas type 2N is inherited in an autosomal recessive fashion. The majority of mutations identified in type 2 VWD are missense mutations and these mutations are clustered in specific areas of VWF. In Type 2A VWD, the
majority of mutations are in the A2 domain, in type 2B and type 2M the majority are found in the A1 domain and in type 2N mutations are located in the D’D3 domains.\textsuperscript{129}

Platelet-type VWD (PT-VWD) is a rare autosomal dominant bleeding disorder that can be mistaken for type 2B VWD.\textsuperscript{130,131} The genetic abnormality underlying PT-VWD are mutations in the GP I\textbeta\alpha gene (GP1BA), as opposed to in the VWF gene, resulting in an increased affinity of GP I\textbeta\alpha for VWF.\textsuperscript{132,133} Similar to type 2B VWD, PT-VWD is characterized by the spontaneous binding of HMW VWF multimers to platelets which leads to increased clearance in the circulation.\textsuperscript{133} The “gold standard” for differentiating between PT-VWD patients and type 2B VWD patients is genetic testing, as phenotypic testing is often not sufficient to discriminate between the two bleeding disorders because it is poorly applied and results are often misinterpreted.\textsuperscript{134,135}

1.10.3 Type 3 VWD

Type 3 VWD is the most severe form of VWD, characterized by moderate to severe bleeding symptoms, resulting from a complete absence or only trace amounts of VWF being present. A parallel decrease in FVIII levels is also observed as a result of there being little or no VWF in the circulation to protect and stabilize FVIII.\textsuperscript{136} Type 3 VWD is rare, with variable prevalence estimates worldwide ranging from 0.11 – 5.3 per million.\textsuperscript{137} The higher estimates come from countries where there is an increased prevalence of consanguinity among parents of affected individuals.\textsuperscript{137} The inheritance of type 3 VWD is classically thought to be autosomal recessive with affected individuals typically homozygous or compound heterozygous for null alleles. Co-dominant inheritance is also observed though in a number of cases, with carriers of
type 3 VWD mutations also exhibiting low VWF levels and bleeding symptoms.\textsuperscript{138,139} In fact, the original type 3 VWD family from the Åland Islands, showed evidence of co-dominance.\textsuperscript{129} Type 3 VWD patients clinically present with moderate to severe mucocutaneous bleeding as well as muscle hematomas and hemarthroses, bleeding episodes similarly observed in individuals with hemophilia.\textsuperscript{137} VWD can also have a significant impact on quality of life (QoL),\textsuperscript{140} especially in females that are affected by menorrhagia, with some females reporting a QoL similar to HIV-positive severe hemophiliacs.\textsuperscript{141}

1.11 Bleeding assessment tools (BAT) for the diagnosis of VWD

The past decade has seen a surge in the research conducted on the use of bleeding assessment tools (BAT) for the evaluation of hemorrhagic symptoms in a number of different clinical settings.\textsuperscript{142} The occurrence, frequency and severity of various bleeding symptoms are best assessed using a standardized bleeding tool and each symptom is evaluated using a common scoring system. Many of the published BAT generate bleeding scores by summing the score of all bleeding symptoms for a given individual. BAT are useful tools as they provide a quantitative measure of bleeding that can be used to correlate with laboratory tests such as VWF levels, and mutational status. As well, BAT provide a unified means of communication between researchers and health care professionals. The consensus BAT which is currently endorsed by the ISTH is the ISTH-BAT\textsuperscript{143} which can be found at:


As would be expected, bleeding scores are higher in patients with type 2 and type 3 VWD versus type 1 VWD.\textsuperscript{144,145} A major limitation of the current BAT is that the severity of a
symptom takes precedence over frequency so for individuals with severe bleeding, such as type 3 VWD, the score can become saturated.

1.12 Diagnostic assays for VWD

Initial analyses of VWF include VWF:Ag, VWF:RCo, and FVIII coagulant activity (FVIII:C) tests. Plasma protein levels of VWF are quantified using the VWF:Ag ELISA. The mean VWF:Ag is 1 IU/mL, which is approximately 10 μg/mL. Normal VWF:Ag levels range from 0.50-2.00 IU/mL and a VWF:Ag below 0.30 IU/mL-0.50 IU/mL is generally considered pathologic. The VWF:RCo assay is a functional assay which tests the ability of VWF to bind GP Ibα using ristocetin as an agonist to induce the interaction. ELISA-based methods are now being employed, including one method that tests GP Ibα binding independent of ristocetin. Because of the relationship between VWF and FVIII, FVIII:C is also measured.

Additional tests which aid in determining VWD disease subtype include collagen binding (VWF:CB) and FVIII binding (VWF:FVIIIB) assays, the VWFpp assay, ristocetin induced platelet agglutination (RIPA) assay, and multimer analyses. The VWF:CB and VWF:FVIIIB are functional assays that test the ability of VWF to bind to each of these substrates. The VWFpp assay is an ELISA-based assay which determines the plasma concentration of the VWFpp and VWF:Ag from which a VWFpp/VWF:Ag ratio can be determined. The normal range of VWFpp is 0.55-2.19 IU/mL, and the normal range for the VWFpp/VWF:Ag ratio is 0.54 to 1.98. An elevated ratio is indicative of enhanced VWF clearance from the plasma. Ristocetin is also used in the RIPA assay, which measures the ability of platelets in platelet rich plasma to aggregate in the presence of ristocetin. Finally, multimer analysis can be performed to assess the overall size distribution of VWF multimers. Loss of VWF multimerization that may arise as a
result of either increased ADAMTS13 cleavage or increased VWF clearance can be observed. While this is a qualitative test, relative quantitative assessments can be made using densitometry. 149

1.13 Treatment of VWD

The management of VWD can consist of treatment with the vasopressin analog, DDAVP, or VWF/FVIII concentrates. DDAVP is administered subcutaneously or intranasally and increases plasma VWF levels by stimulating endothelial release of endogenous VWF. DDAVP is the preferred course of treatment given to responders for non-severe bleeding episodes or as a preventative measure prior to invasive procedures or minor surgery. The majority of patients with type 1 VWD and some type 2 VWD patients respond to DDAVP. 150 The alternative approach is to replace VWF with virally inactivated human plasma-derived clotting factor concentrates. Patients that do not respond to DDAVP or for whom DDAVP is contraindicated are treated with these VWF/FVIII concentrates. 151 A rare complication, in a small number of type 3 VWD patients (7.5-9.5%) is the development of inhibitory antibodies to VWF following replacement therapy that can lead to ineffective treatment and/or severe and life-threatening anaphylaxis. 152

Patients with severe bleeding, such as a joint bleeds, are at risk of long-term debilitating consequences including arthropathy. Prevention of severe bleeding episodes by prophylactic regimens has been shown to be beneficial; however widespread use of these regimens in VWD has not yet been achieved. Long-term prophylaxis for VWD is relatively uncommon; ~22% of type 3 VWD patients are on prophylaxis, but there are significant differences between treatment centers and countries, largely based on access to treatment. 153 Only a handful of studies have investigated the efficacy of this approach to date. 154 An international collaboration, the VWD
Prophylaxis Network (VWD PN), has been initiated to investigate the role of prophylaxis in severe VWD and the VWD International Prophylaxis (VIP) study is ongoing.153

1.14 Cellular models to study VWD

1.14.1 Heterologous cellular systems

Traditionally, in order to study the molecular pathogenesis of VWD mutations, researchers have relied on in vitro experiments by transfecting recombinant VWF into heterologous mammalian cell systems such as human embryonic kidney (HEK) cells. These cells are preferred to other cell types (i.e. AtT-20 or COS cells) because they are able to form elongated pseudo-WPB, are capable of regulated exocytosis, and drive the recruitment of CD63 and P-selectin, both integral membrane proteins of WPB.155 The molecular pathogenesis of a number of VWF mutations has been investigated using this cellular system.155-158 These cells have been useful in furthering our knowledge of the contribution of specific VWF mutations to VWD but have inherent limitations. These heterologous cell systems cannot account for other factors such as post-translational modifications (i.e. glycosylation) and may lack intracellular chaperones that are present in the native cells, which may influence VWF biosynthesis.157 As well, co-transfections in heterologous systems may not accurately mimic the heterozygous and compound heterozygous states in vivo.

1.14.2 Endothelial cellular systems

Human umbilical vein endothelial cells (HUVEC) have been used to study the molecular
pathogenesis of VWD but access to patient-derived HUVEC containing specific VWF mutations of interest is rare.\textsuperscript{159-161} More recently, patient-derived blood outgrowth endothelial cells (BOEC) have been used to study VWD because they are representative of the native vascular endothelium and are accessible by the collection of a peripheral blood sample.\textsuperscript{44,162-164} BOEC, also referred to as late outgrowth endothelial progenitor cells (EPC) or endothelial colony forming cells (ECFC), were first described about a decade ago.\textsuperscript{165} BOEC are derived from circulating EPC obtained from the mononuclear cells (MNC) isolated from a peripheral blood sample. BOEC are able to maintain their differentiated phenotype through multiple passages, contain WPB, and are positive for cell surface markers such as VE-cadherin and platelet endothelial cell adhesion molecule (PECAM).\textsuperscript{165,166} BOEC thus provide an accessible endothelial cell model for studying the cellular and molecular pathogenesis of VWD. These cells can be used to specifically look at defects in WPB storage and release, as well as providing a potential system for studying therapeutic targets in an \textit{ex-vivo} manner.

\subsection*{1.15 Thesis hypothesis and objectives}

The Canadian type 3 VWD population is unique in nature, due to the ethnic heterogeneity and may provide insight into the complexity of this bleeding disorder. We hypothesize that in this population obligate carriers of type 3 VWD mutations are not always asymptomatic, with some exhibiting low VWF levels and bleeding symptoms. We also hypothesize that \textit{VWF} mutations which are recurrent in types 1 and 3 VWD in the Canadian population affect VWF structure and function through a number of different mechanisms including impaired secretion, storage, trafficking and multimerization. The mechanisms that underlie these mutations may be evaluated
using both *in vitro* and *ex vivo* cellular models. The overall objective of this thesis is to characterize a Canadian cohort of type 3 VWD patients and to elucidate the molecular mechanisms of VWF mutations identified in these families using *in vitro* and *ex vivo* cellular systems. The knowledge gained from this work will further our understanding of this heterogeneous, complex, and severe bleeding disorder.

1.15.1 **The genetics of Canadian type 3 VWD**

While a number of type 3 VWD cohorts world-wide have been investigated, the Canadian population of type 3 VWD patients has not. The objective of chapter two is to evaluate the phenotype-genotype correlations of a cohort of Canadian type 3 VWD patients. We hypothesize that the Canadian type 3 VWD population exhibits co-dominant inheritance with some obligate carriers being symptomatic and having low VWF levels.

1.15.2 **The contribution of VWF propeptide (VWFpp) mutations to type 3 VWD**

The VWFpp is responsible for storage and multimerization and acts as an intra-molecular chaperone for the mature VWF protein. The significance of VWFpp mutations identified in Canadian type 3 VWD index cases was investigated in chapter three to elucidate the pathogenic mechanisms of VWFpp mutations using both *in vitro* and *ex vivo* cellular models. We hypothesize that due to the location of the mutations in the VWFpp, secretion and multimerization will be affected.
1.15.3 The pathogenic mechanism of the VWF mutation c.8419_8422dupTCCC

The most frequently identified mutation, c.8419_8422dupTCCC, in the Canadian type 3 VWD population exhibits unique molecular characteristics, such as a resultant elongated protein and resulting milder bleeding phenotypes. The objective of chapter four is to evaluate the underlying pathogenic mechanisms of this VWF mutation using both in vitro and ex vivo cellular models. We hypothesize that this mutation affects dimerization due to its location in the VWF cysteine knot (CK) domain.
Chapter 2

The Genetics of Canadian Type 3 von Willebrand Disease (VWD):
Further Evidence for Co-dominant Inheritance of Mutant Alleles

2.1 Summary

Type 3 von Willebrand disease (VWD) is the most severe form of the disease and is classically inherited in an autosomal recessive fashion. The aim of the current study was to investigate the molecular pathogenesis of a Canadian cohort of type 3 VWD patients. 34 families comprised of 100 individuals were investigated. Phenotypic data, including bleeding scores (BS), von Willebrand factor (VWF) laboratory values, and anti-VWF inhibitor status were included as well as sequence analysis. We identified 31 different mutations (20 novel): 8 frameshift, 5 splice site, 9 nonsense, 1 gene conversion, 6 missense, and 2 partial gene deletion mutations. The majority of mutations identified were in the propeptide (42%); index cases (IC) with these mutations exhibited more severe bleeding (BS=22) than those with mutations elsewhere in VWF (BS=13). 62 of 68 (91%) mutant alleles were identified. Twenty-nine IC (85%) had a VWF null genotype identified; 17 homozygous, 12 compound heterozygous. In five IC (15%), two mutant VWF alleles were not identified to explain the type 3 VWD phenotype. In four ICs only one mutant VWF allele was identified and in one IC no mutant VWF alleles were identified. We have investigated the molecular pathogenesis of a Canadian cohort of type 3 VWD patients. Obligate carriers are not phenotypically silent in the Canadian population; 48% have been diagnosed with type 1 VWD. In ~50% of families in this study the inheritance pattern for type 3 VWD is codominant and not recessive.
2.2 Introduction

von Willebrand disease (VWD) results from quantitative or qualitative deficiencies of von Willebrand factor (VWF), a large, multimeric glycoprotein that plays a critical role in primary and secondary hemostasis. VWD is recognized as the most common inherited bleeding disorder in humans, with symptomatic prevalence estimates of approximately 1 in 1,000 (0.1%). Type 3 VWD is the most severe form of the disease resulting from markedly decreased or undetectable VWF and reduced factor VIII (FVIII) activity. The prevalence of type 3 VWD varies between countries, ranging from 0.1-5.3 per million, with increased prevalence in areas with more frequent consanguineous marriages. Individuals with type 3 VWD present clinically with moderate to severe mucocutaneous bleeding symptoms as well as muscle hematomas and hemarthroses. Classically, inheritance of type 3 VWD follows an autosomal recessive pattern with affected individuals being homozygous or compound heterozygous for null alleles and with obligate carriers (OC) of the type 3 VWD mutations being phenotypically normal. More recently, however, it is becoming clear that this is not always the case and co-dominance is observed, with OC exhibiting low VWF levels and a bleeding diathesis. Castaman et al. (2006) showed that OC of type 3 VWD report more bleeding symptoms than healthy controls, but are less symptomatic than carriers of type 1 VWD. A commentary written in the same issue of the journal titled “When it comes to von Willebrand disease does 1+1=3?” further highlights the need for additional studies looking at the differences between OC of type 3 VWD and individuals with type 1 VWD in order for that question to be answered.

The majority of type 3 VWD mutations are small deletions and insertions, nonsense mutations or other mutations, located throughout the VWF gene that interfere with VWF synthesis
and secretion.\textsuperscript{137} This is in contrast to type 1 VWD in which the majority of mutations are missense changes (70-75%), with only about 10-15% of mutations identified which lead to null alleles.\textsuperscript{121,123,167} There are presently 108 different reported type 3 VWD mutations according to the International Society on Thrombosis and Haemostasis (ISTH) SSC VWF database (http://www.vwf.group.shef.ac.uk/, accessed May 13\textsuperscript{th}, 2012).

Partial and total VWF deletions have been reported previously including deletions of single exons\textsuperscript{168}, multiple exons\textsuperscript{169-173} and the entire VWF gene\textsuperscript{174-177}; these deletions however only constitute approximately 10% of all reported type 3 VWD mutations (http://www.group.shef.ac.uk/). Conventional sequencing of PCR-amplified DNA does not provide an adequate strategy for the detection of all mutations, as partial/total deletions and large duplications may not be apparent in the heterozygous state because the alternate normal allele is amplified and masks the deletion or duplication present on the other allele. Therefore, additional strategies are required in cases where no complete pathogenetic explanation has been obtained through conventional methods. Multiplex ligation-dependent probe amplification (MLPA) has been used recently to identify partial and large gene deletions in VWD patients.\textsuperscript{178-180}

Identification of both mutant VWF alleles resulting in type 3 VWD has been reported in ~80-90% of cases. Importantly, the majority of type 3 VWD populations reported to date have been homogeneous populations.\textsuperscript{172,181-184} In contrast, the Canadian type 3 VWD population is distinct, with areas of significant population homogeneity, as well as areas with heterogeneous populations due to immigration in large Canadian urban centres.

In this paper we report the mutational spectrum of a cohort of Canadian type 3 VWD patients. While a number of type 3 VWD studies have been previously reported, our report here of the Canadian VWD population represents one of the largest and most thoroughly investigated
cohorts of type 3 VWD patients and their family members. Our paper also highlights distinct features of the Canadian population of type 3 VWD patients.

2.3 Patients, materials and methods

2.3.1 Patients

Eligible subjects were enrolled in the Canadian Type 3 VWD study from the Inherited Bleeding Disorders / Hemophilia Clinics of the Association of Hemophilia Clinic Directors of Canada (AHCDC). Inclusion criteria included an index case (IC) with a documented history of excessive mucocutaneous bleeding and plasma levels of VWF antigen (VWF:Ag) and/or VWF ristocetin cofactor (VWF:RCo) < 0.05 IU/ml on at least two occasions and a factor FVIII coagulant activity (FVIII:C) of <0.10 IU/ml. A positive family history of bleeding was not required for enrollment because of the recessive inheritance pattern of type 3 VWD. When possible, samples from the IC and both parents were collected, as well as any available siblings and/or other family members. Venous blood samples were collected by phlebotomy in both 3.2% sodium citrate (at a ratio of 9:1 vol/vol) and EDTA. All study participants gave informed consent and study approval was obtained from the Research Ethics Board of Queen’s University, Kingston, Canada and from each of the source institutions.
2.3.2 Bleeding questionnaire

A standardized bleeding questionnaire was administered to IC and available family members. The occurrence, frequency and severity of various bleeding symptoms including mucocutaneous symptoms as well as muscle hematomas and hemarthroses were assessed using the questionnaires. Bleeding questionnaires were administered by an experienced person and bleeding scores were generated by summing the score of all bleeding symptoms for a given subject.

2.3.3 Coagulation studies

Laboratory VWF and FVIII tests were conducted at the patients’ source clinics as per local methods. In order to confirm the type 3 VWD diagnosis, all tests were repeated on frozen plasma samples at the Clinical and Molecular Hemostasis Research Laboratory, Queen’s University, Kingston, Canada. The values reported here are from the central laboratory in Kingston.

2.3.4 Alloantibodies to VWF

A rare complication of type 3 VWD is the development of inhibitory antibodies to VWF following replacement therapy (incidence of 7.5-9.5%) which can lead to ineffective treatment and/or severe and life-threatening anaphylaxis. To test for alloantibodies to VWF in the type 3 patients (IC and type 3 VWD family members) an ELISA-based assay was used. Briefly, plates were coated with either Humate-P®, Wilate®, or locally produced recombinant human VWF
(~0.7U/ml) and were run in parallel. The recombinant human VWF was produced by transient transfection into HEK293T cells and secretion into serum-free media. Normal plasma pool (NPP) and “positive” anti-VWF antibody plasma, from an individual with acquired VWD, were run as negative and positive controls respectively for the anti-VWF antibody ELISA on each plate. We acknowledge that the use of an acquired VWD patient sample as a positive control is not ideal, and is a limitation of this methodology, but we did not have access to an alloantibody positive control. Plasma samples were diluted 1:200. Anti-Human IgG peroxidase conjugate (Sigma A2290) diluted 1:5000 was used as a secondary antibody.

An assay negative cut off value was determined by taking the mean absorbance value plus three standard deviations of the test samples. Any sample with absorbance values above this cut-off were identified as inhibitor-positive and were further analyzed using mixing studies with NPP and analysis by VWF functional assays. VWF:RCo was analyzed using a standard agglutination test with fixed platelets. ELISA-based FVIII binding and collagen binding assays were also conducted as per previously published methods. 185,186

2.3.5 DNA sequencing

Blood samples from IC and available family members were collected and genomic DNA was extracted from leukocytes using a salt-extraction method. 187 DNA analysis was performed by direct sequencing of the VWF gene as previously reported, 123 including exons 1-52 (including exon/introns boundaries and flanking intronic sequence) as well as approximately 1.5 kb of the VWF promoter region. Primer sequences are listed in Appendix B. If a mutation was identified in an IC, an additional template was PCR amplified and the opposite strand sequenced in order to
confirm the sequence variation. All chromatograms were reviewed by at least two experienced technologists and PCR samples were re-run if there were any doubts in interpretation. Once a mutation was identified in an IC then all enrolled family members were sequenced for that mutation in order to confirm familial transmission.

IC for whom no complete pathogenic explanation could be elucidated through direct sequencing were evaluated for partial/total gene deletions. As well, IC found to be homozygous for a missense mutation through direct sequencing were also investigated to rule out the possibility that the missense mutation was in cis with a partial gene deletion. The SALSA-MLPA VWF assay was performed using the P011-B1 and P012-B2 VWF kit (MRC-Holland, Amsterdam, the Netherlands) according to the manufacturer’s directions. The amplification products were identified on the ABI 3130xl sequencer (Applied Biosystems, Foster City, CA, USA) in the DNA Diagnostic Laboratory, Kingston General Hospital, Kingston, ON, Canada. MLPA analysis spreadsheets (National Genetics Reference Laboratory (NGRL), Manchester, UK) were then used to estimate the DNA dosage. Familial transmission of an identified partial gene deletion was confirmed in available family members by MLPA.

2.4 Results

2.4.1 Patients

Thirty-five families comprised of 102 individuals were initially submitted to the study. After phenotypic confirmation in the central laboratory, one family (one IC and one FM) was excluded due to a FVIII level >0.10 IU/ml. Therefore, the study population includes 100
individuals: 42 type 3 VWD patients (34 IC and 8 type 3 VWD siblings), 21 OC (defined as either being the offspring of an individual with type 3 VWD or having offspring with type 3 VWD), 30 individuals diagnosed at their enrolling institution with type 1 VWD (19 OC and 11 siblings or other family members), and 7 unaffected family members (UFM). Characteristics of all study participants are presented in Table 2.1.

Table 2.1 Characteristics and phenotypic data for the 100 study subjects.

<table>
<thead>
<tr>
<th></th>
<th>Type 3 VWD (N=42)</th>
<th>OC (N=21)</th>
<th>Type 1 VWD (N=30)</th>
<th>UFM (N=7)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Females (%)</td>
<td>23 (55)</td>
<td>11 (52)</td>
<td>17 (57)</td>
<td>4 (57)</td>
<td>NS</td>
</tr>
<tr>
<td>Mean age, years (range)</td>
<td>29 (1-72)</td>
<td>51 (32-83)</td>
<td>39 (2-85)</td>
<td>49 (22-68)</td>
<td>0.001</td>
</tr>
<tr>
<td>No. Blood group O (%)</td>
<td>16 (39)</td>
<td>6 (29)</td>
<td>10 (33)</td>
<td>0 (0)</td>
<td>NS</td>
</tr>
<tr>
<td>Mean VWF:Ag, IU/ml</td>
<td>0.02 (0.01-0.07)</td>
<td>0.74 (0.24-1.42)</td>
<td>0.35 (0.11-0.52)</td>
<td>1.40 (0.95-2.04)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(range)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean VWF:RCo, IU/ml</td>
<td>0.02 (0.00-0.05)</td>
<td>0.66 (0.15-1.15)</td>
<td>0.30 (0.05-0.57)</td>
<td>1.04 (0.66-1.96)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(range)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean FVIII:C, IU/ml</td>
<td>0.03 (0.00-0.09)</td>
<td>0.88 (0.37-1.56)</td>
<td>0.53 (0.02-1.32)</td>
<td>1.23 (0.52-2.12)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(range)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median bleeding score</td>
<td>13 (3-30)</td>
<td>1 (-1 to 6)</td>
<td>4 (0-12)</td>
<td>0 (-1-4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(range)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

OC = obligate carrier; UFM = unaffected family member; NS = no significance. One-way ANOVA followed by Tukey’s post hoc testing for all linear variables. For age: post hoc analysis P=0.001 between type 3 and OC, all other groups =ns. For bleeding score: Kruskal-Wallis followed by Mann-Whitney U post hoc testing Post hoc testing BS: P<0.001 between UFM and T1, T1 and OC, and between T3 and all other groups. Chi-squared testing for categorical variables.
The majority of families are comprised of two generations; however there are two 3-generation families and four single IC represented in this study. The majority of IC were Caucasian (N=21, 62%), however the remaining IC were of a number of different ethnicities (South Asian 12%; Middle Eastern 6%; and Lebanese, Scottish/Jamaican, South American, Trinidad/Ethiopian, Asian East Indian, West Asian, Hispanic ~ 3% each). Eighteen (53%) of the families have both individuals diagnosed with type 1 VWD and individuals diagnosed with type 3 VWD.

2.4.2 Bleeding questionnaire

Bleeding scores (BS) are reported in Table 2.1. The BS showed an overall inverse correlation with plasma VWF:Ag, VWF:RCo and FVIII levels (P < 0.001, Spearman’s rho = -0.746, -0.739, and -0.721, respectively). The BS reported for the type 3 VWD patients in this study (median BS=13: range=3-30) are similar to those reported in other type 3 VWD populations. Overall, the median BS were significantly different between the groups (P < 0.001, Kruskal-Wallis test), with the BS being higher in the type 3 VWD group compared with each of the other three groups (Mann-Whitney U post hoc testing). OC of type 3 VWD have similar BS compared to UFM (P = 0.394; median BS=1 versus median BS= 0). The OC of type 3 VWD had significantly lower median BS compared to the family members diagnosed with type 1 VWD (P < 0.001; median BS=1 compared to BS=4). IC with mutations identified in the propeptide region of VWF (N=9) had a significantly higher BS (median=22), compared to IC with mutations in other regions of VWF (median=13) (P = 0.012, Mann-Whitney U).
2.4.3 Phenotypic analyses

Table 2.1 summarizes the phenotypic data of all study participants. VWF:Ag, VWF:RCo and FVIII levels were significantly different between all groups (P < 0.001, one-way ANOVA followed by Tukey's post hoc testing). Post-hoc testing showed significant differences between each of the groups for all parameters with the exception of FVIII levels, which were not significantly different between OC and UFM.

2.4.4 Alloantibodies to VWF

The 42 type 3 VWD patients (34 IC+ 8 type 3 VWD family members) were tested for alloantibodies to VWF. One IC (T018) had absorbance levels greater than the assay negative cut-off on all anti-VWF ELISA assays. When the plasma of T018 was mixed 50/50 with NPP, VWF:RCo and FVIII binding function were restored to levels within the normal range. However, collagen binding activity was only restored to 40% of normal upon mixing with NPP.

2.4.5 Genotypic analyses

Mutations were identified in 33 (97%) IC. Sixty-two of 68 (91%) mutant alleles were identified. Twenty-nine IC (85%) had two mutant VWF alleles identified; 17 were homozygous, and 12 were compound heterozygous. Of note, these were not always null mutations; three IC were homozygous for missense mutations and one IC was compound heterozygous for a null mutation and a missense mutation. For four IC (12%) only one VWF mutation was identified, all of which were null mutations. No VWF mutations were identified in one IC.
A total of 31 different mutations (20 novel mutations) were identified, comprised of 8 frameshift, 5 splice site, 9 nonsense, 1 gene conversion, 6 missense, and 2 partial gene deletion mutations. All VWF sequence variations identified, both putative and polymorphic, are listed by IC in Table 2.2. Figure 2.1 shows the location of the mutations scattered throughout the VWF gene. While mutations were identified throughout VWF, 42% of the non-splicing mutations were located in the propeptide region. This is in contrast to only 7% of non-splicing mutations being found in the propeptide region in the Canadian Type 1 VWD study.123

Four mutations (exon 18 c.2438dupG (p.Gly813fs), exon 30 c.5180_5181insTT (p.Leu1727fs), exon 43 c.7399C>T (p.Gln2467*), and exon 52 c.8418_8419insTCCC (p.Ser2807fs)) were identified in more than one family. The most frequent mutation identified in this cohort was the frameshift mutation in exon 52, p.Ser2807fs, found in 12 IC (35%), six of whom were homozygous and six heterozygous. Additionally, 14 family members were found to be either homozygous (N=3) or heterozygous (N=11) for this mutation. This frameshift mutation was previously identified and reported in two IC from the Canadian type 1 VWD population123. The individuals with this mutation are from Eastern Canada and can all be linked to a larger pedigree. The insertion of the four nucleotides (TCCC) results in a frameshift, which abolishes the original stop codon and adds 16 amino acids prior to a new stop codon being created. The molecular pathogenesis of this mutation is currently under further investigation. The Baltic founder mutation in exon 18, c.2435delC (p.812Argfs*31), identified in the original VWD family from the Åland Islands,189 was not found in this population.
Table 2.2 Phenotypic and genotypic data for the 34 index cases.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Gender/age (M/F year)</th>
<th>Bleeding Score</th>
<th>VWF:Ag (IU/ml)</th>
<th>VWF:RCo (IU/ml)</th>
<th>FVIII:C (IU/ml)</th>
<th>Nucleotide Change, HGVS</th>
<th>Amino Acid Change, HGVS</th>
<th>Exon</th>
<th>Genotype</th>
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</thead>
<tbody>
<tr>
<td>T001</td>
<td>M/42</td>
<td>19</td>
<td>0.01</td>
<td>0.02</td>
<td>0.02</td>
<td>c.221-977_532+7059del</td>
<td>p.As75_Gly178del</td>
<td>4-5</td>
<td>Homozygous</td>
</tr>
<tr>
<td>T006B</td>
<td>F/11</td>
<td>11</td>
<td>0.01</td>
<td>0.01</td>
<td>0.02</td>
<td>c.4006C&gt;T</td>
<td>p.Arg1336*</td>
<td>28</td>
<td>Homozygous</td>
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<tr>
<td>T015</td>
<td>M/72</td>
<td>8</td>
<td>0.02</td>
<td>0.02</td>
<td>0.07</td>
<td>c.8418_8419insTCCC</td>
<td>p.Ser2807fs</td>
<td>52</td>
<td>Homozygous</td>
</tr>
<tr>
<td>T018</td>
<td>F/18</td>
<td>12</td>
<td>0.04</td>
<td>0.00</td>
<td>0.03</td>
<td>c.7399C&gt;T, c.8418_8419insTCCC</td>
<td>p.Gln2467*, p.Ser2807fs</td>
<td>43/52</td>
<td>Compound heterozygous</td>
</tr>
<tr>
<td>T028</td>
<td>M/34</td>
<td>16</td>
<td>0.01</td>
<td>0.01</td>
<td>0.03</td>
<td>c.7730-1 G&gt;T, c.7906A&gt;T</td>
<td>-</td>
<td>46/48</td>
<td>Homozygous Heterozygous</td>
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<tr>
<td>T030</td>
<td>M/66</td>
<td>7</td>
<td>0.03</td>
<td>0.01</td>
<td>0.09</td>
<td>c.-2328T&gt;G, c.2220G&gt;A, c.3379+7A&gt;C, c.3539-33G&gt;A, c.3539-49C&gt;T, c.4414dupC, c.5620+33-40delA, c.6554G&gt;A, c.6902-5T&gt;A, c.8418_8419insTCCC</td>
<td>p.Met740Ile, p.His1472fs, p.Arg2185Gln, p.Ser2807fs</td>
<td>28/52</td>
<td>Compound heterozygous</td>
</tr>
<tr>
<td>T032</td>
<td>F/19</td>
<td>13</td>
<td>0.01</td>
<td>0.00</td>
<td>0.01</td>
<td>c.3379+1 G&gt;A</td>
<td>-</td>
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</tr>
<tr>
<td>T036</td>
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<td>0.01</td>
<td>0.01</td>
<td>c.5180_5181insTT</td>
<td>p.Leu1727fs</td>
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<td>Homozygous</td>
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<tr>
<td>T040</td>
<td>F/4</td>
<td>11</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>c.874+5 G&gt;A</td>
<td>-</td>
<td>7</td>
<td>Homozygous</td>
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<tr>
<td>T046</td>
<td>F/16</td>
<td>21</td>
<td>0.01</td>
<td>0.04</td>
<td>0.02</td>
<td>c.385C&gt;A, c.813C&gt;A</td>
<td>p.Leu129Met, p.Tyr271*</td>
<td>5/7</td>
<td>Compound heterozygous</td>
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<tr>
<td>T050</td>
<td>F/21</td>
<td>24</td>
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<td>0.00</td>
<td>0.01</td>
<td>c.876delC, c.1255C&gt;T</td>
<td>p.Ser293fs, p.Gln419*</td>
<td>8/11</td>
<td>Compound heterozygous</td>
</tr>
<tr>
<td>T058</td>
<td>F/1</td>
<td>n/a</td>
<td>0.01</td>
<td>0.03</td>
<td>0.02</td>
<td>c.2443-?_2685+?del, c.5180_5181insTT</td>
<td>p.Val815_Gln895 del p.Leu1727fs</td>
<td>19-20/30</td>
<td>Compound heterozygous</td>
</tr>
<tr>
<td>T065</td>
<td>M/15</td>
<td>22</td>
<td>0.01</td>
<td>0.02</td>
<td>0.01</td>
<td>c.3303C&gt;A</td>
<td>p.Cys1101*</td>
<td>25</td>
<td>Homozygous</td>
</tr>
<tr>
<td>T076</td>
<td>M/12</td>
<td>23</td>
<td>0.01</td>
<td>0.03</td>
<td>0.01</td>
<td>c.1897T&gt;C</td>
<td>p.Cys634Arg</td>
<td>15</td>
<td>Homozygous</td>
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<tr>
<td>T078</td>
<td>F/5</td>
<td>9</td>
<td>0.01</td>
<td>0.03</td>
<td>0.01</td>
<td>c.5455+2 T&gt;C</td>
<td>-</td>
<td>31</td>
<td>Homozygous</td>
</tr>
<tr>
<td>ID</td>
<td>Sex/Age</td>
<td>Gender</td>
<td>A1</td>
<td>A2</td>
<td>A3</td>
<td>A4</td>
<td>A5</td>
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<tr>
<td>T085</td>
<td>M/17</td>
<td>23</td>
<td>0.04</td>
<td>0.03</td>
<td>0.01</td>
<td></td>
<td></td>
<td>c.1926G&gt;A, c.2438dupG</td>
<td>p.Trp642*, p.Gly813fs</td>
</tr>
<tr>
<td>T086</td>
<td>M/1</td>
<td>3</td>
<td>0.01</td>
<td>0.02</td>
<td>0.01</td>
<td></td>
<td></td>
<td>c.1A&gt;G, c.2377C&gt;T</td>
<td>p.Met1Val, p.Gln792*</td>
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<tr>
<td>T090</td>
<td>M/2</td>
<td>8</td>
<td>0.01</td>
<td>0.04</td>
<td>0.01</td>
<td></td>
<td></td>
<td>c.1117C&gt;T, gene conversion (c.3931C&gt;T, c.4027A&gt;G, c.4079T&gt;C, c.4105T&gt;A)</td>
<td>p.Arg373*, p.Gln1311*, p.Ile1343Val, p.Val1360Ala, p.Phe1369Ile</td>
</tr>
<tr>
<td>T093</td>
<td>M/8</td>
<td>10</td>
<td>0.02</td>
<td>0.02</td>
<td>0.04</td>
<td></td>
<td></td>
<td>c.6709T&gt;C, c.220+20G&gt;T</td>
<td>p.Cys2237Arg, -</td>
</tr>
<tr>
<td>T099</td>
<td>F/3</td>
<td>18</td>
<td>0.04</td>
<td>0.02</td>
<td>0.06</td>
<td></td>
<td></td>
<td>c.1750_1765delinsCG</td>
<td>p.Cys584_Ser589 delinsArg</td>
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<td>13</td>
<td>0.07</td>
<td>0.01</td>
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<td></td>
<td></td>
<td>c.4146G&gt;T</td>
<td>p.(=)</td>
</tr>
<tr>
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<td>F/41</td>
<td>23</td>
<td>0.01</td>
<td>0.01</td>
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</tr>
<tr>
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<td>p.Ser2807fs</td>
</tr>
<tr>
<td>T114</td>
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<td>8</td>
<td>0.03</td>
<td>0.02</td>
<td>0.05</td>
<td></td>
<td></td>
<td>c.7399C&gt;T, c.8418_8419insTCCC</td>
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<tr>
<td>T116</td>
<td>F/29</td>
<td>6</td>
<td>0.02</td>
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<td>c.8418_8419insTCCC</td>
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<td>0.02</td>
<td>0.00</td>
<td>0.05</td>
<td></td>
<td></td>
<td>c.8418_8419insTCCC</td>
<td>p.Ser2807fs</td>
</tr>
<tr>
<td>T121</td>
<td>M/62</td>
<td>7</td>
<td>0.02</td>
<td>0.05</td>
<td>0.06</td>
<td></td>
<td></td>
<td>c.7399C&gt;T, c.8418_8419insTCCC</td>
<td>p.Gln2467*, p.Ser2807fs</td>
</tr>
<tr>
<td>T136</td>
<td>M/24</td>
<td>24</td>
<td>0.01</td>
<td>0</td>
<td>0</td>
<td>0.02</td>
<td></td>
<td>c.2438dupG</td>
<td>p.Gly813fs</td>
</tr>
<tr>
<td>T141</td>
<td>F/16</td>
<td>13</td>
<td>0.04</td>
<td>0.04</td>
<td>0.06</td>
<td></td>
<td></td>
<td>c.817T&gt;C</td>
<td>p.Arg2737Trp</td>
</tr>
<tr>
<td>T143</td>
<td>F/64</td>
<td>24</td>
<td>0.04</td>
<td>0.04</td>
<td>0.03</td>
<td></td>
<td></td>
<td>c.8043_8044delAG</td>
<td>p.Arg2681fs</td>
</tr>
<tr>
<td>T151</td>
<td>M/27</td>
<td>29</td>
<td>0.02</td>
<td>0.04</td>
<td>0.01</td>
<td></td>
<td></td>
<td>c.3939G&gt;A, c.5842+1G&gt;C</td>
<td>p.Trp1313*, -</td>
</tr>
<tr>
<td>T154</td>
<td>M/16</td>
<td>24</td>
<td>0.01</td>
<td>0.03</td>
<td>0.01</td>
<td></td>
<td></td>
<td>c.1656_1657insT, c.8418_8419insTCCC</td>
<td>p.Trp553fs, p.Ser2807fs</td>
</tr>
<tr>
<td>T166</td>
<td>M/20</td>
<td>10</td>
<td>&lt;0.10</td>
<td>&lt;0.10</td>
<td>0.06</td>
<td></td>
<td></td>
<td>c.8418_8419insTCCC</td>
<td>p.Ser2807fs</td>
</tr>
</tbody>
</table>
Figure 2.1 The location of the 31 mutations identified.

Diagram is based on historically annotated domains of VWF. Human Genome Variation Society (HGVS) nomenclature; * represents a nonsense mutation.
2.4.5.1 Missense mutations

A total of six missense mutations (19% of all mutations) were identified in this study; c.1A>G (p.Met1Val), c.385C>A (p.Leu129Met), c.817T>C (p.Arg273Trp), c.1897T>C (p.Cys633Arg), c.6709T>C (p.Cys2237Arg), and c.7906A>T (p.Asn2636Tyr). Three IC were found to be homozygous for missense mutations and had no other mutations identified throughout VWF. The predicted impact of these mutations was examined using PolyPhen-2 and SIFT in silico programs (Table 2.3). These individuals were negative for partial gene deletions.

Table 2.3 In silico analysis of six missense mutations with PolyPhen-2 and SIFT comparison scores.

<table>
<thead>
<tr>
<th>Amino Acid Change</th>
<th>PolyPhen-2 comparison score</th>
<th>SIFT comparison score</th>
<th>Expression Studies, reference</th>
<th>Patient ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>p.Met1Val^</td>
<td>Possibly damaging (0.924)</td>
<td>Affect protein function (0.00)</td>
<td>no</td>
<td>T086</td>
</tr>
<tr>
<td>p.Leu129Met</td>
<td>Probably damaging (0.999)</td>
<td>Affect protein function (0.00)</td>
<td>no</td>
<td>T046</td>
</tr>
<tr>
<td>p.Arg273Trp</td>
<td>Probably damaging (1.000)</td>
<td>Affect protein function (0.00)</td>
<td>Allen et al. 2000</td>
<td>T141</td>
</tr>
<tr>
<td>p.Cys633Arg^</td>
<td>Probably damaging (0.996)</td>
<td>Affect protein function (0.00)</td>
<td>Under investigation</td>
<td>T076</td>
</tr>
<tr>
<td>p.Cys2237Arg^</td>
<td>Probably damaging (1.000)</td>
<td>Affect protein function (0.00)</td>
<td>no</td>
<td>T093</td>
</tr>
<tr>
<td>p.Gln2636Tyr^</td>
<td>Benign (0.200)</td>
<td>Tolerated (0.30)</td>
<td>no</td>
<td>T028</td>
</tr>
</tbody>
</table>

^Novel missense mutations, not previously reported.

The missense mutation p.Met1Val found in the heterozygous state in T086 replaces the invariant initiator methionine with a valine. This substitution will inevitably result in a null allele due to the marked suppression of VWF translation from the mutant transcript.
The missense mutation \( p.\text{Arg273Trp} \), identified in the homozygous state in IC T141, has been previously reported. Expression studies showed that this mutation was responsible for the VWF deficiencies noted in their patients and the aberrant multimer patterns observed.\(^{190}\)

In IC T076 the only mutation identified was a single homozygous nucleotide change in exon 15 resulting in an arginine residue replacing a cysteine at amino acid 633 (\( p.\text{Cys633Arg} \)). Due to the location of this mutation in the propeptide region and the known importance of cysteine molecules in VWF biosynthesis and the proper folding of VWF, the molecular pathogenesis of this mutation is currently under investigation.

A third IC (T093), was found to be homozygous for a different missense mutation which again results in a cysteine residue being replaced by an arginine, this time at position 2237 (\( p.\text{Cys2237Arg} \)). This mutation is located in exon 38 which encodes part of the D4 domain; the function of which is not well understood.

### 2.4.5.2 Partial/total gene deletions

We identified two different partial VWF deletions in two IC. One IC (T001) was identified as being homozygous for the previously reported exon 4-5 deletion mutation (c.221-977_532+7059del; \( p.\text{Asp75_Gly178del} \)).\(^{173}\) The deletions of exons 4 and 5 were confirmed using the deletion-specific PCR described by Sutherland et al. (2009) and MLPA. Two enrolled family members, a sibling and mother, diagnosed with type 1 VWD (T002 and T156), were screened and determined to be heterozygous for this deletion. The haplotype of these individuals was analyzed and found to be different than the common haplotype reported in the UK population.\(^{173}\) Similarly, no common haplotype was found between individuals with the same mutations in the Canadian and European type 1 VWD studies.\(^{123,167}\)
T058, for whom only a heterozygous frameshift mutation (p.Leu1727fs) had been identified after direct sequencing was also found to be heterozygous for a deletion of exons 19 and 20 (c.2443-?_2685+?del; p.Val815_Gln895). The deletion of exons 19 and 20 removes 81 amino acids and remains in-frame. The locations of the breakpoints have yet to be determined. Both mother and father were subsequently analyzed for this partial gene deletion. The insertion mutation was inherited from the father (T060) and the partial gene deletion from the mother (T059).

2.4.5.3 Gene conversion

Gene conversions between VWF and its pseudogene have been previously reported. One IC (T090) in this study was found to have a heterozygous gene conversion, a minimum 175bp and maximum 395bp in length, which included the nonsense mutation c.3931C>T (p.Gln1311*), and the missense mutations c.4027A>G (p.Ile1343Val), c.4079T>C (p.Val1360Ala), and c.4105T>A (p.Phe1369Ile). Upon sequencing the family members, it was determined that T090 inherited the gene conversion from the father.

2.4.6 Phenotype-Genotype Correlations

The five individuals for whom complete pathogenic explanations could not be elucidated had similar VWF:Ag, VWF:RCo and FVIII levels compared to the 29 individuals for whom two mutant VWF alleles were identified (P = NS, Mann-Whitney U testing). As well, these five IC did not have significantly lower bleeding scores when compared to the other IC (P = 0.119, Mann-Whitney U test) (BS of the five IC=13, 13, 18, 24, and 24).
2.5 Discussion

This report comprises one of the largest type 3 VWD studies to date, with information on 42 Type 3 VWD patients from 34 different families in Canada. Type 3 VWD is classically inherited in an autosomal recessive fashion with individuals homozygous or compound heterozygous for null alleles. However, this is not always the case and interestingly, the original type 3 VWD family from the Åland Islands did not exhibit recessive inheritance and showed evidence of co-dominant inheritance. We have shown that in the Canadian type 3 VWD population as well this is not always the case, with 53% of the families reported in this study having both type 1 VWD and type 3 VWD individuals. Many OC in this cohort are not phenotypically silent, manifesting low VWF levels and mucocutaneous bleeding symptoms. The Canadian type 3 VWD population is comprised of a few homogeneous populations with evidence of founder alleles, yet is also very heterogeneous in nature with approximately 40% of IC from nine different ethnicities. This heterogeneity may account for the distinct nature of this cohort.

Alloantibodies to VWF are a rare complication of type 3 VWD and are frequently associated with homozygous large deletions, however a few cases of alloantibodies to VWF in patients with homozygous gene conversions and homozygous nonsense mutations have also been reported. The one individual (T018) in this study that is positive on the anti-VWF ELISA is compound heterozygous for our commonly reported mutation, p.Ser2807fs, and the nonsense mutation p.Gln2467*. The inhibition of collagen binding activity after mixing suggests that the alloantibody in this patient is affecting this specific function of VWF, however further investigation is required to better define the nature of this inhibitory influence. T018 is not on prophylactic treatment but has been treated with Humate-P® (5000 IU)
at times of nosebleeds, surgery or trauma. The patient responds clinically to treatment and has not
developed any anaphylactic reactions and/or become refractory to replacement therapy.

As with previous type 3 VWD studies we have identified a number of novel mutations
scattered throughout the VWF gene that result in null alleles. An important finding within this
study is the high prevalence of mutations located within the propeptide region of VWF (42%). As
well, we have shown that IC with mutations in the propeptide have higher BS than IC with
mutations in other areas of VWF. Further investigation of the contribution of propeptide
mutations to type 3 VWD is warranted and is under investigation by our group.

While the number of missense mutations (~19%) identified in this study is consistent
with other reported type 3 VWD populations\textsuperscript{170} we believe the contribution of these missense
mutations warrants further investigation. Recently, other groups have begun to investigate the
contribution of missense mutations to type 3 VWD.\textsuperscript{194,195} Investigations of the two missense
mutations that were found in the homozygous state are ongoing.

In the majority of type 3 VWD studies, pathogenic mutations are identified in 80-90% of
patients.\textsuperscript{172,181-184,196} In this study we were able to identify both pathogenic mutations in 85% of
IC (62 of 68 (91%) mutant alleles identified). In the remaining five IC we were unable to find two
mutant VWF alleles that could explain their VWD disease state. A number of possibilities may
exist for this and warrant investigation. Pathogenic possibilities include apparently silent
sequence variations in the VWF gene located outside of consensus splice sites that disrupt the
normal VWF mRNA splicing, deep intronic mutations that may only be identified through whole
gene sequencing, and distant regulatory elements outside of VWF.

In conclusion, our study represents one of the largest and most comprehensive reports of
type 3 VWD patients and their family members. We have made important observations regarding
the contribution of mutations in the propeptide region to the type 3 VWD phenotype including the increased severity of bleeding in these cases. As well, the phenotype-genotype correlations made in this paper further highlight the differences between OC of type 3 VWD and those diagnosed with type 1 VWD. We have also shown that in the Canadian type 3 VWD population, 48% of OC of type 3 VWD are not phenotypically silent and have been diagnosed with type 1 VWD. This Canadian type 3 VWD population further emphasizes that in a significant proportion of cases the genetic transmission of the VWD phenotype is co-dominant in nature and not recessive.

2.6 Acknowledgements

The authors acknowledge the contributions of the members of the Association of Hemophilia Clinic Directors of Canada (AHCDC) and the Canadian Association of Nurses in Hemophilia Care. This project was funded by a Canadian Hemophilia Society Research Grant and through the Zimmerman Program for Molecular and Cellular Biology of von Willebrand Disease by The National Institutes of Health Program Project Grant HL081588. MB is the recipient of an Ontario Graduate Scholarship and a Queen’s University Graduate Scholarship. DL holds a Canada Research Chair in Molecular Hemostasis. PJ held a Bayer Hemophilia Awards Program Early Career Investigator Award at the time of this study and currently holds a Clinician Scientist Award from the Southeastern Ontario Academic Medical Association (SEAMO).
Chapter 3

Investigation of the Contribution of von Willebrand Factor (VWF) Propeptide Mutations to Type 3 von Willebrand Disease (VWD)

3.1 Summary

The VWF propeptide (VWFpp) is critical for multimerization and acts as an intramolecular chaperone of the mature VWF protein in sorting to storage organelles. In the Canadian type 3 von Willebrand disease (VWD) study, 42% of mutations were found in the VWFpp and index cases (IC) with these mutations had a more severe bleeding phenotype. These observations led us to further investigate the underlying molecular mechanisms of two of these mutations. The exon 4-5 deletion mutation and the p.Cys633Arg mutation were investigated in this study using both patient-derived blood outgrowth endothelial cells (BOEC) and a heterologous cellular system. Both mutations resulted in a dramatic reduction in secretion in the heterozygous state and in virtual abolishment in the homozygous states. Both caused a lack of multimerization in the BOEC lysates in the heterozygotes. Confocal immunofluorescence (IF) images showed quantitative and qualitative defects in Weibel-Palade body (WPB) formation, a lack of co-localization with other proteins in WPB, and localization within the endoplasmic reticulum (ER). Stimulation with secretagogues PMA and calcium ionophore gave variable results but indicated that both heterozygous mutants responded to stimulation. Co-transfection of each VWF mutant with the wild-type VWFpp was not sufficient to restore secretion or multimerization. This study highlights the importance of VWFpp mutations to type 3 VWD and the utility of incorporating experiments with patient-derived BOEC for examining these mutations.
3.2 Introduction

von Willebrand disease (VWD) is the most common inherited bleeding disorder in humans and results from genetic alterations in the von Willebrand factor (VWF) gene, which encodes for the VWF protein. VWF is a large, adhesive glycoprotein that functions in primary and secondary hemostasis.\(^1\) VWF is synthesized and secreted from endothelial cells and megakaryocytes as a large pre-pro-VWF of 2813 amino acids containing a 22 amino acid signal peptide, a 741 amino acid propeptide (VWFpp), and a 2050 amino acid mature VWF molecule. Extensive post-translational modifications of VWF occur including N- and O-linked glycosylation, sulfation, carboxy-terminal dimerization, amino-terminal multimerization, and proteolytic cleavage of the VWFpp from mature VWF.\(^1\) The VWFpp and mature molecule are stored together in the Weibel-Palade bodies (WPB) of endothelial cells and dissociate upon release into the circulation.\(^54\)

The VWFpp has been shown to be critical for both multimerization and storage of VWF. Both the D1 and D2 assemblies of the VWFpp contain vicinal cysteines (\(^{159}\)Cys-Gly-Leu-Cys\(^{162}\) and \(^{521}\)Cys-Gly-Leu-Cys\(^{524}\)) which provide the substrate for disulfide oxidoreductase activity that is required for N-terminal disulfide bond formation in the acidic environment of the Golgi apparatus.\(^17\) Multimerization is abolished when the VWFpp is deleted \textit{in vitro};\(^55\) however, expression of the VWFpp in \textit{trans} is sufficient for multimer assembly.\(^16\) The VWFpp acts as an intra-molecular chaperone, facilitating the storage of mature VWF protein. When the VWFpp is expressed alone, sorting to storage organelles is observed. In the absence of the VWFpp, however, the mature VWF will not sort to storage organelles. The expression of the VWFpp in \textit{cis} or \textit{trans} does result in co-storage of the VWFpp and mature VWF.\(^33\)
In a Canadian cohort of type 3 VWD patients, 42% of identified mutations were located in the VWFpp,\textsuperscript{197} this is in contrast to the Canadian type 1 VWD study where only 7% of mutations were located in the VWFpp.\textsuperscript{123} These results are higher compared to reports of other type 3 VWD populations, such as the Swedish and Finnish (31%),\textsuperscript{181} Indian and Greek (29%),\textsuperscript{184} and Hungarian (23%)\textsuperscript{183} populations.

Type 3 VWD index cases (IC) with mutations in the VWFpp also exhibited a more severe bleeding phenotype compared to IC with mutations elsewhere in VWF, as assessed by the bleeding score (BS = 22 vs. 13, respectively).\textsuperscript{197} For the present study we sought to further investigate two of these mutations. The first mutation, the exon 4 and 5 deletion mutation (c.221-977_532+7059del; \textit{p}. Asp75_Gly178del), was previously identified and described in type 1 and 3 VWD patients from the United Kingdom.\textsuperscript{173} This mutation is an in-frame deletion of 103 amino acids in the D1 assembly of the VWFpp.\textsuperscript{173} The second mutation, c.1897T>C; \textit{p}. Cys633Arg, is a novel missense mutation in the D2 assembly identified in one family in the Canadian type 3 VWD population.\textsuperscript{197} This mutation is of interest because of the lack of information on how missense mutations in the homozygous state lead to type 3 VWD and because of the known importance of cysteines in VWF.\textsuperscript{3} Both of the type 3 VWD IC with these VWFpp mutations of interest had bleeding scores of 23.

Conventionally, studies on the underlying pathogenesis of VWF mutants have been primarily performed \textit{in vitro} using transfections of plasmid VWF in non-endothelial cell systems, which do not entirely recapitulate the physiological endothelial environment. Recently, studies have used an \textit{ex vivo} approach, isolating blood outgrowth endothelial cells (BOEC) from VWD patients to investigate the cellular mechanisms of VWD.\textsuperscript{44,164} The procedure to establish BOEC is
minimally invasive (only requires phlebotomy) and provides a cellular model to study VWD which more accurately reflects the native endothelial environment.

The objective of the current study was to investigate the exon 4-5 deletion and p.Cys633Arg mutations using both in vitro and ex vivo cellular systems to further elucidate their contribution to type 3 VWD. Additionally, we sought to investigate whether the VWFpp in trans would restore multimerization of one or both of these mutations, as this has previously been shown to be successful with a different VWFpp mutation (p.Asp437-Arg442del).156

3.3 Patients, materials and methods

3.3.1 Patients

Two families from the Canadian type 3 VWD study were chosen for further investigation in this study. Each family had a type 3 VWD index case (IC) who is homozygous for a VWFpp mutation and a heterozygous family member with type 1 VWD. Figure 3.1A shows the pedigree for the family with the exon 4 and 5 deletion mutation (ex4-5del), including previously determined VWF levels and bleeding scores. Figure 3.1B shows the pedigree for the family with the p.Cys633Arg (Cys633Arg) mutation. All study participants gave informed consent and study approval was obtained from the Research Ethics Board of Queen’s University, Kingston, Canada.

3.3.2 BOEC isolation and culture

Peripheral blood samples (~ 48 mL) were collected by phlebotomy into Cell Preparation Tubes (CPT)™ (BD Biosciences). Detailed methods are similar to those published.198 Briefly, a series of washing and centrifugation steps were followed by the resuspension of mononuclear
Figure 3.1 Pedigree and laboratory data for study families.

Pedigree and laboratory values of the Canadian type 3 VWD family with the ex4-5del mutation (A) and the Cys633Arg mutation (B). Affected patients with type 3 VWD are represented by black symbols; affected patients with type 1 VWD are represented by half-filled symbols; unaffected persons are open symbols; squares are males; and circles are females. * indicates individuals further investigated as part of this study.
cells (MNC) in Endothelial Cell Growth Media-2 (EGM-2) BulletKit™ (Lonza) supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 1% penicillin (10,000 U/mL)/streptomycin (10,000 μg/mL)/ amphotericin (25 μg/mL) (Invitrogen) (complete EGM-2 media, cEGM-2). Cells were seeded at a density of 4x10^7 MNC/well of a 6-well tissue culture plate pre-coated with rat-tail collagen type I. After 24 hours, non-adherent cells and debris were removed and adherent cells were washed once with cEGM-2, and then cEGM-2 was added to each well. Culture media was refreshed daily for 7 days, followed by every other day. For all experiments BOEC were used at passage 3-9, unless stated otherwise.

3.3.3 Confirmation of endothelial cell phenotype

The endothelial cell phenotype of isolated cells was confirmed by: 1) the formation of confluent monolayers of cells with characteristic endothelial cobblestone morphology and 2) staining of cell surface markers followed by flow cytometry. Specifically, this involved identifying the presence of cell surface markers CD31 (Platelet endothelial cell adhesion molecule, (PECAM-1) and CD144 (VE-cadherin) and/or CD146 (melanoma cell adhesion molecule (MCAM)/cell surface glycoprotein MUC18), as well as the absence of leukocyte markers CD14 and CD45 (Protein tyrosine phosphatase receptor type C, PTPRC) in the isolated cells. The following fluorescently conjugated antibodies were used: FITC CD31, Pe CD144, PerCp Cy 5.5 CD14, Pe-Cy 5 CD 45 (all from Ebioscience). Human umbilical vein endothelial cells (HUVEC) were used as control cells for CD31, CD144, and CD146 and human acute monocytic leukemia cells (THP1) were used as a control for CD14 and CD45. Unlabeled cells and cells with isotype control antibodies were also used as controls. Samples were analyzed using
an Epics Altra HSS flow cytometer (Queen’s Cytometry and Imaging Facility, The Cancer Research Institute at Queen’s University, Kingston, Canada).

3.3.4 VWF expression

VWF expression in patient-derived BOEC media and lysates was measured by VWF:Ag enzyme-linked immunosorbent assay (ELISA) using polyclonal antibodies (A0082 and P0226, DAKO). Three sets of independent measures, with three replicates each, were obtained. VWF secreted into serum-free media (OptiMEM® supplemented with 100mg/L CaCl₂) was concentrated using Ultra-70 100K MWCO centrifugal filter devices (Millipore) and multimers analyzed by sodium dodecyl sulfate (SDS)-agarose gel electrophoresis.¹⁹⁹

3.3.5 BOEC confocal immunofluorescence microscopy

To visualize the intracellular location of VWF in the patient BOEC, confocal immunofluorescence (IF) imaging was performed. Confluent BOEC from either VWD patients or a healthy control were seeded on glass coverslips pre-coated with collagen type I at a density of 5x10⁵ cells per well. After 24 hour incubation, BOEC were fixed, permeabilized and stained. Polyclonal rabbit anti-human VWF (A0082, DAKO), and anti-rabbit immunoglobulin-FITC (F0054, DAKO) were used to visualize VWF. The negative control rabbit immunoglobulin fraction (X0936, DAKO) was used as an isotype control. Phalloidin-TRITC (Sigma, P1951) and DAPI (D9542, Sigma) were also used to visualize actin filament and nuclei, respectively. To observe if VWF co-localized with other proteins stored in WPB, staining for P-selectin, IL-8, and CD63 (sc-6941, sc-1269, sc-7080, Santa Cruz) was performed. Cells stained for IL-8 were
stimulated for 24 hours post-seeding with IL-1β (I9401, Sigma) in order to induce appreciable secretion of IL-8. Additionally, BOEC were stained with the endoplasmic reticulum (ER) marker calnexin (sc-6465, Santa Cruz) to observe the localization of the mutant VWF. Donkey anti-goat rhodamine (sc-2094, Santa Cruz) was used as the secondary antibody and normal goat IgG (sc-2028, Santa Cruz) as a control for the additional IF experiments. Slides were analyzed using a Leica TCS SP2 Multi Photon confocal microscope (Queen’s Cytometry and Imaging Facility, The Cancer Research Institute at Queen’s University, Kingston, Canada). Image analysis was performed using MetaMorph® Image Analysis Software and/or Image J. To quantify the number of WPB present in the BOEC, approximately 100 VWF-positive cells were randomly selected from two independent experiments and analyzed.

### 3.3.6 Stimulation of Weibel-Palade bodies (WPB)

For the measurement of regulated VWF release from WPB, BOEC from VWD patients or healthy controls were seeded onto collagen-coated 6-well plates at a density of 1x10^6 cells/well. Twenty-four hours post-seeding, the media was replaced with serum-free media (OptiMEM® supplemented with 100mg/L CaCl₂). BOEC were incubated for an additional 24 hours and then stimulated with 100nM phorbol 12-myristate 13-acetate (PMA, Sigma), 10µM calcium ionophore A23187 (Sigma), or dimethylsulfoxide (DMSO) control, for 75 minutes at 37°C. Conditioned media and cell lysates were then collected. Three sets of independent measures for PMA and two for calcium ionophore, with three replicates each, were obtained. Secreted VWF:Ag before and after stimulation are reported as the percentage of secreted VWF:Ag, expressed as a fraction of the total amount of VWF:Ag (i.e. % of total = VWF:Ag in the media/[VWF:Ag in the media + VWF:Ag in the cell lysate] X 100).
3.3.7 Western blot analysis of BOEC lysates

Immunoblotting of BOEC lysates for VWFpp and mature VWF were performed as follows. Equal amounts of VWF in the cell lysates were electrophoresed under reduced conditions on an SDS-polyacrylamide precast gel (Bio Rad). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. PVDF membranes were blocked in 5% skim milk in TBS buffer, rotating for one hour. Polyclonal rabbit anti-human VWF/HRP antibody was added at a dilution of 1:1000 in 5% skim milk/TBS. After several washes of the blot in TBS with 0.1% Tween-20, chemiluminescent detection and development was performed.

3.3.8 Plasmid constructs

Site-directed mutagenesis (Quick Change® II XL Site-Directed Mutagenesis kit; Stratagene) was performed on the full-length human VWF expression vector, pCIneohuVWFES, to create pClneoVWFdel4-5 and pClneoVWFC633R as per the manufacturer’s protocol. The human pCIneoVWFpp plasmid was constructed using site-directed mutagenesis to insert a stop codon (TGA), followed by a MluI restriction site (ACGCGT) at the end of the VWFpp, after amino acid 763 of the pCIneohuVWF expression vector. BmtI and MluI were used to excise the VWFpp and the fragment was then ligated into the pCI-neo mammalian expression vector (Promega). Therefore, the pCIneoVWFpp expression vector contains the signal peptide, the VWFpp sequence, followed by a stop codon with the mature VWF sequence deleted. The resultant expression plasmids were sequenced to confirm the presence of each mutation and the VWFpp plasmid, and plasmid DNA was purified for transfection.
3.3.9 Cell culture and transient transfections

Human Embryonic Kidney (HEK)-293T cells were cultured in Dulbecco modified Eagle medium (DMEM) containing 2mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10% (vol/vol) FBS, and 0.25 μg/mL fungizone at 37% in 5% CO2. Cells in the log phase of growth were seeded onto pre-coated poly-L lysine 10 cm plates at a density of 3x10⁶ cells/plate. Transient transfections of wild-type (WT; pClneohuVWF), heterozygous (1:1 molar ratio; WT:mutant of interest) and homozygous plasmids using the calcium phosphate method were performed. Approximately 16 hours after transfection, the media was replaced with serum-free media (OptiMEM® containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 1X Insulin-Transferrin-Selenium (ITS)). An additional 48 hours later media was harvested and cells were lysed. Transfection efficiency was determined by measuring βgal reporter transcript using Berthold Lumat LB 9501 luminometer (Fisher Scientific) and the Galacto Light Plus reporter gene assay (Tropix). Quantification of the VWF:Ag present in the media and cell lysates was determined by ELISA. Three independent sets of experiments, with four replicates each, were performed. Recombinant VWF (rVWF) from the media was concentrated using Ultra-70 100K MWCO centrifugal filter devices (Millipore) and multimers analyzed by SDS-agarose gel electrophoresis.

3.3.10 Transient transfection confocal immunofluorescence microscopy

HEK293 cells have been shown to form pseudo-WPB, which are similar to the WBP in endothelial cells. For confocal IF microscopy experiments, HEK293 cells were cultured in Minimum Essential Medium (MEM) containing Earle’s salts and 2mM L-glutamine, and supplemented with 10% (vol/vol) FBS. Cells were seeded onto poly-L lysine coated glass
coverslips in 6-well plates at a density of 350,000 cells/well. Transient transfections of WT (pClneohuVWF), heterozygous (1:1 molar ratio; WT:mutant of interest) and homozygous mutant plasmids using Lipofectamine® (Invitrogen) were performed according to the manufacturer’s protocol. Forty-eight hours post-transfection cells were fixed, permeabilized and stained for VWF as previously described in section 3.3.5.

### 3.3.11 Transient transfection cell treatments

For the measurement of VWF release from pseudo-WPB, HEK293 cells were seeded onto poly-L-Lysine coated 6-well plates at a density of 350,000 cells/well. Twenty-four hours post-seeding, cells were transiently transfected using Lipofectamine®. Forty-eight hours post-transfection cells were stimulated with 100nM PMA (Sigma), 10μM calcium ionophore A23187 (Sigma), or DMSO control, for 75 minutes at 37°C. Additionally, to determine if there was proteasomal degradation of the mutant VWF, the addition of 10μM of the proteasomal inhibitor MG-132 (Cayman Chemical) or DMSO control for 60 min at 37°C was performed. Conditioned media and cell lysates were then collected. Three independent sets of experiments, with three replicates each, were performed. To calculate secretion, secreted VWF before or after stimulation was expressed as a fraction of total VWF (amount in the media + lysate).

### 3.3.12 Co-transfections with VWF propeptide

In order to observe if the VWFpp in trans would restore normal VWF secretion, multimerization and/or storage in these mutations, we performed transient transfections into HEK293 cells using calcium phosphate transfections as previously described. 100% of each
mutation and 1:1 molar ratios of each mutation and the VWFPp were performed. WT VWF (pClneohuVWF) was also transfected to use as a positive control. VWF:Ag was measured by ELISA in the media and cell lysates. Three independent sets of experiments, with four replicates each, were performed. Protein was concentrated using Ultra-70 100K MWCO centrifugal filter devices (Millipore) and multimer analysis of the concentrated protein was performed.199

3.3.13 Graphing and statistical analyses

Data and statistical analyses were performed with GraphPad Prism for Windows (version 4). Statistical analyses were performed using the Kruskal-Wallis non-parametric test; P < 0.05 was considered statistically significant.

3.4 Results

3.4.1 Patient-derived BOEC

BOEC were successfully isolated from the type 3 VWD IC T001 and his mother, T156 who are homozygous and heterozygous for the ex4-5del mutation, respectively. From the Cys633Arg family, BOEC were isolated from the type 3 VWD IC T076, his type 3 VWD sister (T076-S) and their mother (T077), who has type 1 VWD. T076 required two separate isolation attempts with the second one being successful. BOEC from all patients appeared on days 9-14.
3.4.2 Endothelial cell phenotype

All isolated cells were able to form confluent monolayers of cells with cobblestone morphology. To confirm the endothelial cell phenotype of the isolated cells, flow cytometry was performed. Table 3.1 gives a summary of the flow cytometry staining data for the patient-derived BOEC. T076 and T076-S were both positive for CD31 but had low positivity for CD144. Both cell lines were negative for CD14 and CD45, indicating cultures free of monocytes. Staining for CD146 was performed and positivity for this cell surface marker was confirmed in both lines.

<table>
<thead>
<tr>
<th>Patient</th>
<th>CD31(%)</th>
<th>CD144(%)</th>
<th>CD146(%)</th>
<th>CD14(%)</th>
<th>CD45(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T001</td>
<td>93</td>
<td>90</td>
<td>n/a</td>
<td>0.6</td>
<td>0</td>
</tr>
<tr>
<td>T156</td>
<td>96</td>
<td>68</td>
<td>n/a</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>T077</td>
<td>93</td>
<td>72</td>
<td>n/a</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>T076</td>
<td>94</td>
<td>43</td>
<td>90</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>T076-S</td>
<td>90</td>
<td>16</td>
<td>87</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Data are given as percentage positive cells of live cells, corrected for the corresponding IgG control.

3.4.3 VWF phenotype of ex4-5del BOEC

To determine the VWF expression in the media and cell lysates of the patient-derived BOEC, VWF:Ag was measured by ELISA. Secretion of VWF from the heterozygous and homozygous ex4-5del BOEC was decreased by 85% (P < 0.0001) and 98% (P < 0.0001),
respectively, relative to the secretion of VWF from BOEC of a healthy control (N = 9 each) 
(Figure 3.2A). Increased intracellular retention was observed with an increase of 72% (P < 
0.0001) and 26% (P =0.0031), respectively, relative to the VWF in the BOEC lysates of a healthy 
control (Figure 3.2B).

Multimer analysis is shown in Figure 3.2C. A full range of multimers was observed in the 
heterozygous patient plasma and concentrated media secreted from the BOEC, however there was 
an absence of multimerization in the BOEC lysates. A complete lack of multimers was observed 
in the plasma, media and lysates of the homozygous BOEC.

Confocal IF microscopy was performed in order to observe the intracellular localization 
of VWF in the patient-derived BOEC. Figure 3.3 shows the confocal IF images for normal 
healthy BOEC, the heterozygous ex4-5del, and the homozygous ex4-5del BOEC. IF staining for 
VWF in the heterozygous ex4-5del BOEC demonstrated a combination of diffuse staining and 
VWF stored in WPB; however there were 63% fewer WPB (average = 26, SD = 12, P<0.0001) 
and they appeared more rounded rather than cigar-shaped compared to normal BOEC. Staining of 
the homozygous ex4-5del BOEC demonstrated a complete lack of VWF stored in WPB and only 
diffuse, ER-like staining was observed. The absence of WPB in the homozygous ex4-5del BOEC, 
led us to investigate the fate of other proteins normally co-stored with VWF in WPB. P-selectin, 
IL-8, CD63 staining in the homozygous BOEC demonstrated a lack of co-localization with VWF; 
the limited staining observed we speculate is indicative of targeting to lysosomes (Figure 3.4 
shows P-selectin staining; IL-8 and CD63 data not shown). To see if the diffuse VWF staining in 
the ex4-5del BOEC was localized to the ER, BOEC were co-stained with VWF and the ER 
marker calnexin. The diffuse VWF staining co-localized with calnexin, confirming ER retention 
of the mutant VWF (Figure 3.5).
Figure 3.2 VWF expression of ex4-5del BOEC media, lysates, and multimer analysis.

Panel A shows the VWF:Ag secreted into the media from the heterozygous and homozygous ex4-5del patients, relative to normal BOEC (set at 100%). Panel B shows the VWF:Ag for the cell lysates of the heterozygous and homozygous ex4-5del patients, relative to normal BOEC (set at 100%). *** P < 0.0001, **P < 0.001, *P < 0.01. (N=9 each). Panel C shows the multimer analysis for the plasma, BOEC media and lysates of the heterozygous and homozygous ex4-5del patients compared to normal.
Figure 3.3 Qualitative and quantitative defects in Weibel-Palade body (WPB) formation in ex4-5del patient BOEC.

Compared to normal BOEC (A), the heterozygous BOEC (B) show fewer and more rounded WPB and some diffuse VWF staining. There is a complete absence of VWF in WPB in the homozygous BOEC (C), with only diffuse VWF staining. Green = VWF, Red = actin cytoskeleton, Blue = nucleus.
Figure 3.4 Lack of co-localization of ex4-5del VWF with P-selectin in patient BOEC.

In normal BOEC, VWF and P-selectin co-localize together in WPB (merge). In the heterozygous ex4-5del BOEC, there is partial co-localization of VWF and P-selectin. A complete lack of co-localization of VWF and P-selectin is observed in the homozygous ex4-5del BOEC. Green = VWF, Red = P-selectin, Yellow = merge. Arrows point to rounded P-selectin staining which is speculated to be lysosomal.
Figure 3.5 Diffuse ex4-5del VWF co-localizes with the endoplasmic reticulum (ER) marker calnexin.

In normal BOEC, there is minimal ER staining of VWF. In the heterozygous and homozygous ex4-5del BOEC, there is co-localization of the diffuse VWF and calnexin, indicating ER retention of the mutant protein (merge). Green = VWF, Red = calnexin, Yellow= merge.
The regulated secretion of VWF from BOEC was assessed using the secretagogues PMA and calcium ionophore A23187. Stimulation with PMA resulted in a 1.8-fold increase in VWF secretion in the heterozygous ex4-5del BOEC, and while the fold increase is greater than that seen with the normal BOEC (1.2-fold increase), there was a great deal of variability in the levels and the total secretion after stimulation was still much lower compared to normal (N=9 for each) (Figure 3.6A). A 1.3-fold increase in VWF secretion was observed in the homozygous BOEC, however the total VWF secreted was still very low. We repeated the stimulation experiments using the secretagogue calcium ionophore A23187 and observed less variability in the results than with PMA. The normal BOEC (from a different normal healthy individual than those used for the PMA experiments) had a 3.6-fold increase in VWF secretion upon stimulation. The VWF secretion from the heterozygous and homozygous ex4-5del BOEC increased by 1.6-fold and 1.4-fold, respectively. Once again though the total secretion after stimulation was still much lower than in the normal BOEC (N=6 for each) (Figure 3.6B).

3.4.4 VWF phenotype of Cys633Arg BOEC

To evaluate the effect of the Cys633Arg mutation on VWF secretion and intracellular retention, VWF:Ag was measured in the media and lysates from the patient-derived BOEC. A significant decrease in VWF secretion was observed in the heterozygous and the two homozygous patients by 45% (P = 0.0040), 94% (P < 0.0001), and 92% (P < 0.0001) respectively, relative to the VWF secretion from BOEC of a healthy control (N=9 each) (Figure 3.7A). Despite the decrease in secretion, there was no significant increase in intracellular retention observed in either the heterozygous (P = 0.3247) or one representative homozygous Cys633Arg BOEC (P = 0.5546) compared to normal BOEC (Figure 3.7B).
Figure 3.6 Stimulated secretion of ex4-5del patient-derived BOEC.

BOEC from a normal healthy control, the ex4-5del heterozygote and homozygote were stimulated with or without 100nM of PMA (A) or 10μM calcium ionophore A23187 (B) for 75 min and the response in VWF secretion measured. Each bar represents VWF secreted into the media as a fraction of total VWF (media + lysate) X 100 with (grey bars) or without (black bars) stimulation. N=9 (A) and N=6 (B).
Multimer analysis of the Cys633Arg patient plasma and BOEC media and lysate are shown in Figure 3.7C. A full range of multimers was observed in the heterozygous patient plasma and BOEC concentrated media, however there was an absence of multimerization in the BOEC lysates. A complete lack of multimers was observed in the plasma, media and lysates of the homozygous Cys633Arg BOEC. One representative homozygote is shown.

Intracellular staining for VWF demonstrated similar patterns to those observed in the ex4-5del family. The heterozygous Cys633Arg BOEC displayed both diffuse VWF and VWF stored in WPB (Figure 3.8). There were 70% fewer WPB (average = 22, SD = 11, P<0.0001) compared to normal BOEC; however their morphology was similar to the cigar-shaped WPB seen in the normal BOEC. The homozygous BOEC had a complete absence of VWF in WPB and only diffuse VWF staining (a representative homozygous image is shown in Figure 3.8C).

A lack of co-localization of VWF and P-selectin (Figure 3.9), IL-8 and CD63 was observed (data not shown). Additionally, co-staining of the diffuse VWF and calnexin was observed, confirming ER retention of the mutant Cys633Arg protein (Figure 3.10).

Regulated VWF secretion induced by PMA showed a 1.4-fold increase in VWF secretion in the heterozygous Cys633Arg BOEC (N=9 for each) (Figure 3.11A). Interestingly, total % secretion in the heterozygote was comparable to the normal BOEC. No significant increase in VWF secretion was observed in the one representative homozygous BOEC. Experiments were repeated using calcium ionophore A23187 (N=6 each) (Figure 3.11B). The heterozygous Cys633Arg BOEC had a 1.2-fold increase in VWF secretion, compared to a 3.6-fold increase observed in normal BOEC (from a different normal healthy control used for the PMA experiments). The homozygous Cys633Arg BOEC also had a 1.2-fold increase in secretion upon stimulation, however total secretion was still very minimal.
Figure 3.7 VWF expression of Cys633Arg BOEC media, lysates, and multimer analysis.

Panel A shows the VWF:Ag secreted into the media from the heterozygous and one homozygous Cys633Arg patients, relative to normal BOEC (set at 100%). Panel B shows the VWF:Ag for the cell lysates of the heterozygous and homozygous Cys633Arg, relative to normal BOEC (set at 100%). *** P < 0.0001, **P < 0.001, *P < 0.01. (N=9 each). Panel C shows the multimer analysis for the plasma, BOEC media and lysates of the heterozygous and one homozygous Cys633Arg patients compared to normal.
**Figure 3.8** Qualitative and quantitative defects in Weibel-Palade body (WPB) formation in Cys633Arg BOEC.

Compared to normal BOEC (A), the heterozygous Cys633Arg BOEC (B) show fewer WPB and some diffuse VWF staining. There is a complete absence of VWF in WPB in the representative homozygous BOEC (C), with only diffuse VWF staining. Green = VWF, Red = actin cytoskeleton, Blue = nucleus.
Figure 3.9 Lack of co-localization of VWF and P-selectin in Cys633Arg BOEC.

In normal BOEC, VWF and P-selectin co-localize together in WPB (merge). In the heterozygous Cys633Arg BOEC, there is partial co-localization of VWF and P-selectin. A complete lack of co-localization of VWF and P-selectin is observed in the representative homozygous Cys633Arg BOEC. Green = VWF, Red = P-selectin, Yellow= merge. Arrows point to rounded P-selectin staining which is speculated to be lysosomal.
Figure 3.10 ER retention of VWF in Cys633Arg patient BOEC.

In normal BOEC, there is minimal ER staining of VWF. In the heterozygous and the representative homozygous Cys633Arg BOEC, there is co-localization of the diffuse VWF and calnexin, indicating ER retention of the mutant protein (merge). Green = VWF, Red = calnexin, Yellow = merge.
Figure 3.11 Stimulated secretion of Cys633Arg patient-derived BOEC.

BOEC from a healthy control, the Cys633Arg heterozygote and one homozygote were stimulated with or without 100nM of PMA (A) or 10μM calcium ionophore A23187 (B) for 75 min and the response in VWF secretion measured. Each bar represents VWF secreted into the media as a fraction of total VWF (media + lysate) X 100 with (grey bars) or without (black bars) stimulation. N=9 (A) and N=6 (B).
3.4.5 Heterologous cellular expression of ex4-5del

To determine whether or not the mutant recombinant protein was retained within the cell or was inefficiently secreted, VWF:Ag levels were assayed by ELISA in the media and cell lysates from the transfected HEK293T cells. Secretion of the recombinant VWF (rVWF) protein from the heterozygous (1:1 molar ratio of WT VWF:ex4-5del) and homozygous ex4-5del transfections was decreased by 86% (P < 0.0001) and 98% (P < 0.0001), respectively, relative to the secretion of the WT rVWF (N=12 each) (Figure 3.12A). In contrast to what was observed in the ex4-5del BOEC, the decrease in rVWF (heterozygous and homozygous mutant) in the media did not correspond to an increase in intracellular retention; VWF in the cell lysates of the heterozygous and homozygous ex4-5del transfections was decreased by 13% (P = 0.0378) and 18% (P = 0.0161), respectively, compared to WT (Figure 3.12B).

The multimer analysis of the ex4-5del transient transfections is shown in Figure 3.12C. Multimer analysis showed a full range of multimers in the media from the heterozygous transfections. Only dimers were observed in the media of the recombinant homozygous mutant protein, indicating that this mutation interferes with the multimerization process. No multimers were visible in the cell lysates (not shown), which has been observed in other studies of heterologous cellular systems.155,201

HEK293 cells were transfected with WT VWF, ex4-5del, or a 1:1 molar ratio of both to mimic the heterozygous state, stained for VWF and observed using confocal microscopy. Cells transfected with WT VWF were able to form pseudo-WPB (Figure 3.13A). The homozygous ex4-5del transfected cells showed a loss of formation of pseudo-WPB, with only diffuse VWF staining (Figure 3.13C). Co-transfection of the WT and ex4-5del plasmids showed a limited number of pseudo-WPB and diffuse VWF staining (Figure 3.13B).
Figure 3.12 VWF expression from transient transfections of ex4-5del.

Panel A shows the VWF:Ag secreted into the media from the heterozygous and homozygous ex4-5del transfections, relative to wild-type (WT; set at 100%). Panel B shows the VWF:Ag for the cell lysates of the heterozygous and homozygous ex4-5del transfections, relative to WT (set at 100%). *** $P < 0.0001$, *$P < 0.05$, NS = not significant. Panel C shows the multimer analysis for the media of the heterozygous and homozygous ex4-5del transfections compared to WT. N=12.
Figure 3.13 Confocal immunofluorescence microscopy of ex4-5del transient transfections.

Wild-type (WT) VWF, ex4-5del, or 1:1 molar ratio of the two to simulate the heterozygous state, were expressed in HEK293 cells, immunostained, and examined by confocal microscopy. WT VWF was able to form pseudo-WPB (A). The heterozygously expressed cells showed a few pseudo-WPB and diffuse VWF staining (B). The homozygously expressed cells showed only diffuse VWF staining (C). Green = VWF.
The regulated secretion of VWF from the transfected cells was assessed using the secretagogues PMA and calcium ionophore A23187. Stimulation with PMA resulted in a minimal increase of 1.1-fold in VWF secretion in the heterozygous ex4-5del transfections, this is in comparison to a 1.2-fold increase in WT (N=9) (Figure 3.14A). No increase in VWF secretion was observed in the homozygous ex4-5del. Similar results were observed when calcium ionophore was used as a secretagogue, with less variability in the results than with PMA. The heterozygous ex4-5del transfection secretion increased by 1.3-fold, compared to WT which increased only by 1.1-fold. Once again though the total secretion of the ex4-5del heterozygous transfections after stimulation was still much lower than WT (N=6 each) (Figure 3.14B). The homozygous ex4-5del showed a 2.5-fold increase in VWF secretion upon stimulation but total VWF secretion was still negligible.

Due to the lack of increased intracellular retention observed in the initial heterologous cell system expression studies, we sought to investigate if proteasomal degradation was occurring. Degradation of the recombinant ex4-5del by a proteasome was evaluated using the proteasomal inhibitor MG-132 and data is shown in Figure 3.15 (N=9 for each). In these experiments, untreated heterozygous and homozygous ex4-5del transfected cells had VWF levels in the lysates similar to WT. WT expressing cells had a minimal decrease in secretion and increase in intracellular retention in response to the proteasomal inhibitor, similar to other studies. The heterozygous ex4-5del VWF levels increased in the cell lysates with proteasomal inhibition but this was not statistically significant (P=0.0556). There was no increase in the VWF levels in the lysates of the homozygous ex4-5del transfected cells.
Transient transfection of HEK293 cells with wild-type (WT) VWF, heterozygous ex4-5del (1:1 molar ratio of WT:ex4-5del), and homozygous ex4-5del were stimulated with or without 100nM PMA (A) or calcium ionophore A23187 (B) and the response in VWF secretion measured. Each bar represents VWF secreted into the media as a fraction of total VWF (media + lysate) X 100, with (grey bars) or without (black bars) stimulation. N=9 (A) and N=6 (B).
Figure 3.15 Proteasomal inhibition of ex4-5del transfected cells.

WT VWF, heterozygous and homozygous ex4-5del were expressed in the absence or presence of 10μM of the proteasomal inhibitor MG-132 for one hour. Expression was measured by VWF:Ag ELISA and is shown relative to WT expression (set at 100%) in the media (grey bars) and lysates (black bars). N=9 for each. VWF levels in the cell lysates of the heterozygous ex4-5del transfections in HEK293 cells increased with proteasomal inhibition but this increase was not significant (P=0.0556).
3.4.6 Heterologous cellular expression of Cys633Arg

VWF:Ag levels were assayed by ELISA in the media and cell lysates from the transfected HEK293T cells. VWF secretion of the rVWF protein from the heterozygous (1:1 molar ratio of WT:Cys633Arg) and homozygous Cys633Arg transfections was decreased by 76% (P <0.0001) and 98% (P <0.0001), respectively, relative to the secretion of WT (N=15 for each) (Figure 3.16A). While there was an increase in intracellular retention in the heterozygous and homozygous Cys633Arg transfections compared to WT, these increases were not significant (P = 0.0702 and P = 0.3615, respectively) (Figure 3.16B). Multimer analysis of the Cys633Arg transient transfections is shown in Figure 3.16C. Multimer analysis showed a full range of multimers in the media from the heterozygous transfections. Only dimers were observed in the media of the recombinant homozygous mutant protein, indicating that this mutation interferes with the multimerization process. As with the ex4-5del, no multimers were visible in the cell lysates (not shown).

HEK293 cells were transfected with WT VWF, Cys633Arg, or a 1:1 molar ratio of both to simulate the heterozygous state, stained for VWF and observed using confocal microscopy. Cells transfected with WT VWF were able to form pseudo-WPB (Figure 3.17A). The Cys633Arg transfected cells showed a loss of formation of pseudo-WPB, with diffuse VWF staining (Figure 3.17 C). Co-transfection of the WT and Cys633Arg plasmids showed an intermediate of the two with a limited number of smaller pseudo-WPB and diffuse VWF staining (Figure 3.17B).
Figure 3.16 VWF expression from transient transfections of Cys633Arg.

Panel A shows the VWF:Ag secreted into the media from the heterozygous and homozygous Cys633Arg transfections, relative to wild-type (WT; set at 100%). Panel B shows the VWF:Ag for the cell lysates of the heterozygous and homozygous Cys633Arg transfections, relative to WT (set at 100%). *** P < 0.0001, NS = not significant. N=12 each. Panel C shows the multimer analysis for the media of the heterozygous and homozygous Cys633Arg transfections compared to WT.
Wild-type (WT) VWF, Cys633Arg, or 1:1 molar ratio of the two to simulate the heterozygous state, were expressed in HEK293 cells, immunostained, and examined by confocal microscopy. WT VWF was able to form pseudo-WPB (A). The heterozygously expressed cells showed only one or two pseudo-WPB per cell and diffuse VWF staining (B). The homozygously expressed cells showed predominantly diffuse VWF staining (C). Green = VWF.

Figure 3.17 Confocal immunofluorescence microscopy of Cys633Arg transient transfections.
The regulated secretion of VWF from the Cys633Arg transfected cells was measured using the secretagogues PMA and calcium ionophore A23187. Stimulation with PMA resulted in a minimal increase of 1.2-fold in VWF secretion in the heterozygous Cys633Arg transfections, this is in comparison to a 1.2-fold increase in WT (N=9) (Figure 3.18A). The homozygous Cys633Arg showed a 1.4-fold increase in VWF secretion upon stimulation but total VWF secretion was still negligible. When calcium ionophore was used as a secretagogue, similar results, with less variability were observed. The secretion of the heterozygous Cys633Arg transfections increased by 1.3-fold, compared to WT which increased by 1.2-fold. Once again though the total secretion of the Cys633Arg heterozygous transfections after stimulation was still much lower than WT (N=9 each) (Figure 3.18B). No increase in VWF secretion was observed in the homozygous Cys633Arg.

Although there was no increased intracellular retention we still investigated the proteasomal degradation in the Cys633Arg transfected cells. Degradation of the recombinant Cys633Arg by the proteasome was evaluated using the proteasomal inhibitor MG-132 for one hour (Figure 3.19). As observed with the ex4-5del experiments, WT expressing cells had a minimal decrease in secretion and an increase in intracellular retention in response to the proteasomal inhibitor. The heterozygous Cys633Arg VWF levels did not increase in the cell lysates with proteasomal inhibition, however there was a significant increase in the VWF levels in the lysates of the homozygous Cys633Arg transfected cells (P=0.0328).
Figure 3.18 Stimulated release of VWF from transient transfections of Cys633Arg.

Transient transfection of HEK293 cells with wild-type (WT) VWF, heterozygous Cys633Arg (1:1 molar ratio of WT:Cys633Arg), and homozygous Cys633Arg were stimulated with or without 100nM PMA (A) or calcium ionophore A23187 (B) and the response in VWF secretion measured. Each bar represents VWF secreted into the media as a fraction of total VWF (media + lysate) X 100, with (grey bars) or without (black bars) stimulation. N=9 each.
Figure 3.19 Proteasomal inhibition of Cys633Arg transfected cells

WT VWF, heterozygous and homozygous Cys633Arg were expressed in the absence or presence of 10μM of the proteasomal inhibitor MG-132 for one hour. Expression was measured by VWF:Ag ELISA and is shown relative to WT expression (set at 100%) in the media (grey bars) and lysates (black bars). N=9 for each. A significant increase in the VWF levels in the cell lysates of the homozygous Cys633Arg transfected cells (P=0.0328) was observed.
3.4.7 Co-transfections with VWF propeptide (VWFpp)

To determine if the VWFpp in trans would be sufficient to restore normal secretion and multimerization, the VWFpp plasmid was co-transfected with each of the two mutations (1:1 molar ratio) of interest. Co-transfections of the VWFpp and ex4-5del after 72 hours showed no difference in the VWF:Ag in the media (Figure 3.20A) or lysates (Figure 3.20B) compared to that observed with the mutation alone (P < 0.0001 and P <0.001, respectively; N=12). Similar results were observed with the co-transfections of VWFpp and the Cys633Arg mutation (P < 0.0001 and P <0.001, respectively; N=12 for each) (Figure 3.20 C and D). Multimer analysis revealed that the VWFpp transfected in trans with either mutation was not sufficient to restore multimerization (Figure 3.20E).

3.5 Discussion

The present study aimed to elucidate the molecular mechanism of two VWFpp mutations and investigate their contribution to type 3 VWD. To accomplish this, patient-derived BOEC were isolated from type 3 VWD ICs and family members with the ex4-5del and Cys633Arg mutations. All isolated cells were confirmed as endothelial cells because they formed confluent monolayers of cobblestone cells, and showed positivity for cell surface markers CD31, CD144 and/or CD146, and negativity for CD14 and CD45. The weak expression of CD144 in T076 and T076-S was most likely not due to monocyte contamination since these cells lacked CD14 and CD45 expression. In the literature, the presence of CD144 is often assessed using confocal IF imaging,\textsuperscript{163,164,202} while CD146\textsuperscript{202} or endothelial protein C receptor (EPCR)\textsuperscript{44} are more popular
Figure 3.20 Co-transfections of ex4-5del and Cys633Arg with the VWF propeptide.

The media and cell lysates of the co-transfections of ex4-5del and VWF propeptide (VWFpp) are shown in panel A and B. The media and cell lysates of the co-transfections of Cys633Arg and VWF PP are shown in panels C and D. *** P < 0.0001. N=12 for each. The multimer analysis of both sets of co-transfections is shown in panel E.
targets for flow cytometry. When cells were stained for CD146, positivity for this marker was confirmed. Thus, we believe these cells are endothelial cells.

The ex4-5del results in a dramatic reduction in secretion in the heterozygous state and a virtual abolishment of secretion in the homozygous state. We observed discrepancies in the cellular lysate data in the patient-derived BOEC compared to the heterologous cell system; an increase in intracellular retention of the mutant VWF was observed in the BOEC but not in the heterologous cell system. One explanation for the discrepancy may be timing, for our heterologous expression experiments we measured levels after 72 hours and for all other experiments 48 hours was the time point. Additionally, heterologous cell systems do not account for factors that are native to the endothelium; there is possible overexpression of recombinant VWF, and co-transfections in vivo to mimic the heterozygous state may not accurately do so.

Multimerization was observed in the heterozygous ex4-5del patient plasma, concentrated BOEC media, and media from heterozygous transient transfections; however no multimers were observed in the cellular lysates. This is comparable to a recently published study that investigated a number of type 1 VWD patients including a heterozygous ex4-5del patient. The authors speculated that the lack of multimerization in this patient was due to the ER trafficking defect observed with this mutation. Confocal IF imaging showed both qualitative and quantitative deficiencies in WPB formation in the heterozygous ex4-5del BOEC. A complete absence of VWF stored in WPB, was observed in the homozygote. Other proteins stored in WPB, P-selectin, IL-8 and CD63 did not co-localize with the diffuse VWF and we speculate these proteins are targeted to lysosomes. The diffuse VWF co-localized with the ER marker calnexin indicating that the mutant protein is being retained within the ER and is not able to reach the Golgi for further processing. Secretagogue stimulation of the heterozygous ex4-5del BOEC showed a minimal
increase in VWF secretion, an increase which is comparable to this patient’s clinical 1-deamino-8-D-arginine vasopressin (DDAVP) response (Pre-DDAVP: VWF<0.10 IU/mL, FVIII=0.38 IU/mL; 1 hour post-DDAVP: VWF=0.30 IU/mL, FVIII=1.0 IU/mL), that has been sufficient to prevent bleeding during invasive procedures.

The Cys633Arg mutation showed some similarities to the ex4-5del mutation. Secretion was reduced in the heterozygous Cys633Arg state, but not as drastically as with the ex4-5del, and there was a virtual abolishment of secretion in the homozygous state. Increased intracellular retention was observed in the Cys633Arg BOEC lysates but not in the heterozygous transfections. Multimerization in the heterozygous Cys633Arg patient plasma, concentrated BOEC media, and media from heterozygous transient transfections was observed; however no multimers were observed in the cellular lysates. Confocal IF imaging showed both qualitative and quantitative deficiencies in WPB formation in the heterozygous Cys633Arg BOEC. Stored VWF did resemble more normal cigar-shaped WPB. A complete absence of VWF stored in WPB, was observed in the homozygous Cys633Arg. Other proteins stored in WPB, P-selectin, IL-8 and CD63, did not co-localize with the diffuse VWF and we speculate these proteins are targeted to lysosomes. The diffuse VWF co-localized with the ER marker calnexin indicating that the mutant protein is being retained within the ER and is not able to reach the Golgi. Stimulation of the heterozygous Cys633Arg BOEC resulted in a response in VWF secretion to levels comparable to normal BOEC. We do not have clinical DDAVP response information for this patient but we would speculate based on these results that she does respond to DDAVP.

Unfortunately, the western blot analyses have not been successfully optimized to date due to issues with the BOEC lysate samples, which were lysed with a potassium phosphate buffer. When samples are added to the sodium dodecyl sulfate (SDS) buffer, an insoluble precipitate
forms. Based on a recently published report,\textsuperscript{164} we wanted to show that there was a predominance of immature, propeptide containing VWF in the BOEC lysates of the ex4-5del and Cys633Arg patients which would provide further evidence that the mutant protein is being retained in the ER.

Limited experiments have been published in the literature using a proteasomal inhibitor to prevent degradation of VWF by the proteasome in certain VWF mutations.\textsuperscript{8,206} The proteasomal inhibition experiments presented here showed promising results, however they should be repeated at a higher concentration since VWF is such a large protein. We did try additional time points with unsuccessful results, especially at 24 hours, which resulted in cell death.

Consideration of the elements lost when exons 4 and 5 are deleted helps to clarify our results. The first N-linked glycosylation site (N99) is lost, which has been shown to be critical for efficient processing.\textsuperscript{8} The authors suggest that the loss of this initial glycosylation site may alter the ability of VWF to interact with the correct chaperone pathway and results in mis-folding of the newly forming peptide.\textsuperscript{8} Additionally one of the vicinal cysteines (\textsuperscript{159}Cys-Gly-Leu-Cys\textsuperscript{162}) which provide the substrate for disulfide oxidoreductase activity that is required for multimerization is also lost when exons 4 and 5 are deleted.

Previously it was not well understood how missense mutations in the homozygous state could cause severe VWD. In the case of the Cys633Arg mutation, the missense change affects a cysteine, and presumably every cysteine in VWF is involved in a disulfide bond\textsuperscript{3} which all play critical roles in the biosynthesis of VWF by correctly folding the protein. Thus the loss of the cysteine at position 633 would disrupt a disulfide bond and result in the improper folding of the protein.
Misfolded proteins are either retained within the ER and accumulate or are subject to degradation by the ER-associated protein degradation pathway. For both mutations, it appears as though the ER quality control mechanism prevents the mutant VWF protein from exiting the ER. As a result, the mutant VWF is unable to reach the Golgi, where furin would normally cleave the VWFPP, thus we speculate there is a predominance of immature VWF in the cells. In our studies, the VWFPP in trans was not sufficient to restore secretion and multimerization. We hypothesize that this is because the propeptide remains attached to mature VWF and therefore sterically hinders the interaction of the VWFPP in trans.

The work presented here further highlights the importance of the VWFPP and the need for future studies on specific mutations of interest located in this region of VWF. As well, this work further highlights the utility of BOEC as a non-invasive approach for investigating VWF mutations in an ex vivo manner which more accurately recapitulates the native cellular environment.
Chapter 4

Investigation of the Pathogenic Mechanisms of the von Willebrand Factor (VWF) Mutation, c.8419_8422dupTCCC
4.1 Summary

The cysteine knot (CK) domain of von Willebrand (VWF) is involved in dimerization; multiple mutations in this region have been described. The molecular pathogenesis of a novel mutation, c.8419_8422dupTCCC (p.Pro2808Leufs*24), in the CK domain of VWF identified in Canadian type 1 and type 3 VWD patients was investigated in this study. This mutation results in the loss of a cysteine (2811) and an elongated protein including a new cysteine residue (2823). We hypothesized that these molecular consequences would result in a dimerization defect. Blood outgrowth endothelial cells (BOEC) were isolated from two type 3 VWD index cases (IC); one patient was homozygous for c.8419_8422dupTCCC and the other compound heterozygous for c.8419_8422dupTCCC and a duplication in exon 14 (c.1657dupT, p.Trp553Lysfs*97). Consistent with a type 3 VWD phenotype, VWF expression in BOEC media and lysates was dramatically reduced in both IC and multimers were absent. Confocal immunofluorescence (IF) microscopy showed similar patterns of VWF staining in both IC, with diffuse VWF and a limited number of round Weibel-Palade bodies (WPB). In vitro expression study experiments were performed using transient transfections into HEK293T cells. The multimer data observed in our heterologous expression systems did not indicate a dimerization defect. Based on our data, we conclude that the c.8419_8422dupTCCC does not lead to a drastic conformational change of C-terminal VWF and thus the effect is minimal; dimerization is not affected, and some VWF molecules are still able to be stored in WPB.
4.2 Introduction

von Willebrand Factor (VWF) is a multimeric glycoprotein that acts as a mediator of platelet adhesion at sites of vascular injury and acts as a carrier of coagulation factor VIII (FVIII) in the plasma. VWF is synthesized in endothelial cells and megakaryocytes. The 2813 amino acid pre-pro VWF is comprised of a number of homologous regions, which have recently been updated as follows: D1 assembly, D2 assembly, D’D3 assembly, A1-A3 domains, followed by six tandem VWC domains and the C-terminal cysteine knot (CTCK) domain. The CTCK domain contains 90 residues, of which eleven are cysteine residues; it is these cysteine residues that partake in the “tail-to-tail” dimerization of proVWF subunits in the endoplasmic reticulum (ER). Dimers are formed through the creation of inter-chain disulfide bonds between paired cysteine residues. Dimers are then transported to the Golgi apparatus where they are assembled into multimers through N-terminal disulfide bonds. Cysteines account for 8% of the amino acid content of VWF. All cysteine residues in the secreted protein appear to be paired in disulfide bonds, some of which are involved in intra-domain bonds while others are involved in inter-domain bonds.

Genetic alterations in the VWF gene lead to the inherited bleeding disorder von Willebrand disease (VWD). VWD is classified into three types; types 1 and 3 VWD result from quantitative reductions in plasma VWF while type 2 VWD results from qualitative defects of VWF. Type 1 VWD represents approximately 75-80% of all VWD cases and is a partial quantitative deficiency of VWF which causes mild to moderate bleeding. Type 2 VWD is characterized by functionally abnormal VWF and is further divided into four subtypes. Type 3 VWD is the most severe form of VWD, characterized by moderate to severe bleeding symptoms, resulting from a complete absence or only trace amounts of VWF.
A novel mutation in the Canadian VWD population was identified in patients with type 1 VWD\textsuperscript{123} and type 3 VWD\textsuperscript{197} from a large extended family in Eastern Canada. The mutation, which is located in the CK domain of VWF, was originally identified as an insertion, c.8418\_8419insTCCC (p.Ser2807fs), however upon further evaluation the TCCC is more likely a duplication of nucleotides 8419-8422 and the nomenclature is more appropriately c.8419\_8422dupTCCC (p.Pro2808Leufs*24). The molecular consequences of this mutation are: 1) cysteine 2811 is lost, 2) an additional sixteen amino acids after the original stop codon are added prior to the new stop codon, and 3) a new cysteine (2823) is created before the stop codon (Figure 4.1).

Figure 4.1 The sequence changes as a result of VWF c.8419\_8422dupTCCC

The termination sequence for the VWF c.8419\_8422dupTCCC (p.Pro2808Leufs*24) mutation. Both nucleotide and amino acids are shown. Duplication of the four nucleotides and the new amino acids, including the new stop codon, are shown in red.
Thus, the c.8419_8422dupTCCC mutation creates an elongated protein, ultimately resulting in two unpaired cysteine residues, which we hypothesize affects dimerization. Therefore we sought to investigate this mutation further using patient-derived blood outgrowth endothelial cells (BOEC) and a traditional heterologous cell system.

4.3 Patients, materials and methods

4.3.1 Patients

To date, thirty-five individuals from the Canadian VWD population have been identified as having this mutation (19 type 3 VWD and 16 type 1 VWD). Eleven individuals are homozygous, 8 are compound heterozygous for this and a second mutation, and sixteen are heterozygous. For the present study we have further investigated two families that descend from common ancestors of the larger family. Each family has a type 3 VWD IC with the c.8419_8422dupTCCC and at least one other immediate family member with the mutation. The pedigree and laboratory data for family #1 is shown in Figure 4.2A. The IC, T154, is compound heterozygous for c.8419_8422dupTCCC and c.1657dupT (p.Trp553Lysfs*97) in exon 14. The pedigree and laboratory data for family #2 is shown in Figure 4.2B. The IC, T105 is homozygous for c.8419_8422dupTCCC.

4.3.2 BOEC isolation

To isolate BOEC from patients harboring the c.8419_8422dupTCCC, peripheral blood samples were collected by phlebotomy according to published methods, with modifications.198
Figure 4.2 Pedigree and laboratory data for the two families with c.8419_8422dupTCCC.

Pedigrees for family #1 (A) and #2 (B) with the c.8419_8422dupTCCC mutation. Type 3 VWD are represented by black symbols; heterozygous patients are represented by half-filled symbols; unaffected or VWD status unknown individuals are open symbols; squares are males; and circles are females. * indicates individuals further investigated in this study.
Approximately 48 mL of venous blood was collected into Cell Preparation Tubes (CPT™) (BD Biosciences) and mononuclear cells (MNC) isolated through a series of washing and centrifugation steps. MNCs were then resuspended in Endothelial Cell Growth Media-2 (EGM-2) BulletKit™ (Lonza) supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 1% penicillin (10,000 U/mL)/ streptomycin (10,000 μg/mL)/ amphotericin (25 μg/mL) (Invitrogen) (complete EGM-2 media). Cells were seeded to 6-well tissue culture plates pre-coated with rat tail collagen type I. For the first week, on a daily basis, adherent cells were washed once with cEGM-2 medium and then refreshed with cEGM-2 medium. From day 8 onward, media was refreshed every other day. BOEC were used at passages 3 to 9 in all experiments unless otherwise stated.

4.3.3 Confirmation of endothelial cell phenotype

The endothelial cell phenotype of isolated cells was confirmed by: 1) the formation of confluent monolayers of cells with characteristic endothelial cobblestone morphology and 2) staining of cell surface markers followed by flow cytometry. We used primary mouse monoclonal antibodies (all from Ebioscience) against human CD31 conjugated to fluorescein isothiocyanate (FITC), human CD144 conjugated to phycoerythrin (PE), human CD14 conjugated to peridinin chlorophyll protein complex (PerCp)-cyanine (Cy) 5.5, and human CD45 conjugated to phycoerythrin-cyanine 5 (Pe-Cy). Human umbilical vein endothelial cells (HUVEC) and human acute monocytic leukemia cells (THP1) were used as controls, as well as unlabeled cells and cells with isotype control antibodies. Samples were analyzed by flow cytometry using an Epics Altra HSS flow cytometer (Queen’s Cytometry and Imaging Facility, The Cancer Research Institute at Queen’s University).
4.3.4 VWF expression in patient-derived BOEC

To quantify VWF expression in the media and lysates from the patient-derived BOEC a VWF:Ag enzyme-linked immunosorbent assay (ELISA) using polyclonal antibodies (A0082 and P0226, DAKO) was performed. Three independent sets of experiments, with three replicates each, were performed. VWF secreted into serum-free media (OptiMEM® supplemented with 100mg/L CaCl₂) for up to 72 hours was concentrated using the Ultra-70 100K MWCO centrifugal filter devices (Millipore) and the multimers were analyzed by sodium dodecyl sulfate (SDS)-agarose gel electrophoresis.199

4.3.5 Confocal immunofluorescence microscopy of BOEC

To visualize intracellular VWF, confocal immunofluorescence (IF) microscopy was performed. BOEC from the two type 3 VWD patients and a normal healthy control were seeded onto collagen coated glass coverslips at a density of 500,000 cells. Confluent cells were fixed, permeabilized and stained with polyclonal rabbit anti-human VWF (A0082, DAKO), and anti-rabbit immunoglobulin-FITC (F0054, DAKO) to visualize VWF. The negative control rabbit immunoglobulin fraction (X0936, DAKO) was used as an isotype control. To observe the degree of co-localization of VWF with P-selectin, a protein also stored in WPB, staining for P-selectin (sc-6941, Santa Cruz) was performed. Additionally, BOEC were stained with the ER marker calnexin (sc-6465, Santa Cruz) to observe the localization of the mutant VWF. Donkey anti-goat rhodamine (sc-2094, Santa Cruz) was used as the secondary antibody and normal goat IgG (sc-2028, Santa Cruz) as a control for the additional IF experiments. Coverslips were mounted onto slides and analyzed using a Leica TCS SP2 Multi Photon confocal microscope (Queen’s
Cytometry and Imaging Facility, The Cancer Research Institute at Queen’s University, Kingston, Canada). Image analysis was performed using MetaMorph® Image Analysis Software and/or Image J.

4.3.6 Regulated secretion of VWF from BOEC

BOEC from the type 3 VWD patients or a healthy control were seeded onto collagen-coated plates at a density of 1x10^6 cells/well. Twenty-four hours post-seeding, the media was replaced with serum-free media (OptiMEM® supplemented with 100mg/L CaCl2) and BOEC were incubated for an additional 24 hours. BOEC were stimulated with 100nM phorbol 12-myristate 13-acetate (PMA, Sigma), or dimethylsulfoxide (DMSO) control, for 75 minutes at 37°C and conditioned media and cell lysates were then collected. Three independent sets of experiments, with three replicates each, were performed. Results of secreted VWF before and after stimulation are reported as the percentage of secreted VWF, expressed as a fraction of the total amount of VWF (i.e. % VWF secretion = VWF in the media/[VWF in the media + VWF in the cell lysate] X 100).

4.3.7 Plasmid construct

To create the expression vector pCIneoVWFdupTCCC, site-directed mutagenesis (Quick Change® II XL Site-Directed Mutagenesis kit; Stratagene) was performed on the full-length human VWF expression vector, pCIneohuVWFES as per the manufacturer’s protocol. The resultant expression plasmid was digested to confirm the integrity of the plasmid and sequenced to confirm the presence of the mutation. Plasmid DNA was purified for transfection.
4.3.8 Cell culture and calcium phosphate transfections

Human Embryonic Kidney (HEK) 293T cells were transiently transfected with wild-type (WT; pClneohuVWF), heterozygous (1:1 molar ratio; WT: pClneoVWFdupTCCC) and homozygous pClneoVWFdupTCCC plasmids using the calcium phosphate method. Cells were initially grown in Dulbecco modified Eagle medium (DMEM) containing 2mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10% (vol/vol) FBS, and 0.25 ug/mL fungizone at 37% in 5% CO₂. Approximately 16 hours after transfection, the media was replaced with serum-free media (OptiMEM® containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 1X Insulin-Transferrin-Selenium (ITS)). Seventy-two hours post-transfection, media was harvested and cells were lysed. The Galacto Light Plus reporter gene assay (Tropix) was used to measure transfection efficiency. The VWF:Ag ELISA was used to quantify the VWF present in the media and cell lysates. Three independent sets of experiments, with five replicates each, were performed. Recombinant VWF (rVWF) secreted into the media was concentrated using Ultra-70 100K MWCO centrifugal filter devices (Millipore) and multimers analyzed by SDS-agarose gel electrophoresis.

4.3.9 Transient transfection confocal immunofluorescence microscopy

HEK293 cells, as opposed to HEK293T cells, are known to form pseudo-WPB that are similar to the WBP in endothelial cells. HEK293 cells were cultured in Minimum Essential Medium (MEM) containing Earle’s salts and 2mM L-glutamine, and supplemented with 10% (vol/vol) FBS. HEK293 cells were seeded onto poly-L lysine coated glass coverslips in 6-well plates at a density of 350,000 cells/well. Transient transfections of WT (pClneoohuVWF), heterozygous (1:1 molar ratio; WT:c.8419_8422dupTCCC) and homozygous
c.8419_8422dupTCCC plasmids using Lipofectamine® (Invitrogen) were performed according to the manufacture’s protocol. Forty-eight hours post-transfection cells were fixed, permeabilized and stained for VWF as previously described in section 4.3.5. Coverslips were mounted onto slides and analyzed using a Leica TCS SP2 Multi Photon confocal microscope. Images were acquired and analyzed using MetaMorph® Image Analysis Software and Image J.

4.3.10 Regulated secretion of VWF

HEK293 cells were transiently transfected with WT VWF, c.8419_8422dupTCCC or a 1:1 molar ratio of the two using Lipofectamine® and then incubated for 48 hours. Cells were then stimulated with 100nM PMA (Sigma), 10μM calcium ionophore A23187 (Sigma), or DMSO control, for 75 minutes at 37°C. Conditioned media and cell lysates were then collected. Three independent sets of experiments, with three replicates each, were performed. VWF secretion in the media, before and after stimulation, was expressed as a fraction of total VWF (amount in the media + lysate).

4.3.11 Graphing and statistical analysis

Data and statistical analyses were performed with GraphPad Prism for Windows (version 4). Statistical analyses were performed using the Kruskal-Wallis non-parametric tests; P < 0.05 was considered statistically significant.
4.4 Results

4.4.1 Patients

The two families investigated provide further information on the phenotype of this mutation. Variable expressivity is observed between heterozygotes carrying the c.8419_8422dupTCCC, as demonstrated by the variability in VWF levels of T106, T107, and T111 (Figure 4.2A). As well, we have observed two cases of incomplete penetrance thus far (T171 and T172) who are heterozygous for the mutation but have normal VWF levels and a normal bleeding score (T171) (Figure 4.2 A and B).

4.4.2 Patient-derived BOEC

BOEC were successfully isolated from two type 3 VWD individuals; T154 who is compound heterozygous for c.8419_8422dupTCCC and c.1657dupT (p.Trp553Leufs*97) in exon 14, and T105 who is homozygous for the c.8419_8422dupTCCC. BOEC from these patients appeared on days 9 (T105) and 15 (T154). We were unable to isolate BOEC from T107, the sister of T105, who is heterozygous for c.8419_8422dupTCCC. The first isolation attempt resulted in cells that appeared elongated, with fusiform morphology; after a couple of passages the cells became scattered and took on a more stellate morphology consistent with fibroblast or fibroblast-like cells. The second attempt to isolate BOEC from T107 was completely unsuccessful.
4.4.3 Confirmation of endothelial cell phenotype

Isolated cells from T154 and T105 were able to form confluent monolayers of cobblestone cells and were positive for cell surface markers CD31 and CD144 and negative for CD14 and CD45. Table 4.1 shows the results of the flow cytometry for the cell surface markers.

**Table 4.1** Endothelial markers on patient-derived BOEC.

<table>
<thead>
<tr>
<th>Patient</th>
<th>CD31 (%)</th>
<th>CD144 (%)</th>
<th>CD14 (%)</th>
<th>CD45 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T154</td>
<td>93</td>
<td>79</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>T105</td>
<td>95</td>
<td>75</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Data are given as percentage positive cells of live cells, corrected for the corresponding IgG control.

4.4.4 VWF expression of patient-derived BOEC

To determine the VWF expression in the media and cell lysates of the patient-derived c.8419_8422dupTCCC BOEC, VWF:Ag was measured by ELISA. Secretion of VWF from the compound heterozygous and homozygous c.8419_8422dupTCCC BOEC was decreased by 98% (P < 0.0001) and 96% (P < 0.0001), respectively, relative to the secretion of VWF from BOEC of a healthy control (N = 9 each) (Figure 4.3A). The significant decrease in secretion was not associated with a concomitant increase in intracellular retention. A dramatic decrease in the cellular lysates of the compound heterozygote and homozygote by 93% (P < 0.0001) and 87% (P < 0.0001), respectively, relative to the VWF in the BOEC lysates of a healthy control (Figure 4.3B) was observed.
Multimer analysis is shown in Figure 4.3C. A complete lack of multimers was observed in the plasma, media and lysates of the compound heterozygous and homozygous c.8419_8422dupTCCC BOEC.

4.4.5 Confocal immunofluorescence (IF) microscopy of BOEC

To examine intracellular VWF in the type 3 VWD patients with the c.8419_8422dupTCCC, confocal IF microscopy was performed. Both the compound heterozygote (Figure 4.4B) and the homozygote (Figure 4.4C) showed similar staining patterns; diffuse VWF staining and a limited number of rounded WPB compared to normal healthy BOEC (Figure 4.4A). Normal BOEC and those from the compound heterozygous patient were additionally stained for P-selectin. The normal BOEC showed co-localization of VWF with P-selectin, while the compound heterozygous BOEC showed only partial co-localization (Figure 4.5). To observe if the diffuse VWF in the compound heterozygote BOEC was retained within the ER, the ER marker calnexin was examined (Figure 4.6). The diffuse VWF of the compound heterozygote BOEC co-localized with calnexin, indicating ER retention of the mutant protein.
Figure 4.3 VWF expression in the BOEC media and lysates.

VWF expression (VWF:Ag) in the BOEC media (A) and lysates (B) measured by ELISA. The VWF:Ag in the media from the compound heterozygote and homozygote was reduced by 98% and 96%, respectively, and in the lysates reduced by 93% and 87%, respectively, relative to normal BOEC (set at 100%). *** P < 0.0001, **P < 0.001. N=9 for each. (C) shows the multimer analysis for the BOEC media and lysates, and plasma of the compound heterozygote and homozygous c.8419_8422dupTCCC patients compared to normal.
**Figure 4.4** Confocal immunofluorescence microscopy of BOEC from type 3 VWD patients with c.8419_8422dupTCCC.

BOEC from a normal healthy individual, the c.8419_8422dupTCCC compound heterozygote and homozygote were stained for VWF. The VWF in normal healthy BOEC is stored in cigar-shaped WPB (A). Both the compound heterozygote (B) and the homozygote (C) show similar staining patterns with diffuse VWF and VWF in rounded WPB. Green = VWF, Red = actin cytoskeleton, Blue = nucleus.
Figure 4.5 Partial co-localization of VWF and P-Selectin in compound heterozygous c.8419_8422dupTCCC.

Normal BOEC and BOEC from the c.8419_8422dupTCCC compound heterozygote were stained for VWF and the WPB membrane protein P-selectin. In normal BOEC, VWF and P-selectin co-localize together in WPB (merge). In the compound heterozygous c.8419_8422dupTCCC, there is partial co-localization of VWF and P-selectin; areas of co-localization are indicated by white arrows. Green = VWF, Red = P-selectin, Yellow= merge.
Figure 4.6 ER retention of VWF c.8419_8422dupTCCC.

Normal BOEC and BOEC from the c.8419_8422dupTCCC compound heterozygote were stained for VWF and the ER marker calnexin. In the normal BOEC, there is minimal ER staining which does not co-localize with the VWF stored in WPB. In the compound heterozygous c.8419_8422dupTCCC BOEC, there is co-localization of the diffuse VWF and calnexin, indicating ER retention of the mutant protein (merge). Green = VWF, Red = calnexin, Yellow= merge.
4.4.6 Regulated VWF secretion

BOEC were stimulated with the secretagogue PMA to observe the degree of regulated release of VWF. Neither the compound heterozygous nor the homozygous c.8419_8422dupTCCC BOEC showed an increase in % VWF secretion when stimulated with PMA, compared to normal BOEC which had a 3.1-fold increase upon stimulation (Figure 4.7) (N=9 for each).

4.4.7 Transient transfection VWF expression

To determine the VWF expression of the mutant recombinant c.8419_8422dupTCCC protein, VWF:Ag levels were assayed in the media and lysates of transfected HEK293T cells by ELISA. Secretion of the rVWF protein from the heterozygous (1:1 molar ratio of WT:mutant) and homozygous c.8419_8422dupTCCC transfections was reduced by 58% (P < 0.0001) and 98% (P < 0.0001), respectively, relative to the secretion of the wild-type (WT) rVWF (N=15 each) (Figure 4.8A). Similarly to what was observed in the patient BOEC, the decrease in rVWF (heterozygous and homozygous mutant) in the media did not correspond to an increase in intracellular retention; VWF in the cell lysates of the heterozygous and homozygous c.8419_8422dupTCCC transfections was decreased by 21% (P = 0.0053) and 48% (P < 0.0001), respectively, compared to WT (N=15 each) (Figure 4.8B). This decrease in the cell lysates was not as dramatic as that seen in the patient BOEC.

Multimer analysis showed a full range of multimers in the media from the heterozygous c.8419_8418dupTCCC transfections (Figure 4.8C). Only dimers were observed in the media of the recombinant homozygous mutant protein. No multimers were visible in the cell lysates (not shown), which has been observed in other studies of heterologous cellular systems.155,201
Figure 4.7 Stimulated release of VWF from patient-derived BOEC.

BOEC from a normal healthy individual, the c.8419_8422dupTCCC compound heterozygote and homozygote were stimulated with 100 nM PMA for 75 min and the response in VWF secretion measured. Each bar represents VWF secreted into the media as a fraction of total VWF (media + lysate) X 100 with (grey bars) or without (black bars) stimulation. N=9 for each.
HEK293T cells were transfected with either wild-type (WT) VWF, c.8419_8422dupTCCC, or 1:1 molar ratio of WT:c.8419_8422dupTCCC. The VWF:Ag in the media (A) and lysates (B) from the heterozygous and homozygous c.8419_8422dupTCCC transfections, relative to WT (set at 100%) is shown. *** P < 0.0001, **P < 0.001, *P < 0.05 (N=15). Panel C shows the multimer analysis for the media of the heterozygous and homozygous c.8419_8422dupTCCC transfections compared to WT from two independent experiments.
4.4.8 Transient transfection confocal immunofluorescence microscopy

HEK293 cells were transfected with WT VWF, c.8419_8422dupTCCC, or a 1:1 molar ratio of both to simulate the heterozygous state, stained for VWF and observed using confocal microscopy. Cells transfected with WT VWF were able to form pseudo-WPB (Figure 4.9A). The c.8419_8422dupTCCC transfected cells showed a loss of formation of pseudo-WPB, with diffuse VWF staining (Figure 4.9C). Co-transfection of the WT and c.8419_8422dupTCCC plasmids showed an intermediate of the two with a limited number of smaller pseudo-WPB and diffuse VWF staining (Figure 4.9B).

4.4.9 Stimulated secretion of VWF from pseudo-WPB

The increase in VWF secretion upon stimulation with PMA and calcium ionophore A23187 was measured in the HEK293 cells transfected with WT VWF, heterozygous (1:1 molar ration of WT:mutant) and homozygous c.8419_8422dupTCCC. A minimal increase in VWF secretion upon stimulation with PMA was observed in the heterozygous (1.1-fold increase) and homozygous (1.6-fold increase) transfections, however, for both total secretion after stimulation was still much lower compared to WT (N=9 each) (Figure 4.10A). Stimulation with calcium ionophore produced similar results with a 1.2-fold increase in both the heterozygous and homozygous transfections (N=9 each) (Figure 4.10B).
Figure 4.9 Confocal immunofluorescence microscopy images from transient transfections of c.8419_8422dupTCCC.

Wild-type (WT) VWF, c.8419_8422dupTCCC, or 1:1 molar ratio of the two to simulate the heterozygous state, were expressed in HEK293 cells, immunostained, and examined by confocal microscopy. WT VWF was able to form pseudo-WPB (A). The heterozygously expressed cells showed fewer pseudo-WPB and diffuse VWF staining (B). The homozygously expressed cells showed predominantly diffuse VWF staining (C). Green = VWF.
Figure 4.10 Stimulated release of VWF from transient transfections of c.8419_8422dupTCCC.

Transient transfection of HEK293 cells with wild-type (WT) VWF, c.8419_8422dupTCCC, and a 1:1 molar ratio of WT:c.8419_8422dupTCCC to simulate the heterozygous state, were stimulated with PMA (A) and calcium ionophore A23187 (B) and the response in VWF secretion measured. Each bar represents VWF secreted into the media as a fraction of total VWF (media + lysate) X 100, with (grey bars) or without (black bars) stimulation. N=9 for each.
4.5 Discussion

VWF is cysteine rich and each of these cysteines is involved in disulfide bonds. The CK domain of VWF contains eleven of these cysteines and is an area of VWF that is known to be critical for dimerization. In this study, we investigated the molecular pathogenesis of the novel mutation, c.8419_8422dupTCCC, identified in the Canadian VWD population using patient-derived BOEC and a traditional heterologous cell system. This mutation is located in the CK domain and not only abolishes a cysteine (at position 2811), but also results in an elongated protein including the addition of a new cysteine (2823). We hypothesized that the consequence of this mutation would be an effect on dimerization.

Our multimer data, however, did not show evidence of a dimerization defect. Mutations affecting dimerization often show a predominance of monomers, with an odd number of multimeric bands and this was not observed in our studies. Katsumi et al. (2000) proposed that Cys2771, Cys2773, and Cys2811 are the only cysteine residues able to form the inter-subunit bonds between VWF monomers in the CK domain. The substitution of Cys2771 or Cys2773 abolished dimerization, whereas the substitution of Cys2811 did not completely prevent dimerization, data in line with ours. The authors also noted that a small (~22kDa) intracellular fragment observed in the mutated Cys2811 might have been a faster migrating species reflective of proteolytic degradation. Wang et al. (2011) proposed that VWF mutations that disrupt intra-chain disulfide bond formation are unable to get past the ER quality control and are retained and either aggregate or are intracellularly degraded; however, some of the VWF molecules are able to escape and can be stored in WPB. The authors suggest that if the ultimate conformational changes to VWF arising from a given mutation in the CK domain are minimal then the observed effects will not be as dramatic. It is possible that C2811 and the new cysteine that is created as
part of the elongated protein, resulting from the c.8419_8422dupTCCC mutation, do not lie in a position that is critical for the conformation and folding of the C-terminal end of VWF and thus do not affect dimerization.

The confocal IF images obtained in our study shows the formation of some WPB in the type 3 VWD patients, which supports the above intra-disulfide bond model. The confocal images also show diffuse ER-like VWF; however, the VWF:Ag measured in the lysates of the BOEC are dramatically reduced perhaps indicating intracellular degradation of the mutant protein in the ER. Future experiments using a proteasomal inhibitor could answer this.

The stimulated secretion experiments showed very little release of VWF upon stimulation with either secretagogue. These experiments on type 3 VWD patients’ BOEC are not ideal as these cells have virtually no endogenous VWF and thus the effect, if any, upon stimulation is small. Unfortunately we were unable to isolate BOEC from a heterozygous c.8419_8422dupTCCC patient. Isolating BOEC and conducting future studies on a heterozygous patient is warranted. We know from communication with the physician of these patients that individuals heterozygous for this mutation respond clinically to 1-deamino-8-D-arginine vasopressin (DDAVP) so we can speculate that their BOEC would respond to stimulated secretion by secretagogues.

While not discussed within this study, this mutation also poses a unique phenotypic characteristic with respect to platelet VWF. Typical type 3 VWD patients have very low or no VWF in their platelets and plasma, however type 3 VWD individuals with this mutation have been found to have relatively more (~10 fold) platelet VWF than plasma VWF. Evaluation of patient megakaryocytes, platelet α- granule storage and release will also be investigated in these individuals (Kahr lab, Hospital for Sick Children, Toronto, Ontario).
Thus, this novel mutation in the CK domain of VWF appears to have little or no effect on
dimerization and still results in minimal storage of VWF even in type 3 VWD patients.
Additional studies are required which include investigations of heterozygous
c.8419_8422dupTCCC patient BOEC and potentially modeling studies which would help to
answer questions on the conformational changes, if any, to VWF associated with this mutation.
Chapter 5

Thesis Discussion

5.1 Summary

Von Willebrand disease (VWD) is a complex, heterogeneous bleeding disorder. Type 3 VWD is the rarest and most severe form of the disease. Traditionally, type 3 VWD is thought to be inherited in an autosomal recessive manner. We have shown in this thesis that in the Canadian population approximately 50% of families exhibit co-dominant inheritance, as opposed to recessive. This thesis also highlights the importance of the VWF propeptide in type 3 VWD as 42% of mutations identified were found in this region and these mutations were associated with a more severe bleeding phenotype. We investigated two of these mutations, the previously reported, recurrent exon 4 and 5 deletion and a novel Canadian missense mutation, c.1897T>C (p.Cys633Arg). Both mutations showed impaired secretion, defective multimerization, qualitative and quantitative defects in Weibel-Palade body (WPB) formation, ER retention and different responses in secretagogue stimulation. Additionally we studied a third mutation, frequent among a subset of individuals in the Canadian VWD population, which is located in the cysteine knot (CK) domain. We investigated the three mutations using two cellular models; the heterologous cellular system and a patient-derived system by isolating blood outgrowth endothelial cells (BOEC) from index cases (IC) with these mutations. BOEC are ideal for studying VWF mutations as they provide a cellular system that mimics vascular endothelial cells. This thesis contributes critical information to the study of quantitative deficiencies of VWD, including information on the underlying molecular mechanisms of the VWF mutations investigated using in vitro and ex vivo cellular models.
5.2 Genetics of Canadian type 3 von Willebrand disease (VWD)

A number of type 3 VWD populations have been characterized world-wide. Our study, chapter 2 of this thesis, characterized a Canadian cohort of type 3 VWD patients. We investigated genotype-phenotype correlations in 42% of the registered type 3 VWD patients in Canada (Canadian Hemophilia Registry, http://www.fhs.mcmaster.ca/chr/data.html, updated May 27, 2013). We also investigated affected and unaffected family members of these patients for a total study population of 100 individuals. Thus, our study to date is one of the largest and most comprehensive studies of type 3 VWD. We highlighted a few important findings within the study: 1) ~50% of the families exhibited co-dominance of mutant alleles as opposed to recessive inheritance; 2) 42% of the mutations identified in the Canadian VWD population were located in the VWF propeptide (VWFpp) region; 3) the index cases (IC) with these propeptide mutations had more severe bleeding than those IC with mutations elsewhere in VWF.

Since the publication of our paper, a group from India has published a rebuttal. This group has investigated 77 type 3 VWD IC within their population and did not observe higher bleeding scores in IC with mutations in the VWF propeptide. We acknowledged this rebuttal with a response suggesting four potential reasons why there may be discrepancies between the Indian and Canadian type 3 VWD populations studied. The first is the age of the IC in the two studies; the Indian study is comprised primarily of pediatric patients (mean age of 13 years) compared to our study in which the mean age of IC is 29 years. It is well known that evaluating hemorrhagic symptoms in children with VWD is difficult as there are age-specific patterns of bleeding and some children lack hemostatic challenges. In general adults have higher bleeding scores than children due to exposure to hemostatic challenges. Additionally it has been shown that bleeding scores are age dependent, particularly in type 3 VWD. Thus it seems reasonable that there
might be further discrimination in bleeding severity between the two study populations. The second potential reason for the different results is the discrepancies in the frequency of mutation type and location between the two studies. The Indian study had more non-propeptide null mutations compared with the Canadian (34/77 vs. 9/31), which may impact the bleeding severity of IC. Thirdly, the Canadian type 3 VWD population is more heterogeneous than the Indian population which likely has higher instances of consanguinity resulting in increased homozygosity for certain mutations. Finally, we also acknowledge that unrecognized genetic modifiers that differ between the Indian and Canadian populations are possible. These modifiers may have an impact on the bleeding phenotype of the patients investigated. We have re-analyzed our bleeding score data, restricting it to only those IC with both mutant alleles in the propeptide and the results showed a similar, although not statistically significant trend (P = 0.09).

5.3 Importance of von Willebrand factor propeptide (VWFpp)

The VWFpp plays a critical role in multimerization and acts as an intra-molecular chaperone in the storage of mature VWF. Our findings that 42% of mutations were located in the VWFpp and the increased bleeding severity in Canadian type 3 VWD IC with those mutations, combined with the aforementioned important roles of the VWFpp led us to further investigate two of these mutations. The ex4-5del, a previously reported in-frame deletion and the novel Canadian missense mutation, Cys633Arg were investigated. Both of these mutations were identified in type 3 VWD families that exhibited co-dominant inheritance. These mutations displayed defective VWF secretion, with a virtual abolishment of secretion in the homozygous states. Impaired multimerization was observed. Despite multimerization in the heterozygous media, no multimerization was observed in the heterozygous cell lysates. Quantitative and
qualitative defects in WPB formation were observed using confocal immunofluorescence microscopy. A complete lack of WPB was observed in the homozygous states of both mutations, with other proteins normally co-stored in WPB with VWF being presumably targeted to lysosomes. Future experiments using a lysosomal marker to confirm this are warranted. Mutant VWF co-localized with the endoplasmic reticulum marker (ER), calnexin, indicative of ER retention.

With the deletion of exons 4 and 5, there is the loss of a critical glycosylation site (N99) and a CGLC motif, essential for multimerization. The Cys633Arg results in the loss of a cysteine, which is likely involved in a disulfide bond. These molecular consequences likely result in mis-folded proteins that are retained within the ER, preventing trafficking to the Golgi where further processing, such as propeptide cleavage, would occur. We wanted to show that in the BOEC lysates of these patients, there is a predominance of immature, propeptide containing VWF. Unfortunately the lysis buffer used to lyse the cells contained potassium, which when mixed with sodium dodecyl sulfate (SDS) buffer, forms and insoluble precipitate. Thus, buffer exchange or repeat experiments with a new lysis buffer are required.

These two different VWFpp mutations, a complete in-frame deletion of two exons and a missense mutation, had similar effects on VWF biosynthesis and secretion. This further highlights the importance of VWFpp mutations in regulating VWF production and the need for additional investigations of VWFpp mutations.

Highlighting differences in VWF and FVIII levels in IC with VWFpp mutations versus IC with mutations elsewhere in VWF may not be possible because VWF and FVIII levels in these individuals are already at the low limit of detection with current assay methods. It is possible though that the differences in bleeding severity between these two groups of IC may extend
beyond VWF to other components of WPB that are affected when VWF is not stored in WPB. The contribution of other WPB components to hemostasis should also be investigated. P-selectin has been shown to play a role in hemostasis through platelet rolling\textsuperscript{214} and P-selectin deficient mice were reported to have an increase in bleeding severity with a prolongation in bleeding time by 40\%.\textsuperscript{215} Evaluation of P-selectin and other WPB components by ELISA, before and after secretagogue stimulation will be performed to help address this.

5.4 Investigations of VWF c.8419_8422dupTCCC mutation

The c.8419_8422dupTCCC is a mutation unique to the Canadian VWD population, recurrent in type 1 and type 3 VWD and is found at a high frequency in a large extended family from Eastern Canada. The mutation is located in the C-terminal cysteine knot (CTCK) domain of VWF, an area involved in dimerization.\textsuperscript{11} The molecular consequences of the c.8419_8422dupTCCC mutation are three-fold: 1) cysteine 2811 is lost, 2) a shift in the reading frame results in a new, delayed stop codon an additional sixteen amino acids after the original stop codon, and 3) a new cysteine (2823) is created before the new stop codon. Therefore, this mutation results in an elongated protein, with two unpaired cysteine residues, which we hypothesized would affect dimerization.

We investigated this mutation using the patient-derived BOEC and heterologous cellular system. BOEC were isolated from a patient homozygous for the c.8419_8422dupTCCC and a patient compound heterozygous for this mutation and a second duplication in exon 14. The multimer data we obtained did not show evidence of a dimerization defect. VWF mutations that affect dimerization show a predominance of monomers, with an odd number of multimeric
bands,\(^{210}\) which was not observed in our studies. We suggest that the c.8419_8422dupTCCC does not lead to a drastic conformational change of c-terminal VWF and therefore the effect is minimal; dimerization is not affected and some VWF molecules are still able to be stored in WPB, as shown in our confocal images.

A number of future experiments are still required to further investigate this mutation. The first would be computer modeling studies to better understand the conformational changes that occur as a result of this mutation. Secondly, isolating BOEC from a heterozygous c.8419_8422dupTCCC patient is warranted, to understand the effect of this mutation in combination with the wild-type allele. Thirdly, western blot analysis under non-reducing conditions is planned, once the issues related to the lysis buffer (as mentioned in section 5.3) are resolved.

5.5 Blood outgrowth endothelial cells (BOEC) as a cellular model for studying VWD

Traditionally in order to study the pathogenesis of VWD, \textit{in vitro} experiments by transfecting recombinant VWF into heterologous mammalian cell systems have been employed. More recently, studies using blood outgrowth endothelial cells (BOEC) isolated from peripheral blood of patients with VWD have been published.\(^{44,162-164}\) Until recently, we believed the lack of endogenous VWF in patients with type 3 VWD might pose as a challenge for isolating and culturing type 3 VWD BOEC. The work presented within this thesis, however, shows that it is possible to isolate and culture these cells from type 3 VWD patients.

BOEC may be considered as the “gold standard” for investigating the molecular pathogenesis of VWD as they provide a system, which is representative of the natural endothelial
environment. For this thesis BOEC were isolated from five type 3 VWD patients and two type 1 VWD patients to investigate three different VWF mutations. Two attempts were required to isolate BOEC from the homozygous Cys633Arg IC and we were unsuccessful in isolating BOEC from the heterozygous c.8419_8422dupTCCC patient on two separate occasions. In general, we have a success rate for BOEC establishment of 80–85%, which is comparable to other laboratories. The number of BOEC colonies obtained appears to be donor-dependent, with the establishment of BOEC from some individuals being more difficult than others. The underlying reason for the lack of BOEC establishment in some individuals is currently not understood; while some people suggest it may be related to age, others have not observed correlations with age or sex.

The establishment of patient-derived BOEC allowed us to make comparisons of VWF biosynthesis with the endothelial cellular system and the traditional heterologous cell system. There are inherent limitations with the latter including possible overexpression of recombinant VWF, the inability of the heterologous system to account for post-translational modifications, potential differences in exocytotic machinery, and potential lack of intracellular chaperones. As well, co-transfections in vivo to mimic the heterozygous or compound heterozygous states of VWF mutations may not accurately do so. While the majority of our data between the two cellular systems was in agreement, we did see some discrepancies specifically with respect to the cellular lysate data, which may be attributed to these limitations. Further studies are necessary using patient-derived BOEC as the cellular model for studying VWF mutations.
5.6 Significance, future directions and final conclusions

The work presented within this thesis has provided further evidence of the co-dominant inheritance pattern of type 3 VWD, and we have provided more insight into the mechanisms of two VWFpp mutations and their importance and contribution to type 3 VWD. As well, we have investigated a novel mutation recurrent in the Canadian type 1 and 3 VWD population. We are one of only a few groups to date to present work using patient-derived BOEC from patients with VWD, specifically patients with type 3 VWD. In total we were able to isolate BOEC from five type 3 VWD patients and two type 1 VWD patients for this thesis. This work highlights the novelty and importance of using BOEC as a cellular model for the study of VWD.

Future studies will be carried out to investigate other VWFpp mutations identified in Canadian type 3 VWD patients using both the heterologous cell system and patient-derived BOEC. BOEC, once established, may also be used for experiments studying the effects of shear, using flow-systems and can be used to test novel treatment options, which may translate to better clinical outcomes for patients. Not all type 3 patients bleed to the same extent; some require long-term prophylaxis while others do not. This thesis begins to make connections between mutation location and bleeding severity in type 3 VWD and further highlights the heterogeneity in this severe bleeding disorder. While additional studies are needed, we may one day be able to use the genetic information to predict bleeding outcomes in patients and ultimately make predictions with respect to long-term prophylaxis.

In conclusion, the work presented within this thesis will contribute critical information to the study of type 3 VWD. The importance of genetic testing in affected individuals varies between subtypes of VWD but in cases such as type 3 VWD where the causative mutation is identified this information may be used for prenatal testing and genetic counseling. The
translation of this information for diagnostic and therapeutic purposes is critical. Elucidating the pathogenic mechanisms involved in three VWF mutations that are recurrent in quantitative deficiencies of VWF will contribute greatly to body of knowledge on the biosynthesis, trafficking and storage of this complex bleeding disorder.
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Appendix A Thesis Protocols

A.1. VWF antigen (VWF:Ag) Enzyme Immunosorbent Assay (ELISA)

A.2. Anti-VWF antibody ELISA

A.3. HEK293T calcium phosphate transfections

A.4. Blood Outgrowth Endothelial Cell (BOEC) isolation

A.5. Flow cytometry staining

A.6. VWF immunofluorescent staining

A.1. VWF antigen (VWF:Ag) ELISA

Purpose: To determine VWF antigen levels in human plasma and cell culture media and lysates.

Reagents:
Coating Antibody: Rabbit Anti-Human von Willebrand Factor (DAKO A0082).
Detecting Antibody: Rabbit Anti-Human von Willebrand Factor HRP (DAKO P0226).
Human CryoCheck Reference plasma (Precision Biologics CCNR-05)
o-Phenylenediamine dihydrochloride (OPD) tablets (Sigma P6912)
ELISA Plates: 96-well microtiter Immulon 4 HBX, Dynex

The following protocol is based on the conditions DAKO has recommended. Buffers A, B, and C are stable at 4°C for 2 mth. Check for growth before using. Reagent E is stable indefinitely.

Buffer A: PBS, pH 7.2

10 mM Na$_2$HPO$_4$ 0.71 g Na$_2$HPO$_4$ or 5 mL 1M solution
145 mM NaCl 4.238 g NaCl or 14.5 mL 5M solution
500 mL with dH$_2$O, pH 7.2
Buffer B: Washing/Dilution Buffer, PBS 0.5 M NaCl, 0.1% Tween 20, pH 7.2

10 mM Na$_2$HPO$_4$ 0.71 g Na$_2$HPO$_4$ or 5 ml 1M solution
500 mM NaCl 14.61 g NaCl or 50 ml 5M solution
0.1% Tween 20 0.5 mL Tween 20 (cut off end of tip for easier pipetting)
500 mL with dH$_2$O, pH 7.2

10X Buffer B:

100 mM Na$_2$HPO$_4$ 8.52 g Na$_2$HPO$_4$
5 M NaCl 175.32 g NaCl
1% Tween 20 6 mL Tween 20 (after pHing, will precipitate if hot)
600 mL with dH$_2$O Microwave 2 min to dissolve. pH 6.15
HPO$_4$ Buffer is not temperature sensitive.

Buffer C: 0.1 M Citric Acid-Phosphate Buffer, pH 5

0.0347 M Citric Acid 3.34 g Citric Acid
0.0667M Na$_2$HPO$_4$ 4.73 g Na$_2$HPO$_4$
500 mL with dH$_2$O, pH 5.0

Reagent D: Color Reagent (for one plate)

15 ml Buffer C
2 - 5 mg tablets OPD *Dissolve before adding the peroxide
6.2 μL 30% H$_2$O$_2$

Reagent E: Stop Solution, 1 M H$_2$SO$_4$

1M H$_2$SO$_4$ 5.56 mL 95% H$_2$SO$_4$
100 mL with dH$_2$O
Method:

Day 1: Coating wells with first antibody

1. Dilute coating antibody (Rabbit Anti-Human von Willebrand Factor, DAKO A0082, 3.1 μg/μL) in Buffer A to 10μg/mL. Add 100 μL/well of plate. Apply plate sealer and store at 4ºC overnight.

Day 2: Dilutions, Binding, Detecting Antibody, and Plate Development.

2. Dilute samples with Buffer B on ice. Prepare the standard curves with reference plasma at a starting dilution of 1:20.

3. Wash the plate with 250 μL of Buffer B, let sit for 3 min, invert, and tap hard until dry. Repeat 2 times.

4. Add 100 μL of diluted samples/well. Cover plate with plate sealer. Let sit at room temperature for at least 2 hr (4ºC O/N will also work if absolutely necessary).

5. Wash plate as above 3 times.

6. Dilute detecting antibody (Rabbit Anti-Human von Willebrand Factor HRP, DAKO P0226, 1.1 μg/μL) in Buffer B ~1:8000.

7. Add 100 μL/well and recover the plate. Incubate at least 1 hour at room temperature.

8. Wash plate as above 3 times.

9. Add 100 μL of Reagent D/well. Cover the plate and incubate 12-30 min, until standard curve is apparent. This takes ~10 min.

10. Stop the reaction with 100 μL/well of Reagent E.

11. Read results at 492 nm using the plate reader. 1 min shake optional.
A.2. Anti-VWF antibody ELISA

Purpose: To determine the total anti-VWF antibody titer in human plasma.

Reagents:
Recombinant human VWF or Humate-P® or Wilate®
Phosphate Buffered Saline (PBS), pH=7.2
Anti-Human IgG peroxidase conjugate (Sigma A2290) (1:5000 dilution)
o-Phenylenediamine dihydrochloride (OPD) tablets (Sigma P6912)
ELISA Plates: 96-well microtiter Immulon 4 HBX, Dynex

Buffers:
Coating buffer (50 mM Carbonate buffer), pH=9.6 : 1L
- Begin with 800 mL dH2O in a graduated cylinder, add
  - 2.93 g NaHCO3
  - 1.59 g Na2CO3
- Fill graduated cylinder up to 1 L with dH2O
- Store at 4°C for up to 1 year, protect from light (check pH if buffer is older)

Washing buffer (0.1% Tween 20-PBS): 500mL
- 0.5 mL Tween 20 or 5 mL 10% Tween 20
- Fill beaker to 500 mL with PBS

Blocking buffer (2% BSA-PBS), pH=7.2: 250 mL
- 250 mL PBS
- 5 g bovine serum albumin (BSA)
- Dissolve 5 g BSA in 200 mL, top up to 250 mL in a graduated cylinder

Sample Diluent, pH=7.2: 250 mL
- Begin with 200 mL dH2O in a graduated cylinder, add
  - 5.95 g HEPES
  - 1.46 g NaCl
  - 0.93 g EDTA
  - 2.5 g BSA
- 0.25 mL Tween-20
- Fill graduate cylinder up to 250 mL dH₂O
- pH to 7.2 with NaOH, store at -20°C

**OPD buffer (0.1 M Citric acid-phosphate buffer), pH = 5.0**
- Begin with 400 mL dH₂O in a graduated cylinder, add
- 0.0347 M citric acid (3.34 g citric acid)
- 0.0667 M Na₂HPO₄ (4.73 g Na₂HPO₄)
- Fill up to 500 mL with dH₂O
- Store at 4°C, protect from light

**Colour Reagent (1 plate)**
- 15 mL of OPD buffer (described above)
- 2x5 mg tablets of OPD, dissolve in OPD buffer
  - Immediately prior to loading on plate, add 6.2 μL 30% H₂O₂

**Stop solution (1.0 M H₂SO₄)**
- 1 M H₂SO₄ (5.56 mL of 95% H₂SO₄)
- Fill to 100 mL with dH₂O
- Store at 4°C, protect from light

**Method:**

**Day 1:**

1. Coat a 96-well microtiter plate with 100 μL of recombinant human VWF or Humate-P® or Wilate® per well diluted in coating buffer to ~0.7 U/mL. Coat wells with VWF as a control blank, control medium, positive control plasma. In the last column of wells just coat with 100ul of coating buffer. Incubate at 4 °C overnight.

**Day 2:**

2. Wash plate 3 times with >250 μL of wash buffer.

3. Block plate with 200 μL of blocking buffer for 2 hr at room temperature.

4. During the incubation in step 3, prepare plasma samples. Dilute plasma samples as required (this depends on the anti-VWF antibody titer in the plasma sample) with sample diluent.
5. Wash plate 3 times with >250 μL of wash buffer.

6. Load 100 μL of diluted plasma samples in duplicate to the plate. To control blank wells just add 100 μL of sample diluent.

7. Incubate plasma samples for 2 hr at room temperature.

8. Wash plate 3 times with >250 μL of wash buffer.

9. Dilute Anti-Human IgG Peroxidase Conjugate (Sigma A2290) in sample diluent, 1:5,000. Add 100 μL of this secondary antibody to each well.

10. Incubate plasma samples for 1 hr at room temperature.

11. Wash plate 4 times with >250 μL of wash buffer.

12. Add 100 μL of colour reagent to each well. Gently and evenly tap the sides of the plate to mix. Incubate for 20 min in the dark.

13. Add 100 μL of stop solution. Gently and evenly tap the sides of the plate to mix. Incubate for 5 min at room temperature.

14. Read the optical density at 492 nm (with a mixing step) on a microplate reader.
A.3. HEK293T calcium phosphate transfections

**Purpose:** To transiently transfect cells with VWF plasmids of interest.

**Reagents:** Solutions should be prepared fresh. pH is critical for this assay. Solutions can also be made, aliquoted and stored at -20°C.

### 2X HEPES Buffered Saline (HBS) pH 7.15

<table>
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<th>Quantity</th>
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<tbody>
<tr>
<td>50mM HEPES</td>
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<tr>
<td>280mM NaCl</td>
<td>0.815g</td>
</tr>
<tr>
<td>H₂O</td>
<td>50 mL</td>
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### 0.07 M Na₂HPO₄

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### 2 M CaCl₂

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<td>50 mL</td>
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<tr>
<td>Filter sterilize 0.22µm</td>
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### Cell Culture Media:

#### Dulbecco’s Modified Eagle Media (DMEM)

To one 500 mL bottle of DMEM add:
- 10% (50 mL) fetal bovine serum (FBS)
- 1X (5 mL) Penicillin/Streptomycin (P/S)
- 1X (5mL) L-glutamine (L-glut)
- 500 µL Fungizone® antimycotic

#### Opti-MEM Reduced Serum Media

To one 500mL bottle of Opti-MEM add:
- 1X (5mL) Insulin-Transferrin-Selenium (ITS)
- 1X (5mL) Penicillin/Streptomycin (P/S)

**Coating Plates with Poly-L Lysine**
*optional but recommended; improves cell adhesion*
Poly-L Lysine hydrobromide (Sigma, P2636)
Resuspend Poly-L Lysine (10mg/mL) and aliquot
Dilute Poly-L Lysine 1:100 in tissue culture grade water (Sigma P7890)
Add 1.5 mL/10 cm plate, swirl to coat
Incubate for 1.5 hr, “rocking” every 15 min
Wash 2X with 5 mL Hank`s Balanced Salt Solution (HBSS)
*best to do on day required but can do the day before, wrap in foil

**Day -1: Plating Cells**
Aspirate off media
Trypsinize cells by washing 1-2X with 10 mL HBSS, aspirate off
Add 1 mL of trypsin. Let sit for 1 min in incubator.
Add 10 mL DMEM/10%FBS/1XP/S/1XL-Glut to stop reaction. Pipette up and down to wash plate, so there are no clumps.
Pool cells together in a conical tube. Spin at 1000g for 8 min. Remove supernatant.
Add 40 mL DMEM/10%FBS/1XP/S/1XL-Glut to cells to resuspend (pipette up and down to resuspend).
Place drop of 10 μL of cells to hemocytometer. Follow protocol for counting cells.
Plate 2-4x10⁶ HEK 293T cells/100mm dish in 10mL DMEM/10%FBS/1XP/S/1XL-Glut.

**Day 0: Transfections**
Cells should be 50-75% confluent. Batch transfections can also be performed, multiply amounts as appropriate, and add 1 mL mix/10 cm dish.

**1.5 mL DNA tubes**
Total DNA: 20 μg, use calf thymus (1 μg) to make up the difference
pβgal plasmid: 3.2 μg
VWF plasmid: 10 μg
* one negative control plate, 1 pβgal control plate
Add 20 μg DNA to 1.5 ml eppendorf tube
Add 440 μL sterile dd H₂O or 0.1X sterile TE
Add 62.5 μL 2 M CaCl₂ to tube. Flick hard to mix.

5 mL HBS Phosphate tubes
Add to 5 mL eppendorf tubes
500 μL 2X HBS
10 μL 0.07 M Na₂HPO₄

Add DNA mixture drop wise to 5mL tubes, and bubble from top to bottom at least 10 times. Do one tube every 1 minute. Let sit for 20 min at room temperature. Very mild milky appearance will form. Add to plates slowly using P1000, gently swirl to mix. Media will become more orange in colour. You should see a fine “grains of salt” precipitate on the cells at the highest magnification.

Day 1: Medium Change * No more than 17 hr after transfections
Aspirate off media.
Add 8-10 mL 1X PBS 1mM EGTA to plates. Wait 2 min, swirling periodically to remove precipitate. Aspirate off PBS. Add 10 mL of OptiMEM with 1X ITS, 1X P/S.

Day 3: Medium and cell harvest
72 hr after transfections:
Pipette off media into a 15 mL conical tube, labeled. Spin 500g for 8 min. Collect 100 μL from each in 0.6 mL tubes for ELISA. Store media at -80 °C for later use.

To concentrate protein:
Pool all wild-type media, all mutant media into 50 mL conical tubes.
Filter in Centricon Plus-70 centrifugal filter device. Spin 3400 g, 18°C, 20 min. Spin more if necessary.
Wash with 25 mL HBSS. Spin 3400 g, 18°C, 20 min. Spin more if necessary.
Invert with collection cup. Spin 1000 g, 18°C, 2 min.
Aliquot concentrated protein from collection cup into 0.6 ml tubes (4x10 μL, 100 μL for rest, estimate last tube). Store at -80°C.

In the meantime:
Wash plates with 10 mL HBSS. Aspirate off HBSS.
Add 0.625 mL of lysis buffer (100mM Potassium Phosphate/ 0.2% TritonX-100, pH=7.8). If desired, you can add 0.5mM DDT to Lysis buffer). Lyse cells for approximately 15 min. Use cell scraper to scrape off cells to bottom. Pipette into 1.5 mL tube (approximately 1 mL lysate).
Spin 13000 rpm, 3 min, remove supernatant to fresh tube. Store lysate at -80°C.

**β-galactosidase reporter assay using Applied Biosytems Galacto-Light Plus kit (T1011)**

* Buffers need to be at room temp.
Dilute Galacton-Plus 1:100 in Galacto reaction buffer diluent.
100 μL reaction buffer/luminometer tubes.
10 μL cell lysates/tube (or lysis buffer control) every 30 sec.
Incubate 1 hr (min. 30 min, must be same for all tubes)
Add 150 μL Accelerator II to the tubes every 30 sec. IMMEDIATELY read in the LB501 Luminometer using protocol 2.
A.4. BOEC isolation

**Purpose:** To isolate blood outgrowth endothelial cells (BOEC) from a patient blood sample.

**Reagents:**
- CPT vacutainer tubes (BD, cat no: 362780)
- Phosphate buffered saline (PBS), pH 7.2
- Complete-EGM-2 (cEGM-2): EBM-2 (Lonza; cat.no. CC-3162) supplemented with the entire growth factor bullet kit (2% FBS, 0.4% hFGF-2, 0.1% VEGF, 0.1% R3-IGF-1, 0.1% hEGF, 0.04% hydrocortisone, 0.1% ascorbic acid, 0.1% heparin, 0.1% GA-100), plus 10% FBS and 1% penicillin (10,000U/mL)/ streptomycin (10,000μg/mL)/ amphotericin (25 µg/mL, Invitrogen; cat.no. 15240-062). Store at 4°C for one month.
- Fetal Bovine Serum (FBS, Hyclone; cat.no.SH30070.03)
- 6 well plates (Costar® 6 Well Plates, Sterile #3516)
- Rat tail collagen I (BD Biosciences; cat. No. 354236); diluted to 0.05mg/mL in dH2O/0.02M glacial acetic acid, filter sterilized.

**Method:**
1. Collect 48 mL blood into 6 CPT vacutainer tubes. Invert tubes 8-10 times to mix anticoagulant additive with blood. (Do not shake as this can cause hemolysis).

2. Store tubes upright at room temperature until centrifugation. (blood samples should be centrifuged within 1 hour of blood collection).

3. Centrifuge at room temperature (18-25°C) in a horizontal rotor (swing-out head) for 30 min at 1600 RCF. (Note: remix the blood sample immediately prior to centrifugation by gently inverting the tubes 8-10 times).

4. Remix the blood sample immediately after the centrifugation by gently inverting the tubes 8-10 times.

   * At this point the CPT tubes can be shipped at ambient temperature in a Styrofoam shipper so the samples will be received in < 24 hr.

5. Remove the serum +MNCs layer from all six CPT tubes and add to 8 mL of PBS+10% FBS (in a 50 mL conical).

6. Centrifuge the MNCs at 520g for 10min at room temperature with a high brake.
7. Aspirate and discard the supernatant. Following this initial centrifugation the pellet of cells is often loose. Care should be taken to avoid aspirating cells.

8. Gently tap loose the pelleted cells and resuspend in 10 mL of PBS (+10% FBS). If there are multiple tubes, cell pellets can be serially combined at this point.

9. Repeat steps 6-8 one time.

10. Remove 10 μL of cell suspension and mix with 90 μL of PBS (1:10 dilution). Remove 30 μL of the diluted sample and mix with 30 μL trypan blue. Count viable cells on a hemocytometer and calculate the total number of MNCs in the sample.

11. Centrifuge the cell suspension at 520g for 10 min at room temperature with a high brake and aspirate the supernatant.

12. Tap loose the cell pellet and resuspend MNCs in cEGM-2. A seeding density in the range of 3-5 x10⁷ MNCs/4 mL is ideal for ECFC colony formation.

13. Pipette 4 mL (3-5x10⁷ MNCs) into each well of a collagen coated 6-well tissue culture plate and place in a 37°C, 5% CO₂ humidifier incubator.

**Media changes**

14. After 24 hr (day 1), slowly remove the media from the well with a pipette. Media is removed at a rate of 1 mL per 4-5 sec. Leave some liquid in the well to prevent drying of the plate surface.

15. Slowly add 2 mL of cEGM-2 to the well.

16. Slowly remove the 2 mL of the media and add 4 mL cEGM-2 to the well. Return the culture plates in the incubator.

17. Change media daily for the first 7 days by removing and adding slowly the media. From day 8, change the media every other day and use 3 mL of media.

18. Anywhere from day 9-21 the characteristic cobblestone cells will hopefully start to appear. Continue to change the media every day until these cells reach confluency or until they begin to clump together a lot. At this point cells can be passaged to larger plates.
A.5. Flow cytometry staining

**Purpose:** To confirm the endothelial cell phenotype of isolated BOEC.

**Reagents:**
- 0.05% trypsin, 0.02% EDTA (Invitrogen)
- Flow cytometry staining buffer (FCSB; PBS, 2% BSA, sodium azide) (Ebioscience)
- Fixation Buffer – BD Cytofix/Cytoperm™ (BD Bioscience)

**Isotype Controls (Ebioscience):**
- FITC conjugated mouse IgG1 isotype control (11-4714-42)
- PE conjugated mouse IgG1 isotype control (12-4714-42)
- PerCp Cy 5.5 conjugated mouse IgG1 isotype control (45-4714-82)
- Pe-Cy5 conjugated mouse IgG1 isotype control (15-4714-42)

**Antibodies (Ebioscience):**
- FITC conjugated Anti-Human CD31 (PECAM1) (11-0319-42)
- FITC conjugated Anti-Human CD146 (MCAM, EndoCAM) (11-1469-42)
- PE conjugated Anti-Human CD144 (VE Cadherin) (12-1449-82)
- PerCp Cy 5.5 conjugated Anti-Human CD14 (45-0149-42)
- Pe-Cy5 conjugated Anti-Human CD45 (15-0459-42)

**Method:**

1. Wash cells with warm HBSS (2 times).
2. Remove cells from the plate with 0.05% trypsin, 0.02% EDTA.
3. Stop the trypsin action with media, then centrifuge and resuspend in HBSS.
4. Determine the cell number. Calculate the number of cells required for the flow experiment (1 × 10⁶ per test – add a couple extra tests for pipette error) and centrifuge at 800g for 5 min at 4°C, or 400 g for 10 min. (500, 000 cells per test will work but best to start off with 1x 10⁶ cells because cells will be lost during subsequent centrifugations).
5. Decant the supernatant and invert the tube over a Kimwipe to remove the remaining supernatant.

6. Resuspend the cells in FCSB at a concentration of $10^7$ cells/mL.

7. Pipette 100 μL cells into 5 mL sterile tubes.

8. Stain surface molecules with fluorescently conjugated antibodies or isotype control antibodies. Pulse vortex to mix and incubate for 30 min in the dark at 4°C.

9. Wash the cells with 4 mL FCSB. Cap and invert several times.

10. Centrifuge at 800g for 5 min 4°C, or 400 g for 10 min, decant and invert to remove excess supernatant.

11. Add 250 μL Fixation Buffer from BD Bioscience and pulse vortex to mix. Incubate 20 min at 4°C in dark.

12. Wash the cells with 4 mL FCSB. Cap and invert several times.

13. Centrifuge at 800g for 5 min 4°C, or 400 g for 10 min, decant and invert to remove excess supernatant.

14. Add 600 μL FCSB, pulse to resuspend.

15. Protect from light until ready to be analyzed.

16. Samples are analyzed using an Epics Altra HSS Flow Cytometer (Queen’s Cytometry and Imaging Facility, The Cancer Research Institute at Queen’s University).
A.6. VWF immunofluorescent staining

**Purpose:** To visualize intracellular von Willebrand Factor (VWF)

**Reagents:**
- Phosphate Buffered Saline (PBS), filter sterilized
- PBS/1% Triton X-100 (500 mL/5 mL), filter sterilized
- PBS/1% BSA (500 mL/5g), filter sterilized
- BD Cytofix/Cytoperm™ (BD Bioscience)
- Serum-free Protein Block (DAKO X0909)
- Primary antibodies:
  - DAKO Rabbit anti-human VWF (A0082, 3.1g/L) 2 μg/mL (1:500) in PBS/1% BSA
  - DAKO Negative Control Rabbit Immunoglobulin Fraction (X0936, 15g/L) 2 μg/mL (1:500) in PBS/1% BSA
- Secondary antibodies:
  - Anti-Rabbit Immunoglobulin-FITC DAKO (F0054, 0.5 g/L) 2 μg/ml (1:500) in PBS/1% BSA
  - Phalloidin-TRITC (Sigma P1951, 1 μg/mL). Make up 1 μg/μL aliquots, diluted 1:1000 (1μL/mL) PBS/1% BSA
- DAPI nucleus stain (Sigma D9542). Make up 5 mg/mL aliquots.
- Mounting media (Vectashield H-1000, Vector Laboratories or DAKO, S3023)

**Methods:**

24 hours in advance, plate 53,000 cells/cm² to collagen coated coverslips

**DAY 1:**

1. Aspirate the media
2. Wash the glass coverslips with 1-2 mL of room temperature PBS (3X)
3. Fix coverslips with BD Fix (1mL/well) for 20 min at room temperature
4. Wash the slides with 1-2mL PBS, 3X (5 min each)
5. Permeabilize the cells in 1 mL PBS-1% TritonX-100 for 10 min. at room temperature
6. Wash the cells with 1-2mL PBS, 3X (5 min each)
7. Block the coverslips with the serum free protein block for 20 min at room temperature (8 drops)

8. Wash the coverslips with 1-2 mL PBS, 3X (10 min each)

9. Incubate with 500 μL 1° antibody and incubate overnight at 4°C in the dark

**DAY 2:**

10. Wash the coverslips with PBS, 3X (15 min each)

11. Incubate the coverslips with 500 μL of 2° antibody at room temperature in the dark for 1 hr

12. Wash the coverslips with PBS, 3X (15 min each)

13. Add 500 μL of DAPI nucleus stain, diluted 1:5000 in water (i.e. 0.2 μL/mL water) and incubate for 10 min at room temperature

14. Wash with 1-2 mL water for 10 min (3X)

15. Let the coverslips air dry for 10 min

16. Drop a small amount of mounting media on the slide

17. Remove coverslips from the wells with bent scraper, grab with forceps

18. Place coverslip upside down on mounting media. Seal edges with clear nail polish. Store slides in a slide book, wrapped in foil at 4°C

19. Slides are visualized using a Leica TCS SP2 Multi Photon confocal microscope (Queen’s Cytometry and Imaging Facility, The Cancer Research Institute at Queen’s University)

20. Image analysis is performed using MetaMorph® Image Analysis Software
Appendix B
Primer Sequences

B.1. Primers used for direct sequencing of \textit{VWF} for Canadian type 3 VWD study

B.2. Primers used for site-directed mutagenesis to create \textit{VWF} mutant expression plasmids
B.1. Primers used for direct sequencing of VWF for Canadian type 3 VWD study

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*forward sequencing primer; **reverse sequencing primer
B.2. Primers used for site-directed mutagenesis to create VWF mutant expression plasmids

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F = forward; R = reverse
Appendix C

Research Ethics Board (REB) approvals

C.1. Genetic Differences Between Obligate Carriers of Type 3 von Willebrand Disease and Individuals with Type 1 von Willebrand Disease

C.2. von Willebrand Disease Plasma and Platelets: Functional Characterization of Quantitative and Qualitative von Willebrand Factor Mutations
C.1. Genetic differences between obligate carriers of type 3 von Willebrand disease and individuals with type 1 von Willebrand disease

QUEEN'S UNIVERSITY HEALTH SCIENCES & AFFILIATED TEACHING HOSPITALS RESEARCH ETHICS BOARD

Queen's University, in accordance with the "Tri-Council Policy Statement, 1998" prepared by the Medical Research Council, Natural Sciences and Engineering Research Council of Canada and Social Sciences and Humanities Research Council of Canada requires that research projects involving human subjects be reviewed annually to determine their acceptability on ethical grounds.

A Research Ethics Board composed of:

Dr. A.F. Clark  Emeritus Professor, Department of Biochemistry, Faculty of Health Sciences, Queen's University (Chair)
Dr. S. Burke  Emeritus Professor, School of Nursing, Queen's University
Rev. T. Deline  Community Member
Dr. M. Evans  Community Member
Dr. M. Green  Assistant Professor, Department of Family Medicine, Queen's University
Ms. T.C. Knott  Research & Evaluation, Southeastern Regional Geriatric Program, Providence Continuing Care Centre – St. Mary’s of the Lake Hospital Site
Dr. J. Low  Emeritus Professor, Department of Obstetrics and Gynaecology, Queen's University and Kingston General Hospital
Dr. H. Murray  Assistant Professor, Department of Emergency Medicine, Queen's University
Dr. W. Racz  Emeritus Professor, Department of Pharmacology & Toxicology, Queen's University
Dr. B. Simchison  Assistant Professor, Department of Anesthesiology, Queen's University
Dr. A.N. Singh  WHO Professor in Psychosomatic Medicine and Psychopharmacology
Professor of Psychiatry and Pharmacology
Chair and Head, Division of Psychopharmacology, Queen's University
Dr. S. Taylor  Director, Office of Bioethics, Queen's University and Kingston General Hospital;
Associate Professor, Department of Medicine, Queen's University

Dr. K. Weinbaum  L.L.B. and Adjunct Instructor, Department of Family Medicine (Bioethics)

has examined the protocol and revised consent form (May 17, 2005) for the project entitled "Genetic Differences Between Obligate Carriers of Type 3 von Willebrand Disease and Individuals with Type 1 von Willebrand Disease" as proposed by Dr. Paula James of the Department of Medicine and Dr. D. Lillcrap of the Department of Pathology at Queen's University and considers it to be ethically acceptable. This approval is valid for one year. If there are any amendments or changes to the protocol affecting the subjects in this study, it is the responsibility of the principal investigator to notify the Research Ethics Board. Any unexpected serious adverse event occurring locally must be reported within 2 working days or earlier if required by the study sponsor. All other serious adverse events must be reported within 15 days after becoming aware of the information.

Chair, Research Ethics Board

Date

PATH-066-05
2005-05-09
C.2. von Willebrand disease plasma and platelets: functional characterization of quantitative and qualitative von Willebrand factor mutations