MOLECULAR GENETICS OF VON WILLEBRAND DISEASE

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TO

THE SREE CHITRA TIRUNAL INSTITUTE

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS

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DECLARATION BY STUDENT

I, Sumitha E hereby certify that I had personally carried out the work depicted in the thesis, entitled, **"Molecular Genetics of Von Willebrand Disease"**. No part of the thesis has been submitted for the award of any degree or diploma prior to this date.

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* Clearance was obtained from Institutional Ethics Committee for carrying out the study.

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Sumitha E

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ABBREVIATIONS

ADAMTS13	a disintegrin-like and metalloprotease with thrombospondin type 1 motif, 13th member
ADP	Adenosine diphosphate
APC	Activated protein C
aPTT	activated Partial Thromboplastin Time
ASSP	Alternative Splice Site Predictor
AT	Antithrombin
ATCC	American Type Culture Collection
Bp	Base pair
BS	Bleeding Score
BT	Bleeding Time
CBC	Complete Blood Count
CK	Cysteine knot
CLEC4M	C-type lectin domain family four member M
CSGE	Conformation Sensitive Gel Electrophoresis
DAPI	4',6-diamidino-2-phenylindole
DDAVP	1-deamino-8-D-arginine vasopressin
dL	Decilitre
dNTPS	Deoxy ribo Nucleotide TriPhosphate
DTR	Dye Terminator Removal Clean up
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme Linked Immunosorbent Assay
ET-1	Endothelin-1
EPCR	Endothelial Protein C Receptor
ER	Endoplasmic Reticulum
eNOS	Endothelial Nitric Oxide Synthase
ETS	E-twenty six (transcription factor family)
Factor VIII	FVIII
FBS	Fetal Bovine Serum
FVIII:C	Factor VIII procoagulant activity
GATA	A family of transcription factors known as GATA 1 to 6
GLA	Gamma-Carboxyglutamic Acid
GP Ibα	Platelet glycoprotein Ibα receptor
HEK	Human Embryonic Kidney Cells

HMW	High Molecular Weight
HOPE	Have (y)Our Protein Explained
HRP	Horseradish Peroxidise
IC	Index Case
IF	Immunofluorescence
ISTH SSC	International Society for Hemostasis and Thrombosis Scientific and Standardization Committee
IU	International Unit
kDa	Kilo Daltons
LB	Luria-Bertani
LRP	LDL Receptor Related Protein
Mab	Monoclonal Antibody
MCMDM	Molecular And Clinical Markers For The Diagnosis And Management
MEM	Minimal Essential Media
MLPA	Multiplex Ligation-dependent Probe Amplification
MUC	Mucins
NC	Normal Control
NEQAS	National External Quality Assessment Service
nm	Nano Metre
NO	Nitric Oxide
OD	Optical Density
OPD	O-Phenylenediamine Dihydrochloride
PAI 1&2	Plasminogen activator inhibitor 1 & 2
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PC	Protein C
PDI	Protein Disulphide Isomerase
PECAM-1	Platelet Endothelial Cell Adhesion Module-1
PFA	Platelet Function Analyzer
PGI2	Prostaglandin I2
Polyphen	Polymorphism Phenotyping
PPP	Platelet Poor Plasma
ProVWF protein	Unprocessed VWF Containing Both VWF And Mature VWF
PRP	Platelet Rich Plasma
PS	Protein S
PAF	Platelet activating factor

PT	Prothrombin Time
PT-VWD	Platelet Type Pseudo VWD
PZI	Protease Zinc Inhibitor
RFLP	Restriction Fragment Length Polymorphism
RIPA	Ristocetin Induced Platelet Agglutination Assay
rpm	Revolutions Per Minute
RT	Room Temperature
SIFT	Sorting Intolerant From Tolerant
SNAP	Screening For Nonacceptable Polymorphism
SNP	Single Nucleotide Polymorphism
SP	Serine Protease Domain
TE	Tris-EDTA Buffer
TEMED	Tetra methyl ethylenediamine
TF	Tissue Factor
TFPI	Tissue Factor Pathway Inhibitor
TGN	Trans Golgi Network
tPA	Tissue Type Plasminogen Activator
TT	Thrombin Time
TTE	Tris Taurine EDTA
TXA2	Thrombaxane A2
uPA	Urokinase Plasminogen Activator
UPN	Unique Patient Number
VCAM	Vascular Cell Adhesion Molecule
VWD	Von Willebrand Disease
VWF	Von Willebrand Factor
VWF:Ag	Von Willebrand Factor Antigen
VWF:CB	Collagen Binding Assay
VWF:FVIIIB	Von Willebrand Factor FVIII Binding
VWF:RCo	Von Willebrand Factor Ristocetin Cofactor Activity
VWFpp	Von Willebrand Factor Propeptide
VWFpp/VWF:Ag	Ratio of VWF Propeptide Levels to VWF antigen Levels
WPB	Weibel Palade Bodies

PhD SYNOPSIS

Background

Von Willebrand disease (VWD) is the most common congenital bleeding disorder. The disease is classified as type 1, 2 and 3 based on the plasma concentration of Von Willebrand factor (VWF), FVIII levels and multimer assay as recognized by the International Society on Thrombosis and Hemostasis (ISTH). Mutations in VWF gene results in quantitative or qualitative defects leading to the disease. In India, there is limited epidemiological data on the prevalence of different subtypes. Amongst the subtypes, type-3 VWD outnumbers the others due to high degree of consanguinity, in some parts of the country and under diagnosis of mild to moderate subtypes.

Type 3 VWD is an autosomal recessive bleeding disorder with a prevalence of about 0.5 to 1 per million in Western countries. Clinical manifestations in type3-VWD are generally severe but also variable between patients compared to other subtypes leading to higher morbidity and mortality. Given the challenge of treatment in patients with type-3VWD, understanding the basis of this heterogeneity and establishing molecular genetic methods for carrier detection and antenatal diagnosis is important for better clinical care. There is a paucity of data on the molecular genetic pathology of this disease in India. This study was therefore undertaken to evaluate clinical, haematological and molecular profile for type3-VWD patients from India, and to study their genotype-phenotype correlations and also to compare the algorithm for mutation detection with the approach reported in the literature and then establish a clinical service for it.

Objectives

Objective 1: Enrol a large series of patients with type-3 VWD and evaluate the clinical and haematological data.

Objective 2: Develop a practical stratergy for mutation detection in the VWF gene in these patients.

Objective 3: Elucidate the relationship between genetic defects and clinical/ haematological parameters.

Objective 4: Evaluate the functional impact of selected mutations through expression studies.

Materials and methods

Patients: A total of one hundred and two patients with Type 3-VWD were evaluated in the present study (2012-2015). Inclusion criteria included a documented history of excessive mucocutaneous bleeding associated with reduced concentration of VWF and FVIII levels in plasma. Peripheral blood samples were aseptically collected in appropriately labelled EDTA/Citrate tube. All the participants in the study gave informed consent and the approval of the study was obtained from the Institutional Research and Ethics committees (Christian Medical College, Vellore IRB min A13-25-04-2012).

Bleeding history: Demographic profile and information related to patients bleeding history were documented using the ISTH-BAT bleeding questionnaire. The bleeding scores were summed up to reflect the severity of bleeding in a given patient.

Coagulation assays: Laboratory diagnosis of type-3 VWD was based on prolonged clotting times, performed on a CS 2000i (Sysmex, Kobe, Japan) or automated

analyzer (ACL TOP; IL, Milan, Italy). In patients with VWD where aPTT is prolonged, the samples were subjected to mixing studies with pooled normal plasma to assess the presence of a factor deficiency or inhibitors. This test was followed by the measurement of FVIII coagulant activity (FVIII: C). The assay VWF:RCo, was carried out in an automated coagulometer (ACL 10000; IL, Milan, Italy) using commercially available platelets. Antigenic levels of VWF in the plasma and their ability to bind to collagen was measured by the enzyme-linked immunosorbent assay (ELISA) (ICN Biomedicals Inc, Aurora, OH, USA).

Assessement of the VWF gene: Genomic DNA was extracted and amplified by in house designed primers (n=52 pairs). Equal volume of PCR product from patient and control was mixed and heteroduplexed and then loaded in the gel.The PCR products were resolved in a 12.5% CSGE (Conformation Sensitive Gel Electrophoresis)

(i) DNA sequencing*:* Amplicons displaying aberrant pattern were sequenced using fluorescently labelled dideoxy nucleotide triphosphates using the Big Dye Terminator cycle sequencing kit Version 1.1 or 3.1(Applied Biosystems, Foster City, CA). The mutation data was validated by using set of primers received on request from *Hampshire DJ et al,* 2010.

(ii) Gene dosage analysis: In patients where large deletions were suspected, the sample was subjected to gene dosage analysis. Deletion status in the patient was confirmed by co-amplifying a control gene at same annealing temperature. Since the carrier status in the parents cannot be confirmed by the normal PCR approach, gene dosage analysis was performed. This assay was carried out using fluorescently labelled primers, resolved by capillary electrophoresis in an ABI-3130 genetic analyzer (Applied Biosystems). The results were analyzed by Gene Mapper (4.0)

(Applied Biosystems). The ratio obtained between the peak heights of the exon in the VWF gene and albumin control gene in the parent samples was normalized with a normal control.

(iii) Restriction fragment length polymorphism (RFLP): For the screening of novel nucleotide changes identified in the study, we screened in 25 healthy controls (50 normal alleles) to rule out the possibility of a common polymorphism by RFLP.

(iv) Haplotype analysis: Haplotype analysis was carried out for the common mutations identified in VWF gene by using the following polymorphic markers (rs 216902 C>T; rs 216311 A>G, rs 1063857 T>C, rs 1063857 A>G, r s216867 T>C, rs 216321G>A, rs 216868-7C>T and intron 40 VNTR) by direct sequencing of the PCR amplicons or by using fluorescently labelled primers. The PCR products were further resolved by capillary electrophoresis and the data was analysed.

Major findings

One hundred and two type-3 VWD patients from 90 families were evaluated. These patients were characterized by absent VWF antigenic levels in the plasma with a very low ristocetin cofactor activity and FVIII levels. The median age at presentation of bleeding symptoms was 2 years (range: 0-60years). Sixty two patients (61%) gave history of consanguinity. All these patients presented with history of variable skin and mucosal bleeding with a median ISTH bleeding score (BS) of 9 (range 2-19). Based on the established practical strategy, mutations were identified in 93 patients belonging to 81 families. In three patients mutations were identified only in a single allele. Mutations could not be identified in 9 (9%) patients, which is comparable other reports, wherein different sensitivities ranging from 28-90% have been reported using different mutation detection techniques. A total of fifty five

different disease causing mutations were identified, of which 35 (64%) were novel. These included frame shift (n=15, 27.27%) missense (n=13, 23.64%), non-sense (n=12, 21.82%), large deletion (n=2, 3.64%) gene conversion (n=3, 5.45%) and splice site mutation (n=10, 18%). Among the thirteen missense mutations, 7(54%) were novel. The mutations p.Asp47 and p.Gly74 are highly conserved across multiple species and mutations in this region are known to impair the polymerization of the multimers. The residue p.Met1055Lys lies in the D3 domain which may impair FVIII binding. Amino acid change at residues, p.Ala1150Pro, p.Gln2266His & p.Cys2184Tyr is predicted to impair the protein stability, which may affect the secretion of VWF proteins. The amino acid change at residue p.Cys2257Arg is predicted to disturb dimerization. The functional significance of some of these mutations has to be further evaluated and confirmed. Interestingly, in the present study, gene conversion was found to occur at exon-28 in five patients resulting in missense changes p.Pro1266Leu, p.Gln1311* and p.Val1279Ile, and p.Arg1315His. Both single and multi-exonic deletions were observed in two patients. The deletional status was validated by gene dosage analysis in the patients, and the carrier status in the parents was analysed by the same method. Novel splice mutations were analysed using *in silico* analysis tools, alternative splice site prediction finder (ASSP) and splice port analysis etc. On *in silico* analysis, these mutations abolished (c.3675+1G>A) or altered the physiological acceptor site (c.6599-2A>T). Interestingly five common mutations (c.3675+1G>C, n=4; p.Gln1311*, n=5; p.Trp2107*, n=7; p.Arg373*, n=9; c.2443-1G>C, n=12) were highly prevalent (36%) in the present study. Haplotype analysis was carried out to identify the origin of these mutations. A common haplotype was shared among different ethnic group from India only in patients with p.Trp2107*.

In order to understand the relationship between the clinical and hematological parameters, we attempted to study genotype-phenotype correlation as there is paucity of data in the literature. In a total of 52 females, 25 females presented with history of menorrhagia with a median bleeding score 11 (range: 6-19). To negate the effects of menorrhagia on phenotype, we recalculated the bleeding scores excluding menorrhagia. The bleeding scores were segregated into 4 quartiles (group 1= $\leq$ 5; group 2 = 5.1-8; group 3 = 8.1-11 and group 4 = >11). Genetic analysis had shown that the maximum no of patients had mutations clustered in the propeptide region of VWF. However when mutation subtypes were compared with different groups, no correlation was observed.

Significance of the findings

The present study is one of the largest series, carried out to understand the clinical profile and molecular basis of Type3-VWD. The median age at presentation was 2 years, varied from 17 days to 60 years. Commonest clinical manifestations observed in the study include bleeding from minor wounds and cutaneous bleeding. Nearly two third of the patients had consanguineous parents.

Mutations could be identified in 93(91%) patients from 81(79%) families. Among the different domains analyzed maximum number of mutations (27%) was localized in the propeptide region (VWFpp). The mutations identified in patients with VWD are as heterogeneous as reported in other populations. Pathological implications of the novel mutations were evaluated by *in silico* analysis. Interestingly in the present study five common mutations accounted to 36% of cases. Haplotype analysis suggested a common haplotype for p.Trp2107* shared among different ethnic group from India. This suggests a possible founder effect and also that the common mutations could be screened first in patients from

southern India, facilitating a more rapid and cost-effective molecular diagnosis. The molecular data presented here adds significantly to the existing international mutation database of this disease (n=35). The experimental approach designed to study the mutation spectrum in these patients was successful in identifying mutations 93(91%) patients from 81(79%) families. Based on these results, facilities to control VWD through genetic counselling and prenatal diagnosis have been set up.

The present study highlights the phenotypic and genotypic heterogeneity in patients with VWD-type 3 in India.

1. Introduction

Von Willebrand disease (VWD) is the most common inherited bleeding disorder, with a prevalence of about 1% (Rodeghiero et al., 1987, Srivastava and Rodeghiero, 2005) in the general population. In western countries, it is estimated to affect ~0.5–1.6% of the population (Bowman et al., 2010). However, in India, there is limited epidemiological data on the prevalence of VWD (Bowman et al., 2010, Srivastava and Rodeghiero, 2005). Biologic heterogeneity and variability in their clinical presentation complicate the diagnosis of this condition (Srivastava, 2005, Lillicrap, 2013c). Patients with VWD present with a broad range of clinical manifestations including epistaxis, bleeding after dental extraction, gum bleeding, menorrhagia, gastrointestinal bleeding, intracranial bleeding, and hemarthrosis. Inheritance of this disease follows either dominant or codominant pattern (Castaman et al., 2003). In most VWD populations the mutant alleles were found to be inherited in both the sexes with equal frequency. However, females outnumber males in a ratio of about 2:1 (Lillicrap, 2013c), presumably due to the burden of excessive mucocutaneous bleeding during the reproductive age of women. This disease is caused due to deficiency of Von Willebrand factor (VWF), a large multimeric protein which imparts a fundamental role in hemostasis. VWF synthesized by the endothelial cells and megakaryocytes acts both as a mediator of platelet adhesion to subendothelium in response to a denuded endothelium and as a chaperone for coagulation factor VIII (FVIII) in the plasma (Lillicrap, 2013c). Much progress has been made to understand the pathology underlying the cause of the disease in the last two decades (Lillicrap, 2013c, Laffan et al., 2014).

Patients with VWD are currently classified, into one of the three subtypes, Quantitative (Type 1 and Type -3) or Qualitative (type 2[A, B, M, N]) disorders to facilitate diagnosis, treatment, and counseling for these patients (Goodeve, 2010).

Type 1-VWD accounts for about 65% to 80% of the cases, characterized by mild to moderate reduction in VWF (Lillicrap, 2013c). Qualitative deficiency of VWF results in type-2 VWD, which occurs in about 20% to 35% of the subjects (Lillicrap, 2013b). Prevalence of type 3-VWD usually, varies between countries, ranging from 0.1-5.3 million (Rodeghiero et al., 2009), however, increases with consanguinity (Mannucci et al., 2004). Type 3-VWD is characterized by markedly decreased or complete deficiency of VWF. Clinically type-3 VWD is the most severe form of the disease than other types. Hence, the molecular methods are imperative to manage this condition, especially in developing countries, since the care for prevention and management of the bleeding disorder is not optimal (Ghosh and Shetty, 2011, Nair et al., 2011). Limited data exists on the molecular genetics of VWD in patients from India (Baronciani et al., 2000, Baronciani et al., 2003, Ahmad et al., 2013a) and their genotype-phenotype correlation. No cost effective methods have been adapted for the diagnosis VWD in India.

Hence, the purpose of the study was to evaluate their clinical, hematological and molecular profile for type3-VWD patients in India and develop a cost-effective strategy for the diagnosis of VWD further to correlate the observed genotype with the phenotype.

1.1 Objectives of the Study

1. Enrol a large series of patients with type-3 VWD and evaluate the clinical and haematological data.
2. Develop a practical stratergy for mutation detection in the VWF gene in these patients.
3. Elucidate the relationship between genetic defects and clinical/ haematological parameters.
4. Evaluate the functional impact of selected mutations through expression studies.

1.2 Brief Overview of the Thesis Chapters

1.2.1 Literature review and Material and Methods

After the introduction, the next major chapter in this thesis is the literature review. The first chapter begins with the overview of blood coagulation describing its evolution, components involved in maintaining hemostasis, mechanism of coagulation and its regulation. The description of the biology of VWF follows next. In the third chapter clinical features, diagnosis and classification and treatment of VWD are outlined.

In the fourth chapter, the molecular basis of VWD is detailed. The last chapter in the review illustrates the diagnosis and management of VWD in India. In the next chapter, material and methods, the study design and various techniques/ methods used for conducting experiments and analysing the data are described.

1.2.2 Results

The next main chapter describes the results of the study. This chapter has been sub-divided into four parts.

1.2.2.1 Evaluation of clinical and hematological data in patients with Type 3-VWD

First objective of this study was to enroll a large series of patients with type 3-VWD. We recruited 102 patients from 90 families. Clinical performa of the patients were documented and scored based on ISTH-BAT scores. Demographic profile including consanguinity, family history was also documented. Sixty-two patients were born of the consanguineous marriage. Laboratory evaluation of these patients was performed by measuring the VWF: Ag, FVIII: C, and RiCof activity. All these patients represented absent antigenic levels of VWF and decrease FVIII levels.

1.2.2.2 Strategy for mutation detection in patients with VWD

The second part of the study was to design a practical strategy for mutation detection. The algorithm followed was to pre-screen for the mutations by CSGE followed by sequencing. Direct sequencing of the amplicons was carried out, where a causative mutation could not be established.

A total of 55 mutations including nonsense, missense, splice site, large deletions, and gene conversions was identified. Frameshift and nonsense mutations are self-evident they lead to truncation of the protein. For the splice site mutations identified in the study, we performed *in silico* analysis, were these mutations may result in exon skipping or retention. For the large deletions, we performed gene

dosage analysis, to confirm the carrier status in their parents. In five patients gene conversions were identified. For the seven novel, missense, mutations identified *in silico* analysis was carried out. Most of the mutations were found to affect the structure and function of these proteins. Further on structural analysis, these nucleotide changes were found to disturb the process associated with the dimerization or the multimerization of VWF multimers, leading to impaired secretion of VWF. Interestingly, five common mutations were identified in the study. Haplotype analysis was carried out using polymorphic markers to determine the origin of these mutations.

1.2.2.3 Elucidate the relationship between the genetic defect and haematological parameters

In the third part of the study to elucidate the relationship between the observed genotype with the phenotype, quartile analysis was performed. Bleeding scores were segregated and compared with the location of mutations in the VWF gene. On comparison of the mutation subtypes with the BS, no correlation was observed. The maximum no of mutations identified in this study were harboured on to the propeptide region of VWF.

1.2.2.4 Function effect of the mutations by in vitro expression studies

The fourth part of the study was to evaluate the functional effect of the novel missense mutations by *in vitro* expression studies. Site-directed mutagenesis was carried out to create specific nucleotide changes in the plasmid pSVHVWF1. The nucleotide changes were confirmed by sequencing, followed by large-scale extraction of the plasmid. Transfection studies were carried out in HEK-293 cell

line. Forty-eight hours after transfection immunofluorescence studies was performed to quantitate the expression of VWF. The results of the study suggest, when compared to wild type the mutants were suggestive of VWF retained in the endoplasmic recticlum as proposed by the molecular modelling studies.

1.2.3 Discussion

The next main chapter is discussion where the significance of the major results was compared with the existing literature. In short, the observed bleeding score was similar to those reported in other populations. Further for the reported mutations in the study, phenotypic characteristics were compared, with the existing literature. For the novel mutations, identified pathological implications of the mutations were proposed based on *in silico* analysis. For the novel missense mutations identified in the study, based on *in silico* analysis and data from, *in vitro* cell expression studies suggest impaired multimerization or dimerization leading to secretory defect has been hypothesised. This, in turn, explains the phenotype in these series of patients.

1.2.4 Summary and conclusions

In the final chapter, summary, and conclusions, the main findings of the study and their implications in understanding the molecular basis of type3-VWD in these series of patients are summarised. In this study using the strategy, the PCR-CSGE-sequencing mutation was identified in 93 patients from 81 families. The present study highlights the phenotypic and genotypic heterogeneity in patients with type-3 VWD from India. A comprehensive genetic diagnosis including prenatal diagnosis has been established in our centre. *In vitro* expression studies on the missense mutations were suggestive of VWF retained in endoplasmic reticulum when compared to the wild type.

2. Literature Review

2.1 Overview of Blood Coagulation

Hemostasis is defined traditionally as the mechanism by which the bleeding ceases, following vascular disruption, to maintain the integrity of blood vessel (Furie and Furie, 2008). The term hemostasis was coined by Walter Bradford Cannon in 1930. Under normal physiological conditions fluidity of blood is maintained by the endothelial cells, which form the inner cellular lining of blood vessels. Due to damage to the wall of the blood vessel, a series of orderly events takes place at the site of injury to stem the loss of blood resulting in an insoluble fibrin clot (Seegers, 2013). This process necessitates the combined activity of vasculature (endothelial cells), platelets, and coagulation factors. In response to a vascular trigger, initially, the blood vessels constrict to reduce the blood flow at the site of injury. Uncoiling of VWF follows this process were the platelet binds resulting in platelet adhesion and aggregation mechanisms. These aggregated platelets act as a platform for the assembly of coagulation proteins. These proteins are serially activated by proteolytic reactions to form a stable fibrin clot.

2.1.1 Evolution of Blood Coagulation

Every biological organism requires an evolutionary explanation. Life on the earth originated from a common ancestor that thrived approximately 3.5–3.8 billion years ago (Wetherill, 1991). Evidence from microfossils suggests that life first evolved from a single cell organism i.e. prokaryotes. Several lines of evidence indicate that multi-cellularity was observed initially in cyanobacteria-like organisms.

The last common ancestor of vertebrates was the protostome–deuterostome ancestor, which thrived between 600 and 700 million years ago. The blood vascular system has undergone significant modification in individual phyla in response to the selective pressures experienced. Existing lines of evidence suggests that the blood vascular system was evolved in an ancestor Triploblast coelomate (e.g. Limulus) over 600 million years ago (Monahan-Earley et al., 2013). Blood vascular system is regressed (e.g. in flatworms and nematodes) in some cases, while in others the primitive system is transitioned to an open circulation system [Figure 1].

The endothelium is present in all vertebrates but distinctly absent in invertebrates. Studies suggest that endothelium appeared in an ancestral vertebrate through mutation and selection of a small number of pre-existing regulatory genes, between 440 and 510 million years ago, diverging from Urochordates and Cephalochordates (Monahan-Earley et al., 2013). Phenotypic heterogeneity was first observed in hagfish, which suggest that phenotypic heterogeneity as an ancestral feature rather than derived from cell lineage (Yano et al., 2007). Biochemical and sequence analysis tools indicate that serum proteins in blood coagulation [Figure 2] system evolved in parallel with the development of a pressurized compartment, within 50-100 million years window between the appearance of protochordate and vertebrates with the closed circulatory system (Doolittle, 2009).

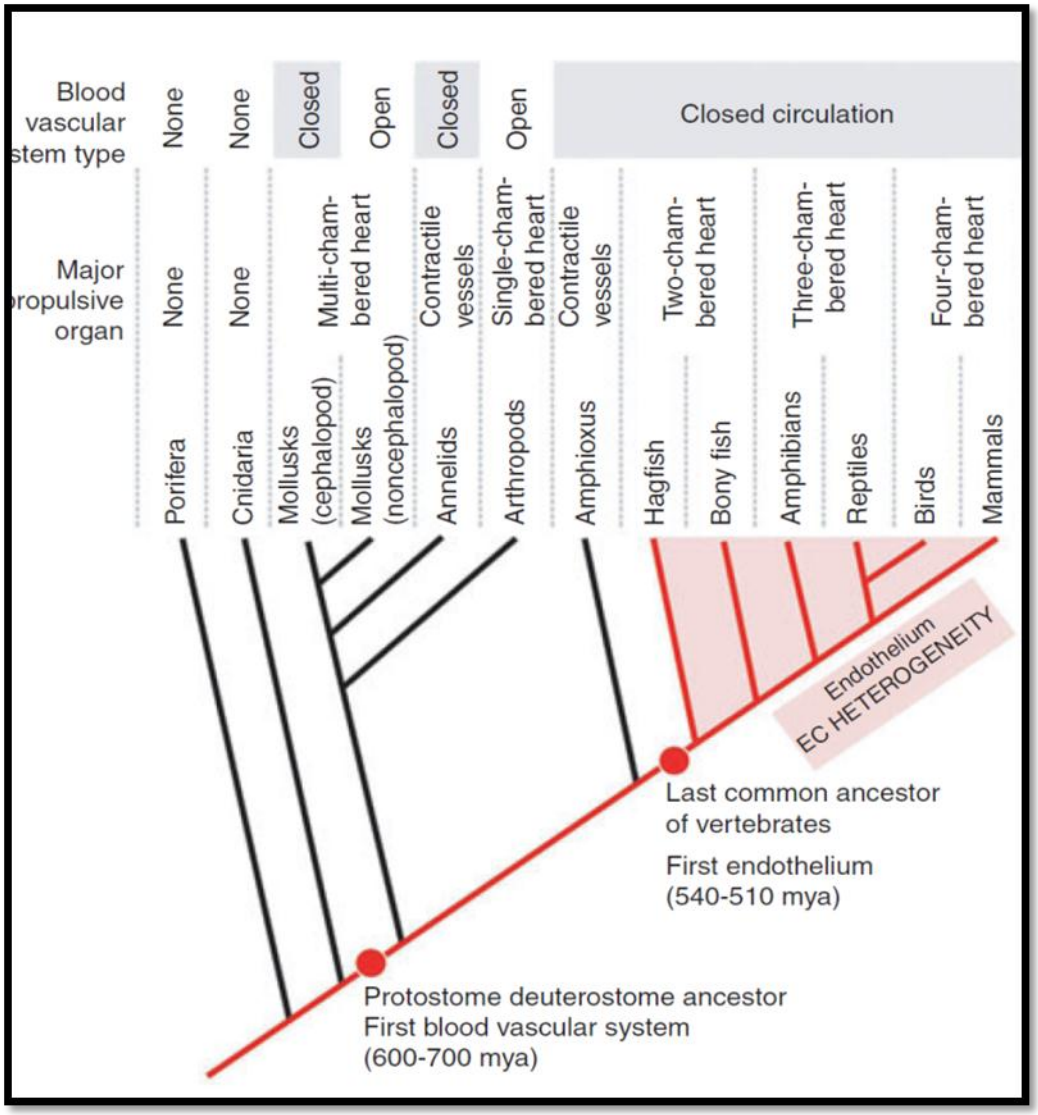


Figure 1: ***The Phylogenetic perspective of the blood vascular system.***

[Reference: Monahan-Earley R, J Thromb Haemost, 2013]

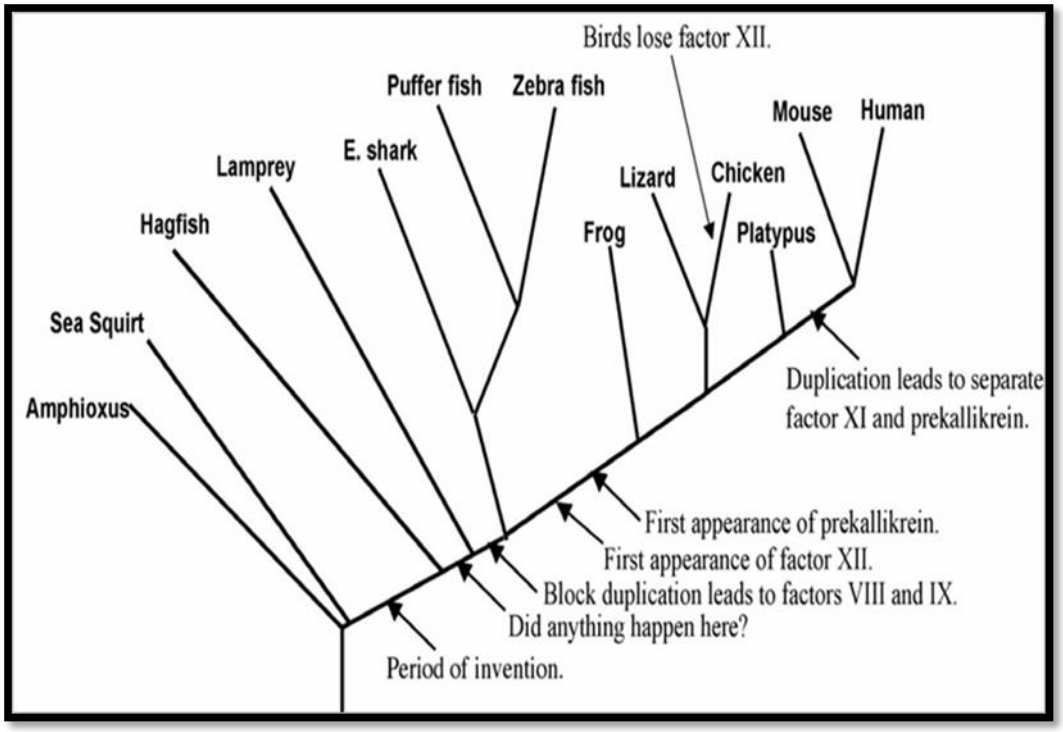


Figure 2: ***Timeline phylogeny for appearance (and disappearance) of various clotting factors during vertebrate evolution.***

[Reference: Monahan R et al, J Thromb Haemost, 2013]

In mammals, blood coagulation is delicately balanced and complex process involving more than two dozen extracellular proteins, which is needed to be converted from its precursor form, where half of the components comprise members of the serine protease family. Biochemical and sequence analysis tools strongly suggest that the network of coagulation factors was present in all jawed vertebrates; hence, it is presumed that they might have evolved before the divergence of tetrapods and teleosts. Sequence based phylogeny predictions on Gla–EGF1–EGF2–SP domains have offered insights into the relative order in which certain factors could have appeared. The proteins were found to be arisen by exon shuffling, or two rounds of gene

duplications to form mosaic proteins with diverse functions (Davidson et al., 2003). This hypothesis is supportive of the fact that subsidiary domains were found to exist in association with the catalytic domains. Gla (γ-carboxyglutamic acid) domains and discoidin domains localise the coagulation factors at the surface of platelets. These critical processes mediate protein-protein interaction leading to clot formation [Figure 3]. Sequence-based phylogenetic studies on numerous serine proteases suggest thrombin to be the earliest GLA-containing proteases. In mammals, thrombin is known to possess a broad range of activities including procoagulant, anticoagulant, and fibrinolytic activity, which suggest that thrombin and fibrinogen could have appeared simultaneously.

Coagulation system in mammals is a highly complex process, as explored by the pathogenic nature of the disease causing mutations which provide new insights to understand the molecular mechanisms of coagulation disorders (Doolittle, 2009). Deficiencies in coagulation proteins lead to nucleotide changes ranging from life threatening to milder variants. The most common bleeding disorders include Haemophilia A, Haemophilia B, and VWD**.** Joint evolutionary mutational studies on FVIII, FIX and FXI suggest that under negative selection mutations, were found to affect primarily the highly conserved residues resulting in instability of the respective proteins.

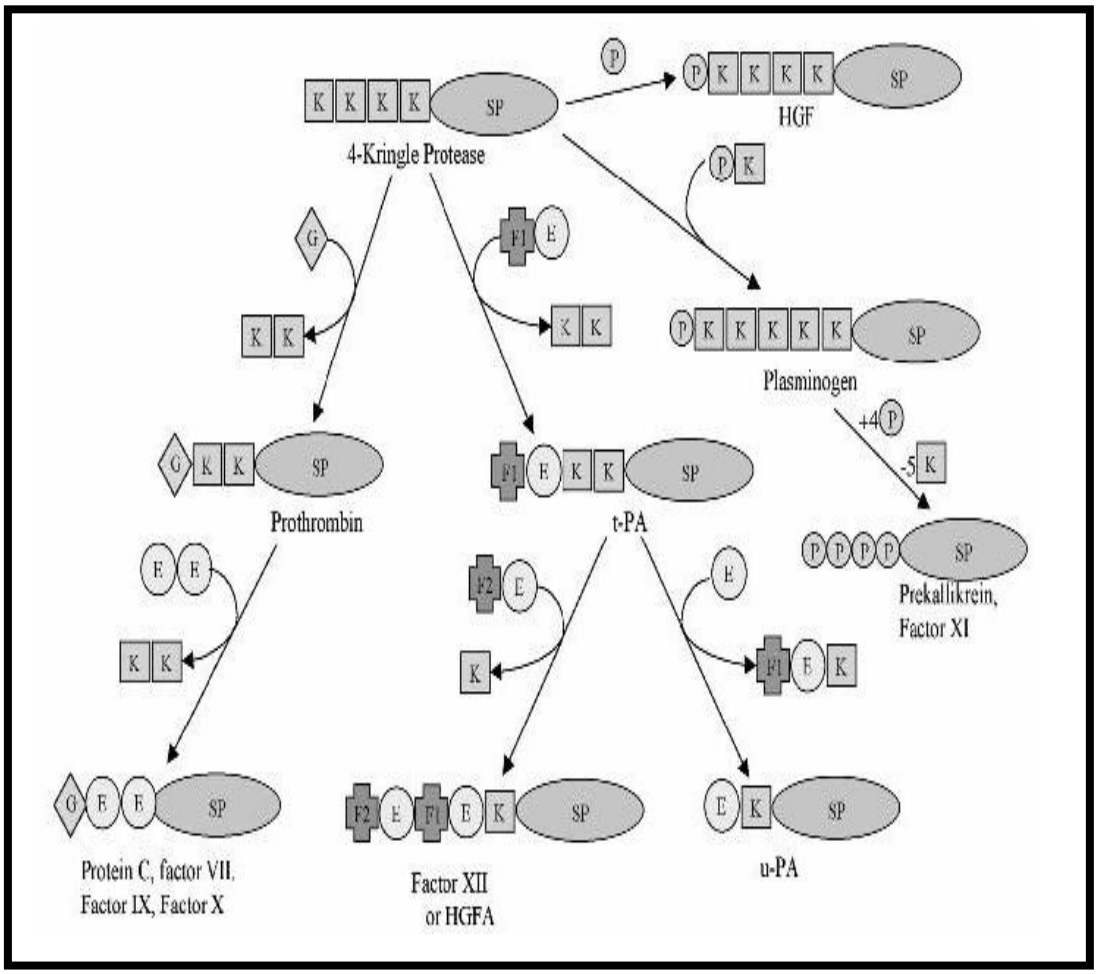


Figure 3: ***During early vertebrate evolution step-by-step rearrangement of domains that had presumably given rise to different clotting factor proteases.*** *(G) GLA domain, (E) EGF domain, (K) Kringle, (SP) serine protease, (P) PAN domain, (F1) fibronectin type I, (F2) fibronectin type II.*
[Reference: Jiang and Doolittle, Proc Natl Acad Sci, 2003]

2.1.1.1 Evolution of VWF

Evolution of VWF is highly complex and widely distributed. The D domains which impart a vital role in the species-specific recognition of extracellular matrix was identified in the zonadhesin, mammalian sperm membrane glycoprotein (Hardy and Garbers, 1995). VWF was found in several invertebrate orders,

indicating that VWF has deep evolutionary roots which predate the vertebrate blood circulation and its vital role in hemostasis (Sadler, 1998). Evolutionary studies on the repeated motifs in VWF suggest that repeated domains are due to a process involving exon shuffling or duplication (Mancuso et al., 1991). Domain A was found to be present in several proteins example: collagen α1 (VI) and α2 (VI) which contains three A domain and 12 in α3 (VI) chain. Studies have shown that in certain integrins including leukocyte adhesion receptors, collagen, and nonfibrillar collagens presence of A domain was observed.

However, the functions of these proteins are not demonstrated, where they are likely to be allosterically regulated, which may be metal ion independent. The domains near the carboxyl-terminal end (VWF B, C, D and CK domains) share with certain epithelial mucins are often associated with B domains or D domains. Homodimerization or oligomerization of these proteins was found to occur, mediated by disulphide bonds which were structurally similar to mucins (Perez-Vilar et al., 1998). The CK domains in the VWF were homologous to the neutrophil family of growth factor, and transforming growth factor. D domains were found to impart a pivotal role in recognition of extracellular matrix as observed in *Plasmodium falciparum*, malaria parasite. In the silk moth, *Bombyx Mori* homologue of VWF was identified. The inducible protein defensin, is also similar to the structure of VWF, except that they lack A domain. The presence of pseudogene, which is 99% similar to the authentic gene, suggests that they might have arisen by partial gene duplication (Mancuso et al., 1991). The observed phenomenon was consistent with the pseudogene in apes, but not in distantly related primates [Figure 4].

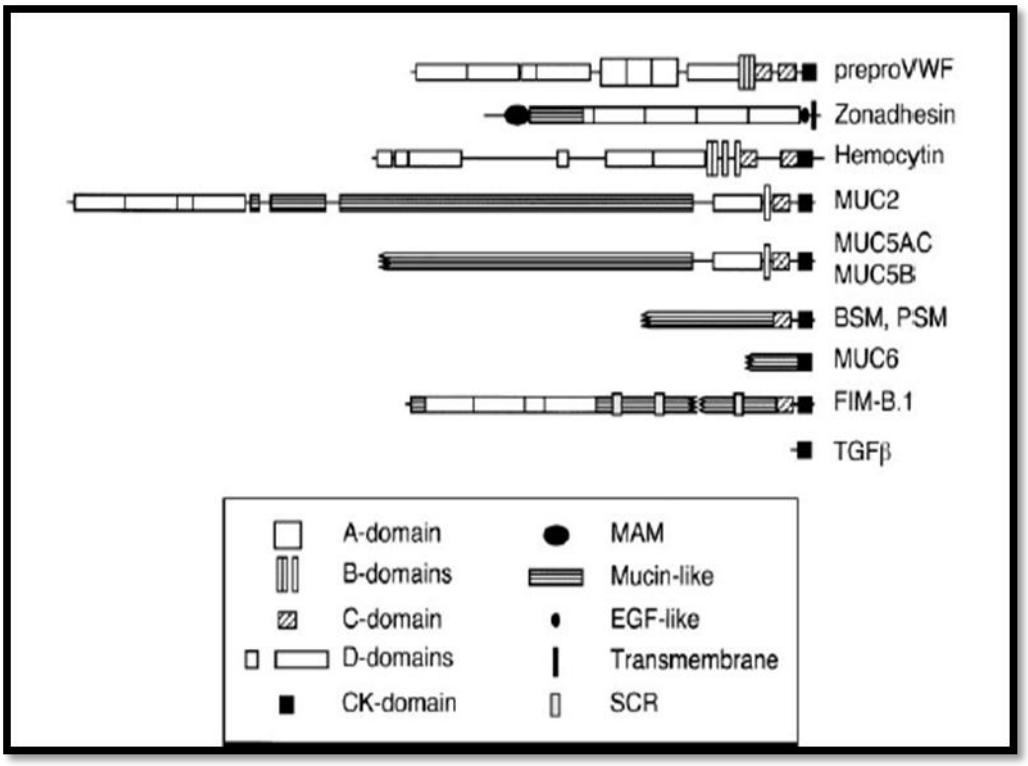


Figure 4: ***Schematic structure of proteins with conserved VWF B, C, D, and CK domains****. In addition to preproVWF, these include Zonadhesin; B. mori Hemocytin; the mammalian epithelial mucins MUC2, MUC5AC, MUC5B, and MUC6; bovine and porcine submaxillary mucins (BSM, PSM); X. laevis integumentary mucin FIM-B.1; and the large family of neurotrophins represented here by transforming growth factor (TGF). In mucins, certain portions are not cloned as illustrated by jagged like interruptions in mucin-like domains. Symbols for structural motifs include VWF A, B, C, and D domains; CK domains; MAM domains named for a common motif found in meprin, X. laevis neuronal protein A5, and protein tyrosine phosphatise mucin like domains, EGF-like domains, transmembrane domains, and complement consensus repeats (SCR). [Reference: Sadler JE, Annu Rev Biochem, 1998]*

2.2 Molecular Basis of Blood Coagulation

Blood coagulation involves cascade of signalling events where the zymogens of serine proteases, are transformed into active enzymes (Furie and Furie, 1988).

These enzymes aid in the conversion of the procofactor substrates to cofactors were these proteases assembles on the cell surfaces leading to the formation of a stable fibrin clot. Under normal physiological conditions, components of the blood coagulation machinery exist as zymogen or inactive enzyme precursor. All resting endothelial cells inhibit coagulation of blood by exhibiting unique non-thrombogenic properties (van Hinsbergh, 2012). In response to a denuded endothelium, primary hemostasis is triggered by a multitude of coordinated events, resulting in dramatic morphological and biochemical changes. Resting platelet exhibits circular disc shape structure. Platelets on activation impede dramatic morphological and biochemical changes. Activation of platelet results in a marked increase in surface area, expression of surface receptors, and release of granule contents. Platelets initially interact with VWF, resulting in adhesion and aggregation (Primary hemostasis) leading to the formation of fragile platelet plug (Broos et al., 2011). This process aids in the presentation of the procoagulant surface were the coagulation factors are sequentially activated, resulting in the formation of a fibrin-rich hemostatic plug at the injury site. Several mechanisms mediate blood coagulation, including dilution of the rate of blood flow, the action of circulating inhibitors TFPI and antithrombin,and so forth. Also, vascular and platelet reactivity is modulated by prostacyclin, thromboxane, and nitric oxide (Tanaka et al., 2009).

2.2.1 Endothelium

Blood fluidity is controlled by a thin layer of cells, endothelium, where the functions are attributed to specific location and tissue. Endothelial cells play a crucial role to regulate hemostasis, including maintenance of fluidity of blood, vasomotor tone,

and trafficking of blood cells between the underlying tissue permeability, angiogenesis, both innate and adaptive immunity (van Hinsbergh, 2012). Endothelium lining the luminal surface are metabolically active and play a central role in the regulation of coagulation. The critical function of endothelium is to maintain non-thrombogenic character. Adequate haemostasis is assured as a result of endothelial heterogeneity in different organs in the vascular system which explains the pathological response elicited in response to breached vascular system. Coagulation of the blood is inhibited by the endothelial cells, mediated by the tissue factor pathway inhibitors (TFPIs). This process inhibits the formation of factor-VIIa–tissue factor complex. Endothelial cells synthesize heparan sulphate proteoglycans which cause bound anti-thrombin III to inhibit thrombin molecules generated by the coagulation cascade. Nitric oxide synthase 3 (NOS3) and ecto-ADPase (CD39) inhibits the interaction between VWF and platelets. Anticoagulant and profibrinolytic actions proceed when activated protein C, in concert with its cofactor protein S, performs proteolytic degradation of Va and VIIIa.

When the wall of the blood vessel is severed, vasoconstriction occurs mediated by enothelin-1 and other vasoconstrictors, thereby diverting the flow of the blood at the site of injury. This process impedes cascade of signalling events leading to a burst of thrombin resulting in stable fibrin clot formation (Pober and Sessa, 2007).

2.2.2 Primary hemostasis

Platelets were first described by Giulio Bizzozero (1880) as small subcellular discoid shape structures (Steinhubl, 2011) which impart a pivotal role in

the process of hemostasis and thrombosis. They circulate in the blood stream with a life span of about 8-10 days. Biogenesis of platelets involves complex series of progression events in the bone marrow environment, where the process is brought by tailoring of cytoplasm and membrane system of megakaryocytes. Thrombopoiesis is regulated by the transcription factors GATA-1, FOG (friend of GATA) and NF-E2. Studies have shown that transcription factor GATA-1 is found to play a vital role in the process of megakaryocyte maturation while NF-E2 regulates thrombopoiesis (Patel et al., 2005). Platelets are characterized by the presence of (1) highly organized cytoskeleton (tubulin and actin polymers) (2) storage granules and (3) receptors which facilitate adhesion and aggregation. Under normal conditions, platelets circulate near the vascular wall. However, they are protected from activation due to the presence of an inhibitory substance such as PGI2 and NO, which are natural barriers to thrombosis (Broos et al., 2011). The binding site for the platelet GPIbα on VWF A1 domain remains cryptic in resting platelets.

In circulation, integrity and shape of the platelets are maintained by three major cytoskeletal components including spectrin, actin-based membrane skeleton, and marginal microtubule coil. Resting platelets were distinguished from the activated platelets by the presence of marginal microtubule coil. Orchestration of the platelets in response to breached vessel wall occurs via platelet-endothelium cell adhesion molecule (PECAM-1) expressed on the cell surface (Mei et al., 2014). Two classes of secretory granules are present in the platelet which includes dense and alpha granules. Dense granules secreted on platelet activation (ADP and calcium) enhance the process of platelet aggregation and their interaction with the

coagulation factors. The second types of granules are the alpha granules, including platelet factor 4, fibronectin, vitronectin, and VWF. Fibronectin and vitronectin play a profound role in matrix assembly and spreading of the platelets. Fibrinogen acquired from the plasma by receptor-mediated endocytosis imparts a vital role in the process of platelet aggregation. Platelets also serve as a source factor for Factor [F] V (Duckers et al., 2010), FXIII (Gosk-Bierska et al., 2011), and so forth.

2.2.2.1 Platelet Adhesion and Activation

In a severed blood vessel when the sub-endothelium, gets exposed the platelets initially undergo the process of tethering, rolling, and spreading over the damaged vessel wall. The platelets are now activated mediated by a series of morphological and biochemical changes were the shape of the platelets changes from disc-shaped structures to long dendritic lesions.

Initial interaction between GPVI and collagen retains the platelets closer to the blood vessel. This process further aids in the release of thromboxane A2 (TxA2) and adenosine diphosphate (ADP), which are secondary agonists. Preceded by this process involves a multitude of events resulting in firm adhesion mediated by the coordinated action of platelet receptors (Broos et al., 2011). The function of these platelets is attributed to the blood flow and shear conditions. Adhesion of the platelets at the vicinity of injury involves key events involving the interaction between the platelet receptors and the ligands. Adhesion of platelets at low shear rate i.e. rates <1000 s^{-1} primarily involves binding to collagen, fibronectin and laminin in arteries and veins (Tokarev et al., 2011).

In the initial phase of primary hemostasis, platelets roll and spread followed by adhesion mediated by platelet receptors. At the high shear rate, i.e. >1000 s^{-1} in microvasculature and arteries interaction occurs between, VWF and glycoprotein Ibα (GPIbα). GPIbα consists of leucine-rich repeats with GPIbβ, GPIX, and GPV in a stoichiometric ratio of 2:4:2:1. Von Willebrand factor a large multimeric and multifunctional protein secreted from the Weibel palade bodies as ultra large multimers, binds to platelets to aid fragile platelet plug formation. Further also with the locally produced thrombin, a cascade of signalling events mediate an array of G-coupled tyrosine kinase receptors. This process results in an increase in calcium levels which in turn guides the impulsive process of platelet activation and granule release (Gibbins, 2004).

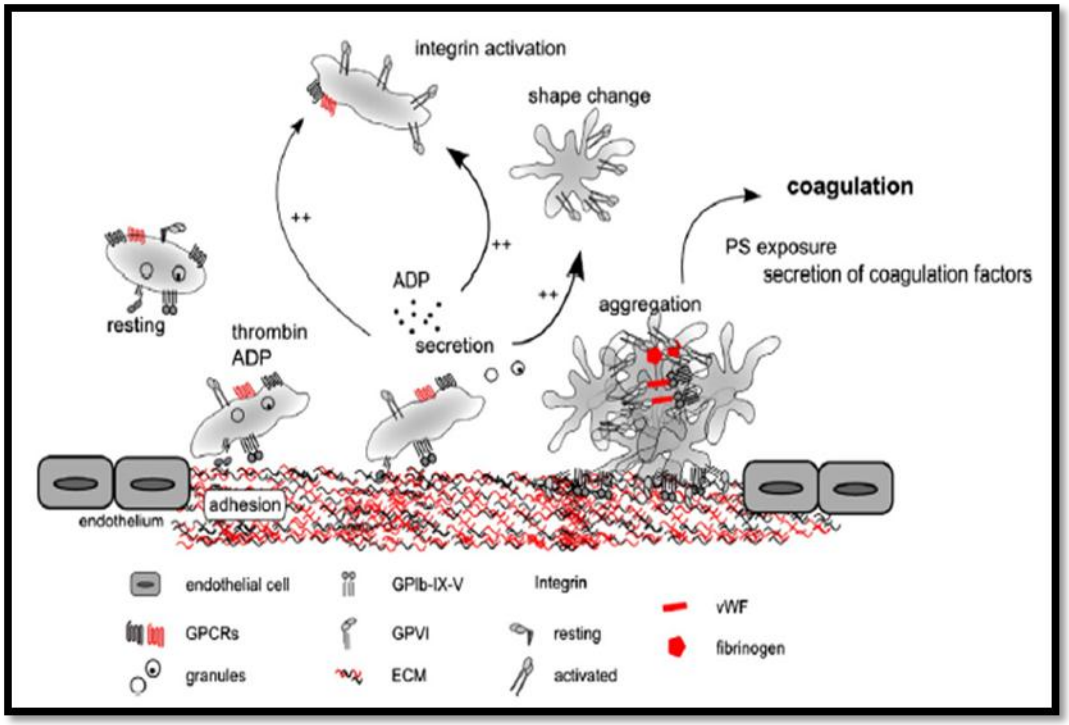


Figure 5: ***Schematic representation of platelet activation***

Exocytosis of the platelets aids in the delivery of the major effector molecules which mediate thrombus formation at the site of injury. These key processes were facilitated by the canalicular system, which is reminiscent of the demarcation membranes. Platelet adherence via GPIbα to VWF is not sufficient to mediate clot formation. Studies have shown that the bond formed between, VWF and platelets are transient with fast on- and off-rates, which permits the platelets to roll in the direction of blood flow (Kumar et al., 2003) [Figure 5].

Platelet activation impedes upregulation of their integrins. Integrin alpha IIb beta 3-integrin being predominant binds to fibrinogen present in plasma, whereby the circulating platelets acquire new adhesive properties. This process aids in the engagement of other receptors in platelets and impedes the process of platelet aggregation (Jackson, 2007). All these critical processes are highly mediated by local rheological conditions. Platelet recruitment continues until thrombus formation occurs to limit the process of haemorrhage. This key process is sufficient when there is a small breach in the vascular wall. Following primary hemostasis, exposure of phosphatidylserine, mediated by the flippase activity occurs on the surface of the platelets. These platelets act as a platform for the coagulation factors to culminate resulting in a stable clot formation.

2.2.3 Secondary Hemostasis

Blood coagulation in mammals is initiated and regulated by a complex array of interactions, where multitude of events occurs to transform zymogens in the plasma to active forms. Core components of the secondary hemostasis include the coagulation proteins which are activated, resulting in the fibrin-rich haemostatic

plug at the injury site. Currently accepted model for coagulation, is the cell-based model of coagulation (Hoffman and Monroe, 2001), which allows a thorough understanding of how haemostasis occurs *in vivo* [Figure 6]. This model proposes that coagulation takes place on different cell surfaces in four key steps: initiation, amplification, propagation, and termination.

Given the current model, the initial haemostatic process is triggered by expression of Tissue Factor [TF] present on the surface of the membrane of cells such as smooth muscle cells, fibroblast, and subendothelial pericytes. Based on the Idling theory which states that TF remains in an active state, however, a small amount of thrombin is generated, even at conditions where the vascular integrity is maintained. At the site of vascular damage, initially serine protease patrols, followed by binding of FVIIa, which circulates at a lower concentration; FVIIa binds to TF to activate Factor X to FXa, but also factor IX. FX generates a trace amount of thrombin (0.1–1 nM). Antithrombin III and TFPI are serine protease inhibitor which neutralizes the initially formed FXa and thrombin (Hoffman, 2003). Hence, the procoagulant activity proceeds when TF was exposed at a level high enough to overcome inhibition by TFPI and AT. Tissue Factor, when bound to FVIIa in the blood, results in the formation of the complex FVIIa/TF, which in turn activates trace amount of FX and FIX (Smith, 2009). Further, this process aids in the formation of prothrombinase complex, on the cell surface. FX, formed initially, generates only trace amount of thrombin which is not sufficient to aid stable clot formation. Haemostatic components in the vasculature including VWF, FVIII and platelets come in contact with the limited amount of thrombin (generated initially

during the initiation phase in a severed blood vessel). Platelets in the presence of thrombin undergo a series of events to form a fragile platelet plug resulting in primary haemostasis.

Thrombin generated initially activates platelets including alteration of permeability of platelet membrane, entry of calcium ions into circulation, followed by the release of chemotactic substance which brings forth the coagulation factors into play (in addition to FV, which is released partially). Thrombin activates FVa, cleavage of FVIII and subsequent activation of VWF (Smith, 2009). This process brings forth the fibrin clot generation mediated by the coagulation factors in the circulation resulting in secondary hemostasis. Also, Thrombin activates FXI on the surface of the platelets. A large number of platelets in circulation now migrates to the site of injury resulting in the formation of tenase and prothrombinase complex, which marks the beginning of amplification phase. Stabilization of the fibrin clot occurs via activated FXIII, a transglutaminase which aids in the formation of a firm clot. Hence, the assembly of enzymes and cofactor on the surface of phospholipid surface is essential to aid propagation phase coupled with regulation of anticoagulant mechanism. Thrombin, a central protease in coagulation cascade mediates various processes in the procoagulant pathway.

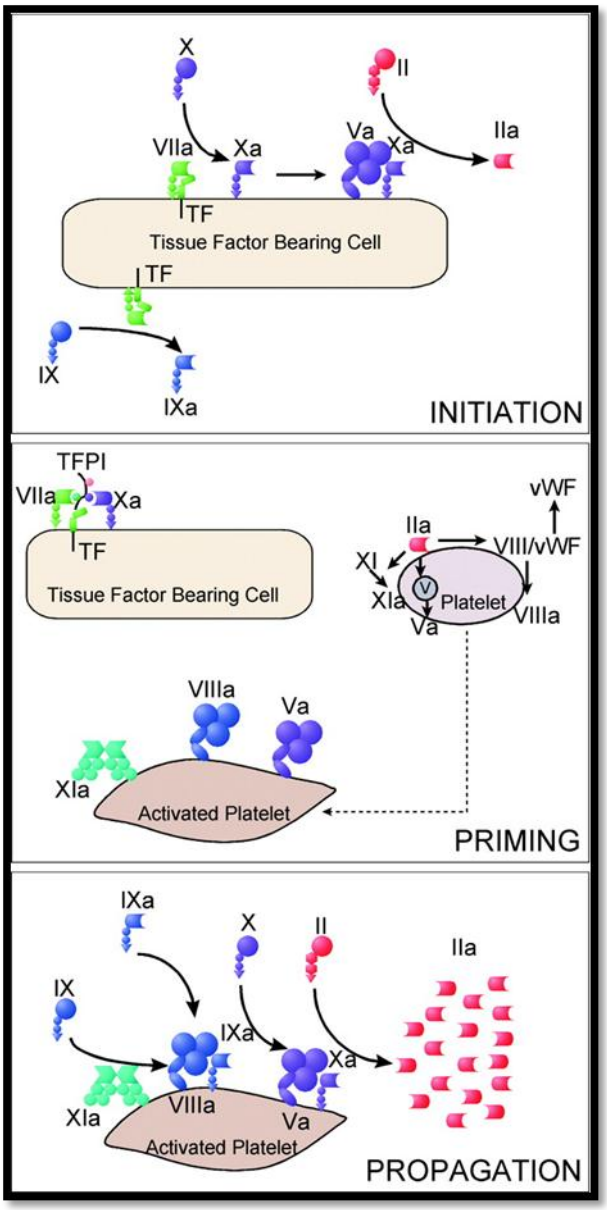


Figure 6: ***Cell-based model of coagulation.***

[Reference: Hoffman and Monroe, Blood Reviews, 2003; Dougald M. Monroe et al, Arterioscler Thromb Vasc Biol, 2002]

2.2.4 Regulation of Blood Coagulation

Blood coagulation in mammals needs to be regulated to prevent massive fibrin deposition. Hence the components associated with the blood coagulation must be active only at the vicinity of injury for a sufficient span of time to form a fibrin clot. Various regulatory mechanisms maintain this key process (Dahlback, 2005). Regulation of

blood coagulation in mammals at each level of the pathway is mediated by complex interactions involving positive and negative feedback mechanisms [Figure 7]. Components including platelets, endothelium, and plasma proteins are involved. Activation of coagulation and formation of complexes occurs only at the surface of activated platelets and cells, in the presence of negatively charged phospholipids. Hence the essential trigger for coagulation process is pronounced in response to a denuded endothelium. Series of anticoagulant proteins proceed to limit their coagulant time span. Finally, the fibrin clot formed is dissolved into fibrin degradable products mediated by the fibrinolysis pathway (Cesarman-Maus and Hajjar, 2005).

2.2.4.1 Tissue factor pathway inhibitor (TFPI)

Formation of TF-VIIa complex marks the beginning of coagulant process, were the activation of FIX and X occurs (Hoffman, 2003). Formation of this complex is complex is inhibited by Tissue factor pathway inhibitor (TFPI), a Kunitz-type inhibitor synthesized by the endothelial cells. TFPI regulates the formation of tenase complex (Girard et al., 1989, Wood et al., 2013). The mode of action follows; TFPI initially forms a complex with one one of the domains in Xa. The complex formed now binds to TF-VIIa complex, resulting inhibition of the cascade. In addition to this process, TFPI aids in the internalization and degradation of the inhibited complex (Wood et al., 2013).

2.2.4.2 Antithrombin

Antithrombin a serine protease inhibitor, serpin inhibits factors including FIXa, Xa, TF-VIIa complex and thrombin (van Boven and Lane, 1997). Heparin sulphate proteoglycans accelerate inhibition of these factors. One of the key players

being thrombomodulin expressed on the surface of endothelium. Thrombin when bound to antithrombin results in the formation of thrombin–antithrombin (TAT) complex [Figure 8]. Antithrombin also aids in the removal of the proteases from the circulation and confines their activity only at the vicinity of the clot (Weitz, 2003).

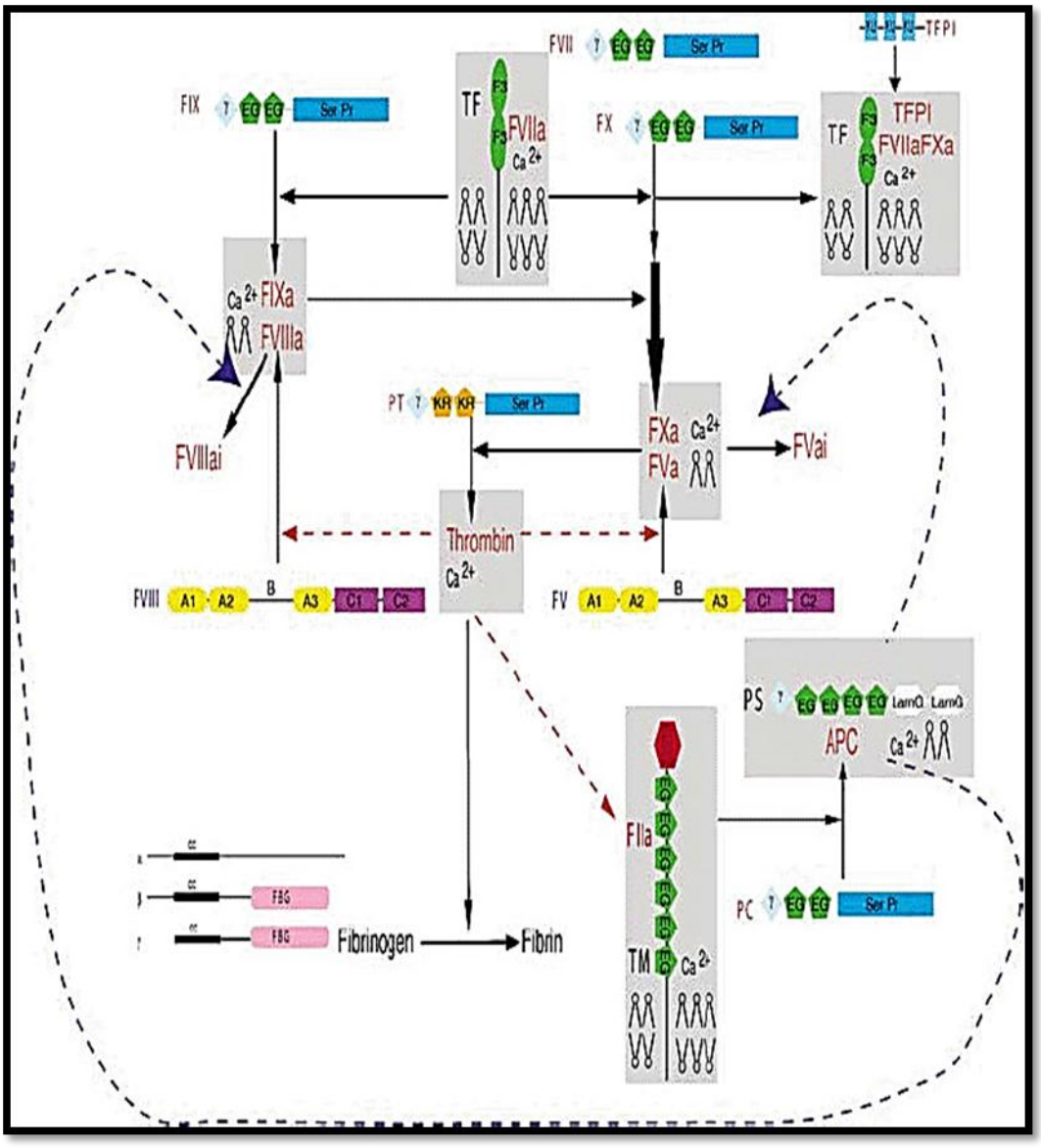


Figure 7: ***The blood coagulation network in mammals****. The coagulation protein modules were illustrated as proposed by Bork and Bairoch [31]. Red dashed line depicts activation by thrombin, and blue dashed line indicates a negative feedback inactivation by activated protein C/protein S.*
[Reference: Davidson et al, J Thromb Haemost, 2003]

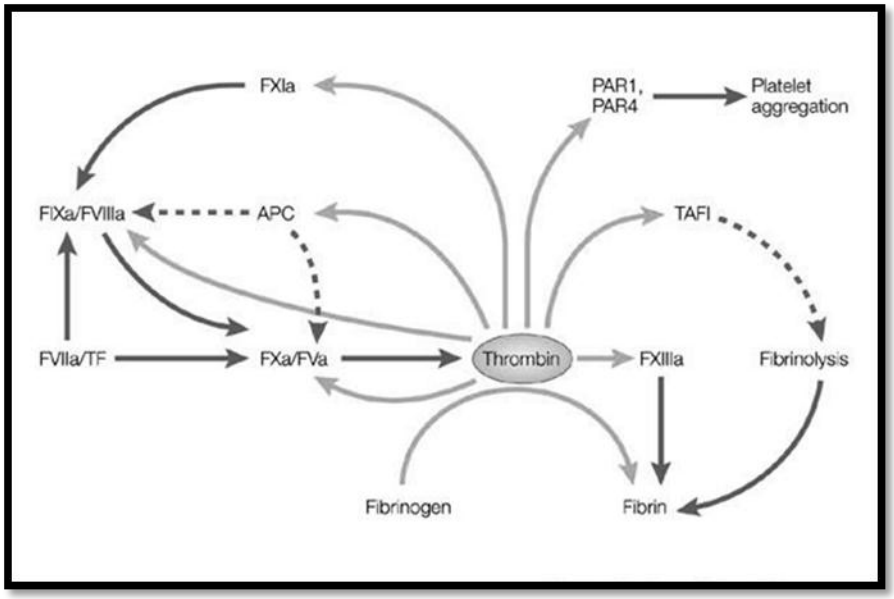


Figure 8: ***The central role of thrombin in coagulation.***

[Reference: Davidson et al, J Thromb Haemost, 2003]

2.2.4.3 The protein C-anticoagulant pathway

In circulation, protein C circulates as an inactive zymogen. It is activated when bound to the endothelial protein C receptor (EPCR) via GLA domain, expressed on the surface of the endothelium (Mosnier et al., 2007). An essential factor for the activation of the protein C (PC) is free thrombin. Free thrombin, when bound to thrombomodulin (endothelial cells), presents PC for activation. Activated PC dissociates from the EPCR, and binds to its cofactor PS. This results in the formation of the complex (APC/S), which inactivates FVa and FVIIIa, and formation of prothrombinase/tenase complex [Figure 9].

Inhibition of the activity of FXa is brought about by protein Z. It circulates in plasma associated with ZPI (Zinc Protease Inhibitor). The structure is identical to that of FVII, FIX, and FX. Protein Z displays pivotal role in regulation of coagulation by forming a complex with FXa and PZI (Broze, 2001)

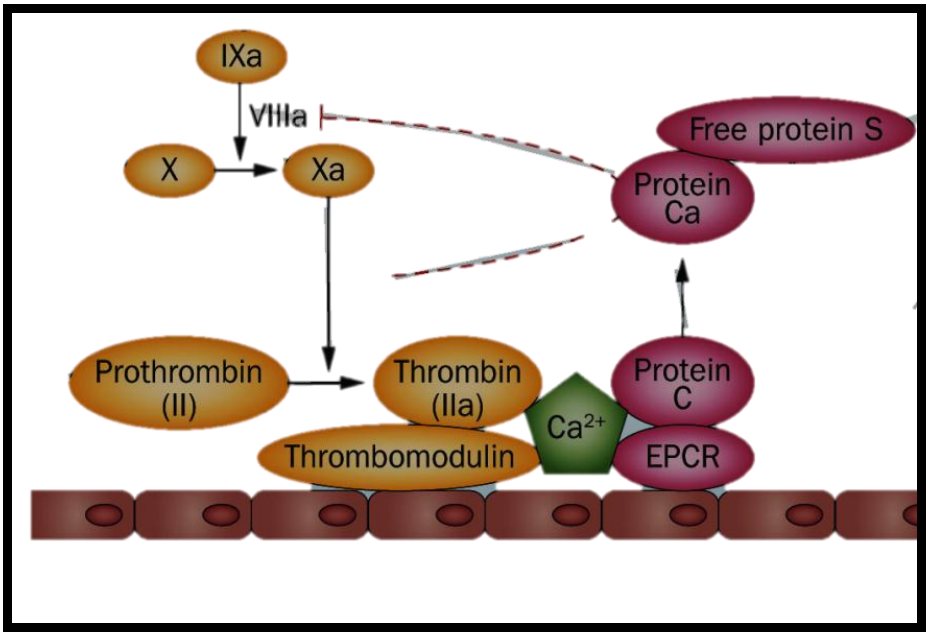


Figure 9: ***Anticoagulant mechanisms of the protein C and protein S system.***

[Reference: Martinelli I et al, Nat Rev Cardiol, 2014]

2.2.4.4 Fibrinolysis

Once the hemostatic function is restored fibrinolysis aids to limit the clot formation at the vicinity of injury. On activation, the fibrinolytic system cleaves the fibrin to fibrin degradation products mediated by series of enzymes. Plasmin, the key enzyme involved in this process circulates as inactive zymogen plasminogen. Formation of plasmin occurs when fibrin binds to the heavy chain of the plasminogen molecule bind to the fibrin via lysine [Figure 10].

Functionally there are two distinct plasminogen activators (Foley et al., 2013). In response to thrombin or occlusion of the venous system tissue-type plasminogen activator (tPA) are released. Urokinase-type plasminogen activator (uPA) and contact factors including FXIIa, plasmin, and kallikrein are also released.

Regulation of fibrinolysis occurs at two levels. α2- antiplasmin inhibits the activity of plasmin, and the inhibitors of plasminogen activators regulate the activity of tPA and uPA (Dejouvencel et al., 2010).

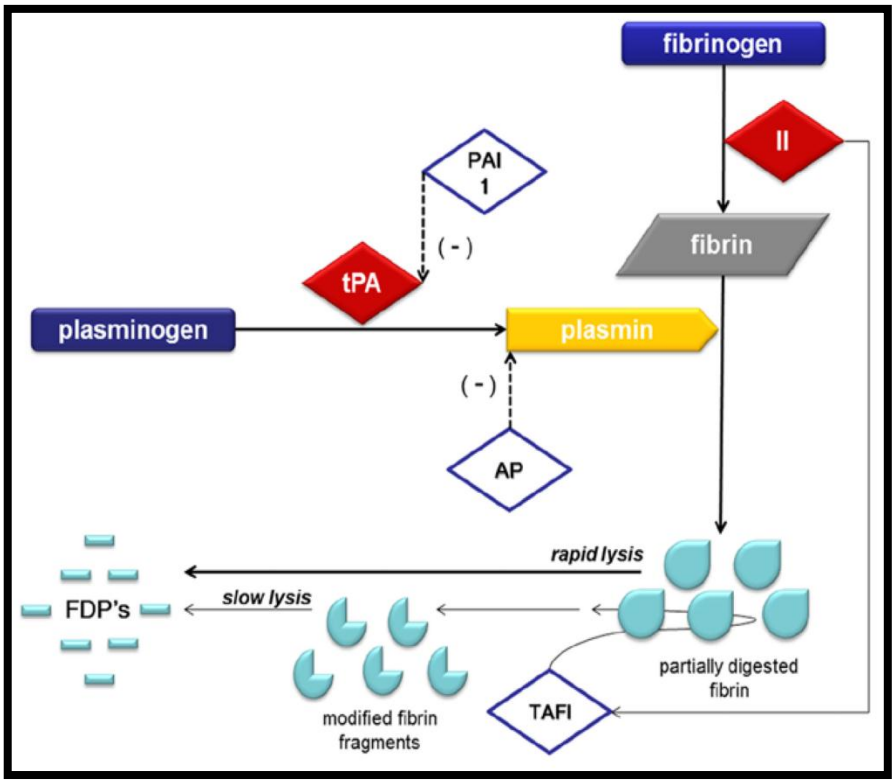


Figure 10: ***Mediators and Inhibitors of fibrinolysis.***

[Reference: Gonzalez Eet al, Scand J Surg, 2014]

2.3 Biology of Von Willebrand Factor

The physiological response elicited, in response to a denuded endothelium involves array of events. The key process involves activation of blood coagulant plasma proteases. This process results in localized burst of thrombin leading to the subsequent conversion of soluble fibrinogen to fibrin (Gale, 2011). Generation of thrombin at the vicinity of injury involves complex interplay involving plasma proteases, protein cofactors, and substrate zymogen, which assembles at the surface

of the damaged cell wall (Mancuso et al., 1991). VWF imparts a vital role in both primary and secondary hemostasis. It circulates in plasma as inactive form, stored in the cytoplasmic granules and released in response to the hemostatic challenge (Shida et al., 2014). It also acts as a chaperone for FVIII thereby protecting it from proteolytic degradation (Sadler, 1998).

Expression of VWF is regulated transcriptionally by positive and negative feedback mechanisms. Upstream to the promoter at -32 position lies TAATTA sequence, TATA box, and downstream being CCAAT sequence (Hough et al., 2005). Existing lines of evidence suggest when VWF binds to the transcription factors ETS, GATA, and YY1, results in upregulation of VWF locus.When VWF binds to NF1, Oct1, YY1 (to a non-CCAAT sequence) and E4BP4 was found to repress the expression of VWF (Mercier et al., 1991). Repetitive sequences observed in VWF includes, 14 Alu repeats and intron 40 spanning ~670bp, with a repeat of TCTA. Studies have shown that polymorphic dinucleotide repeat at position –1234, -1185 and –1051, was found to co-segregate with the haplotype study (Mercier et al., 1991).

2.3.1 Von Willebrand Factor

VWF a complex molecule both structurally and functionally maps to the short arm of chromosome 12 (Sadler, 1998). VWF synthesised as a prepropolypepide, by the endothelial cells and platelets encompasses 2813 amino acids (Lillicrap, 2013b). On synthesis, 95% endothelial VWF (Low, Intermediate, and HMVW (High Molecular Weight Multimers) multimers are secreted constitutively, whereas remaining is stored in the cytoplasmic granules of endothelial cells and platelets (Millar and Brown, 2006).

VWF cDNA was cloned in the year 1985, from the endothelial cells by four groups in US and Europe (Sadler, 1998). The gene comprises the signal peptide (22 residues), a propeptide (741 residues), and a mature subunit (2050 residues). These features implicate that the pre-pro-protein before it enters circulation undergoes two rounds of proteolytic processing during its conversion to mature VWF as follows; (a) Signal peptide cleavage mediated by signal peptidase (b) Cleavage of the propeptide by endoprotease. The precursor protein is highly repetitive in structure (Sadler, 1998). Remarkably the protein is rich in cysteines which constitute about 8.3% of the total residues in VWF (Shapiro et al., 2014). A recent study using mass spectrometry analysis reveals the presence of unpaired cysteines in circulation, is a prerequisite necessary for the proper folding, secretion and formation of disulphide bonds (Shapiro et al., 2014) in the protein. Two factors complicate the analysis of the gene. (i) The Large size and (ii) Existence of a non-functional part of the gene, pseudogene which displays 97% similarity to the authentic gene (Surdhar et al., 2001). Duplication of the pseudogene spans exons 23 to 34.

2.3.2 Domain structure

Domains in the VWF were identified by using various techniques including, synthetic peptides, monoclonal antibodies, deletion or scanning mutagenesis. Analysis of the sequence of VWF reveals, four distinct types of domains in the order D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK [Figure 11]. The presence of repeated domains in VWF implicates the origin of the gene by duplication or exon shuffling mechanisms (Sadler, 1998). D1 and D2 domain characterise the

propeptide, and the remainder corresponds to the mature subunit. Cross-talk between the domains and their functions, were evaluated using, bioinformatics tools have led to propose the following domain structure comprising [Figure 11], D1-D2-D'-D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK (Shapiro et al., 2014). The D domains exhibit a complex structure. D domains are separated from the triplicated A domain by a segment of more than 600 amino acids. Exons span in size from 40 bases to largest spanning 1.4 Kb (exon 28), while the introns vary from 96bp to 19.9Kb [Table 1].

Two remarkable disulphide loops comprising 185 amino acids lie one in the A1 domain and A2 domain. In the A1 domain, disulphide bond lies between Cys 509 and 695, in A3 domain between Cys 923 and 1109. An important proteolytic site within A2 domain includes amino acids between 842 and 843. At the C-terminal of the D3 domain, the disulphide bridges are involved in the process of dimerization and multimerization. On secretion, the cysteines were found to be paired by disulphide bonds, which was not restricted to a single subunit. Multimers range in size from 500 (protomer) to over 15000 kDa. Common to the characteristic of the adhesive proteins the multimers exhibit an RGD (Arg-Gly-Asp) sequence, with a high proportion of cysteine residues (18.7%). These cysteine residues are involved in inter or interchain disulphide bridge formation. Summary of the functions of these domainsis illustrated in [Table 2]

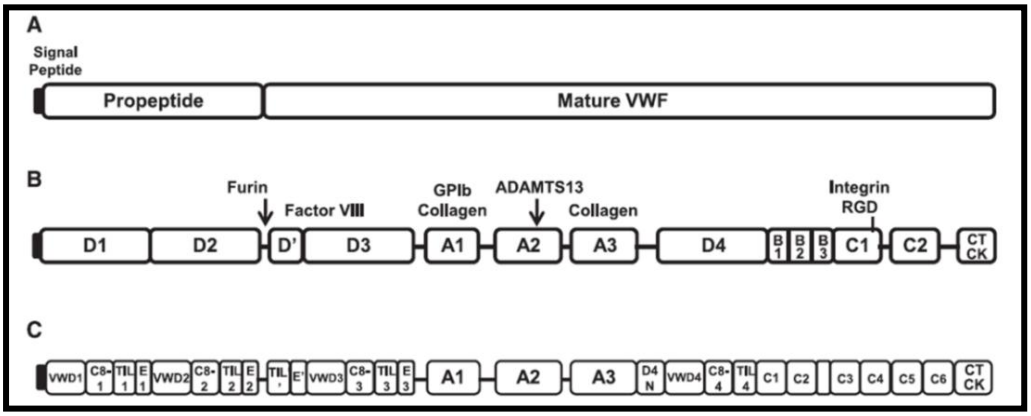


Figure 11: ***VWF structure and binding/ cleavage site (****A) VWF contains a signal peptide, propeptide, and mature VWF subunit. (B) VWF encompasses homologous domains (A-D), where specific binding or cleavage sites are mapped. (C) Refined domain structure of VWF.*

[Reference: Sandra L, Blood, 2015]

Exon	Length (bp)	Intron	Length (bp)
1	360	1274	1274
2	54	1	1804
3	165	2	10205
4	103	3	283
5	209	4	14789
6	127	5	19906
7	217	6	1593
8	123	7	1176
9	112	8	987
10	47	9	60203
11	137	10	752
12	139	11	1191
13	101	11	4909
14	196	13	776
15	216	14	4073
16	441	15	5725
17	95	16	2271
18	161	17	7802
19	122	18	1561
20	139	19	3109

Exon	Length (bp)	Intron	Length (bp)
21	135	20	1955
22	147	21	3295
23	141	22	211
24	114	23	1800
25	157	24	732
26	159	25	704
27	137	26	331
28	445	27	1380
29	1379	28	1494
39	117	29	97
31	141	30	283
32	144	31	2443
33	165	33	1350
34	44	34	292
35	178	35	15394
36	221	36	1394
37	193	37	211
38	342	38	10843
39	200	39	6153
40	103	40	443
41	75	41	3053
42	206	42	5525
43	150	43	4401
44	111	44	2207
45	181	45	1043
46	41	46	524
47	117	47	13891
48	99	48	976
49	129	49	507
50	40	50	1960
51	98	51	582
52	337	52	600

Table 1: ***Exon and Intron size for Human VWF gene.***

[Adapted from Mancuso DJ,J Biol Chem, 1989]

Name	Binding domain	Site of interaction
VWF propeptide	D', D3	Endothelial cells; platelets
FVIII	D', D3	Circulation, some endothelial cells
P-selectin	D', D3	Endothelial cells
ß2-Integrins	D, D3, A1, A2, A3	Leukocytes
Glycoprotein Iba	A1	Platelets
Osteoprotegrin	A1	Circulation, endothelial cells
b2-Glycoprotein I	A1	Circulation
PSGL-1	A1	Leukocytes
Collagen VI	A1	Subendothelial Matrix
ADAMTS-13	A1, A2,A3, D4	Circulation
Collagen I	A3	Subendothelial Matrix
Collagen III	A3	Subendothelial Matrix
Thrombospondin	A3	Circulation
IGFBP7	D4-CK	Circulation, endothelial cells
aIIbb3	C4 (RGD motif)	Platelets
avb3	C4 (RGD motif)	Endothelial, tumour cells
Fibrin	C1–6	Thrombus
CTGF/CCN2	CK	Endothelial cells
Galectin-1	Glycan moieties	Circulation, endothelial cells
Galectin-1	Glycan moieties	Circulation, endothelial cells
Siglec-5	Glycan moieties	Macrophages
Angiopoietin-2	Unknown	Unknown
Interleukin-8	Unknown	Circulation, endothelial cells
LRP1	Unknown	Macrophages

Table 2: ***Human protein ligands for mature VWF.*** *CK (cysteine knot); CTGF/CCN 2 (connective tissue growth factor); IGFBP7 (insulin like growth factor binding protein-7); LRP1 (LDL receptor- related protein 1); PSGL-1(P-selectin glycoprotein ligand-1).*

[Reference: Lenting PJ, J Thromb Haemost, 2012]

2.3.3 Synthesis of Von Willebrand Factor

Synthesis and post-translational modification involved in the processing of VWF is mediated by the multistep process, involving endoplasmic reticulum, golgi apparatus, and post-Golgi compartment. In the endoplasmic reticulum the, monomeric

form of the protein, initially assembles as dimers via an interchain disulphide bond (covalent connection) formation at the C-terminus in a tail-to-tail fashion which results in the formation of 3 interchain cysteine pairs. These cysteine pairs ensure the long-term dimeric configuration of VWF [Figure 12]. In the endoplasmic reticulum, pH is neutral and oxidoreductases are present. These dimers, when transported to endoplasmic reticulum N-linked glycosylation, occurs at 12 potential sites on the propeptide and mature subunit, which is necessary for the proper folding of the protein (Lenting et al., 2015). In a developing propeptide chain, the attachment of asparagine residues occurs mediated by the enzyme oligosaccharyl transferase.

Pro-VWF dimers when transported to the trans-Golgi network (TGN), undergo head-head interchain disulphide bond formation, between the D3 domains of VWF dimers, were high mannose oligosaccharides are processed to complex form (Lenting et al., 2015). Following sulphatation and O-linked glycosylation, the formation of disulphide bonds at the N-termini of adjacent dimers occurs. The mature subunit undergoes extensive glycosylation with N-linked and 10 O-linked oligosaccharides before secretion. This oligosaccharide makes up to 20% of the mass of VWF. They have shown to confer protection to VWF, thereby protecting it from proteolytic degradation and maintain the multimeric structure of VWF (Vlot et al., 1998). Glycosylation accounts for ~18% to 19% of the total protein mass. The majority of the glycans are capped by sialic acid structures (Vlot et al., 1998). At the later stage, VWF undergoes extensive disulphide bond formation, sequenced by novel disulphide isomerase activity within the VWF propeptide. In a recent study were springers group has shown that dimeric bouquet

structure formed was mediated by assembling of B1-CK and six globular domains to adopt flower like arrangement (Lenting et al., 2015) [Figure 12].

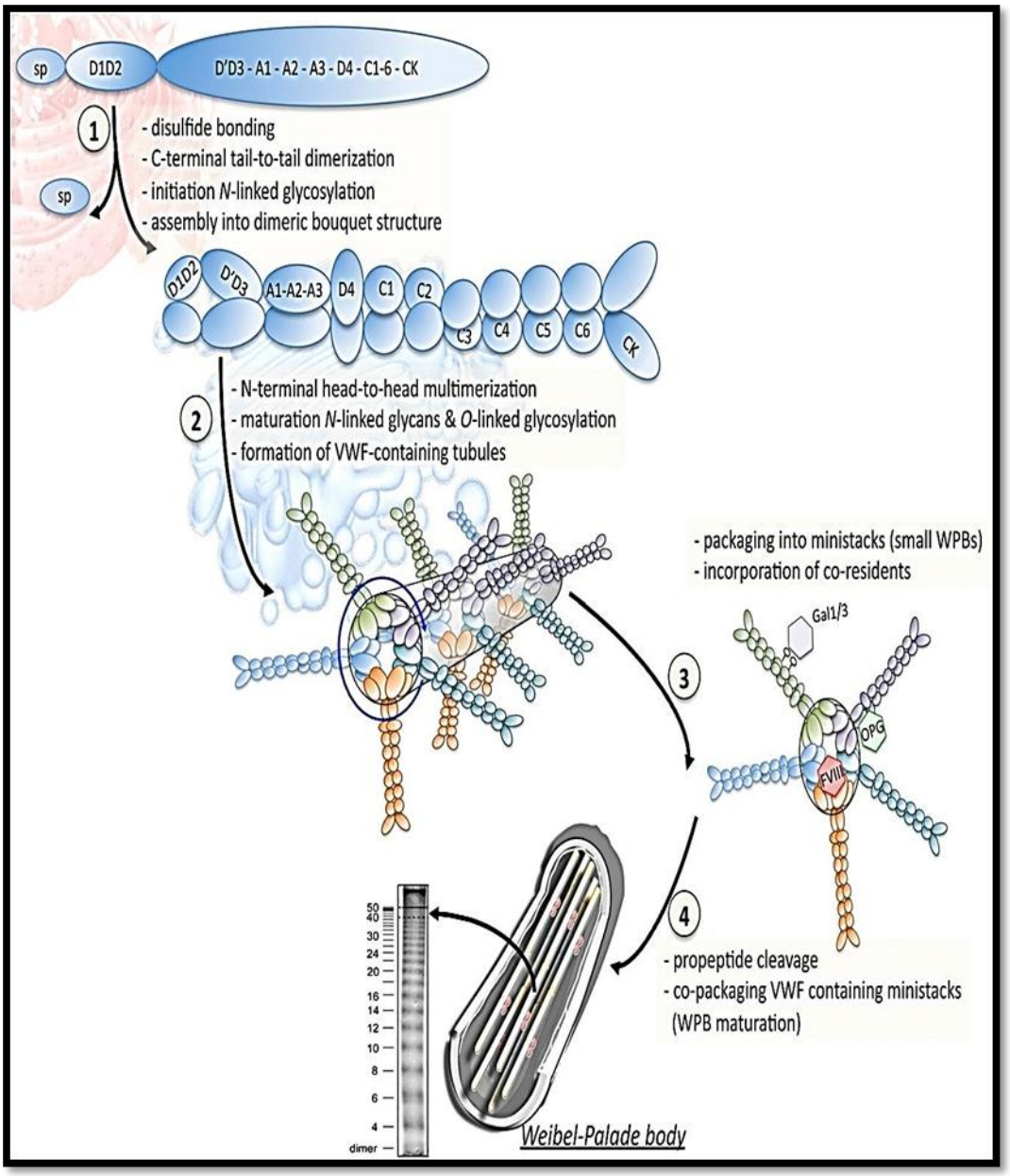


Figure 12: ***Biosynthesis and packaging of VWF in WPBs.***

[Reference: Lenting PJ, Blood, 2015]

2.3.3.1 VWF multimer formation and storage

Formation of multimers occurs at the distal Golgi network, requires slightly acidic environment, where the enzymes necessary for the formation of disulphide formation are lacking. At low pH in the TGN, these dimers assemble into a right-handed helical structure having 4.2 repeating units per turn, a variable pitch of 9–12 nm (Lenting et al., 2015) [Figure 13]. The propeptide self-associates to align VWF to form multimer assembly (Valentijn and Eikenboom, 2013). Enzyme Furin mediates processing of pro-VWF into VWFpp and mature VWF.

The site of propeptide cleavage is characterized by the sequence motif Arg-Xxx-Arg/Lys-Arg at the C-terminal end of the propeptide. Propeptide cleavage is an essential prerequisite for multimerization and secretion, which occurs in the TGN before storage [Figure. 14]. This phenomenon is the characteristic of VWF maturation. Multimerization aids in the generation of a heterogeneous pool of differently sized multimers ranging from 2 to 60 subunits (Valentijn and Eikenboom, 2013) [Figure 14].

Functional Tubules of VWF lie in the TGN, where they lie in the process of budding off with high electron-lucent density. Cutler's group have reported the origin of immature WPBs, where a single tubule can induce membrane protrusions in the membrane. Based on the morphological analysis WPB adopts cigar shaped structure [Figure 14]. They undergo classic maturation involving homotypic fusion, giving rise to multiple microtubules (Tooze et al., 2001). After synthesis, VWF is stored in the WPB in the endothelial cells and alpha granules in platelets. The mean plasmatic concentration of VWF ranges between 5 and 10 $\mu g\ mL^{-1}$. In the normal population VWF, antigen levels show a wide distribution. The ABH blood

group phenotype, the key determinant expressed in the VWF present in plasma, but not in platelets. Studies suggest, individuals with blood group H exhibit plasmatic levels 25-30% lower than non-H individuals (Smith et al., 2010)

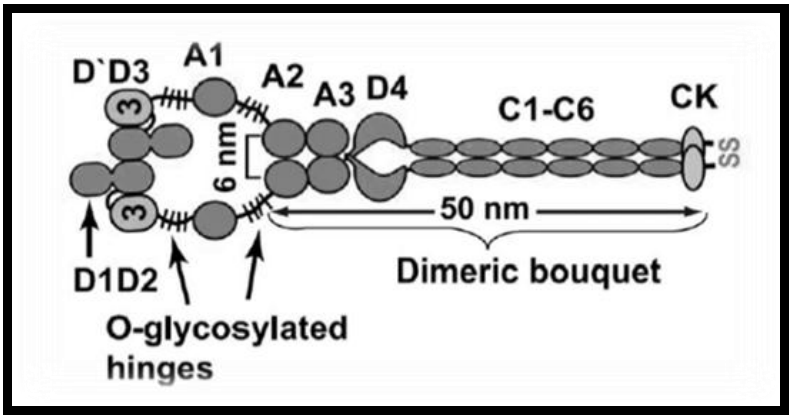


Figure 13: ***Dimeric bouquet structure of VWF.*** *At acidic pH 6.2, VWF zips into "dimeric bouquet" in the Golgi.*
[Reference: Springer TA, Blood, 2014]

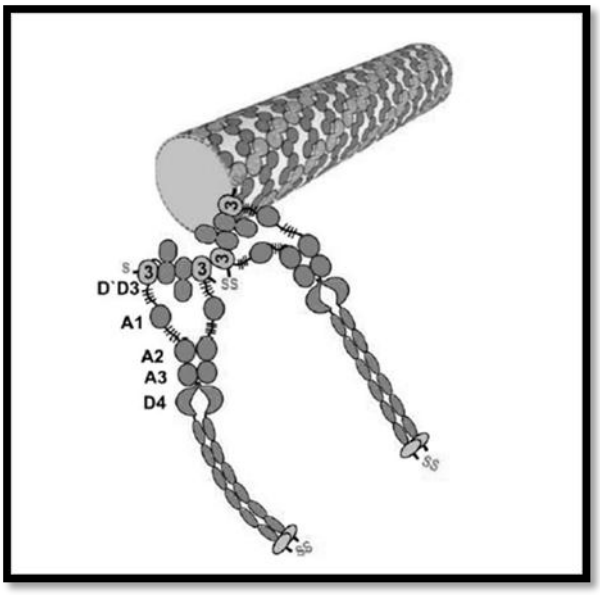


Figure 14: ***Organization of VWF multimers into a helix centred around the proVWF/D'D3 structures with projecting outwards A2-CK domains.***
[Reference: Springer TA, Blood, 2014]

2.3.3.2 Exocytosis of VWF

Secretion of VWF occurs by two modes. 1. Regulated secretion 2. Constitutive secretion. Constitutive secretion requires no cellular stimulation, which is taught to occur by continuous synthesis and exocytosis after processing in the Golgi. Smaller VWF multimers produced on secretion, is sensitive to inhibitors of protein synthesis (Valentijn and Eikenboom, 2013). Regulated secretion requires, stimulus to release VWF from the storage granules. Physiological secretagogues such as thrombin, epinephrine, and vasopressin induce the release of VWF from the endothelial cells. Models proposed for exocytosis of VWF includes the following (a) Multigranular exocytosis (b) lingering-kiss exocytosis (c) Single WPB exocytosis depending on the VWF release in bulk or small amounts (Valentijn and Eikenboom, 2013) [Figure 15].

In the single WPB body exocytosis, fusion of one WPB with plasma membrane occurs whereas in multigranular exocytosis secretory pod formation occurs were several WPBs coalesce to form new structures. In the lingering mode of exocytosis, a small pore is formed between the WPB and membrane, allows selective release cargo molecule release from WPB. VWF on secretion adopts three different conformations. VWF released from the Weibel palade bodies are incredibly long termed as ultra large multimers [Figure 16]. The multimers isolated from the plasma were shorter, displaying globular structure, which undergoes extension only in response to shear conditions (Lenting et al., 2015). The normal plasmatic concentration of VWF ranges from 500 to 1000 mcg/dl. Platelets account for about 15% of the quantity of VWF. The concentration of VWF in the plasma vary among normal individuals, and the polymorphisms appear to impart a pivotal role (Campos et al., 2011).

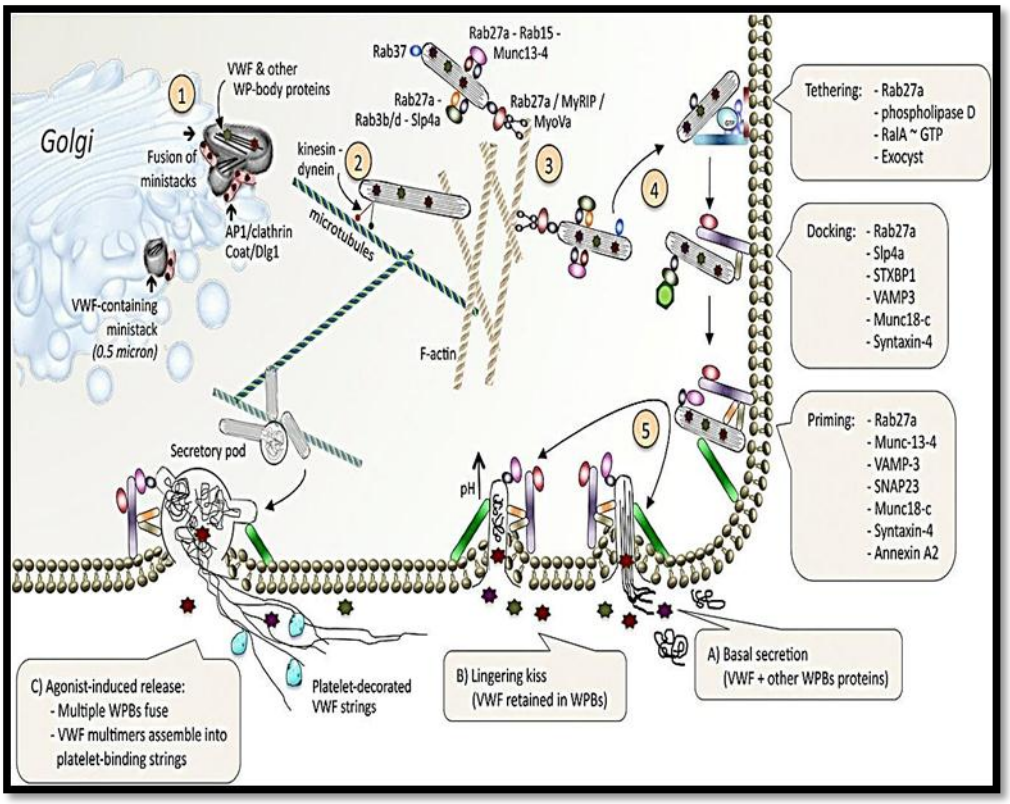


Figure 15: ***Schematic representation of secretion of VWF from the endothelial cells.*** *Following synthesis and packaging in WPBs, VWF follows a complex pathway allowing intra-endothelial storage combined with basal and regulated secretion. VWF formation-containing ministacks and subsequent WPBs requires the presence of clathrin coat, the adaptor protein AP-1, and Dlg1. In the endothelial cells, the WPBs move randomly along microtubules driven kinesin/dynein complex. WPBs adhere onto the filamentous actin network mediated by Rab27a, MyRIP, and MyoVa. Series of other proteins in the secretory machinery, includes Rab3, Rab15, Rab27a, Rab37, Munc13-4, and Slp4a. Protein complexes participate in each of these steps. Secretion of VWF occurs by three modes: (A) In basal secretion a single WPB fuses with the membrane where the contents are released.(B) Under rare circumstances, lingering kiss process occurs. Exposure of the organelle to the extracellular environment results in rapid deacidification thereby provoking the VWF structure to collapse which is pH dependent. As a result, VWF is retained within WPB. However, other WPB protein follows secretion. (C) Multiple WPB's aggregates, upon stimulation of the endothelium resulting in the formation of a large secretory vesicle, called secretory pods. Further secretion of the massive amount of VWF multimers occurs. Multimers assembles as long bundles, which are highly thrombogenic and they efficiently, recruit platelets.*

[Reference: Lenting PJ, Blood, 2015]

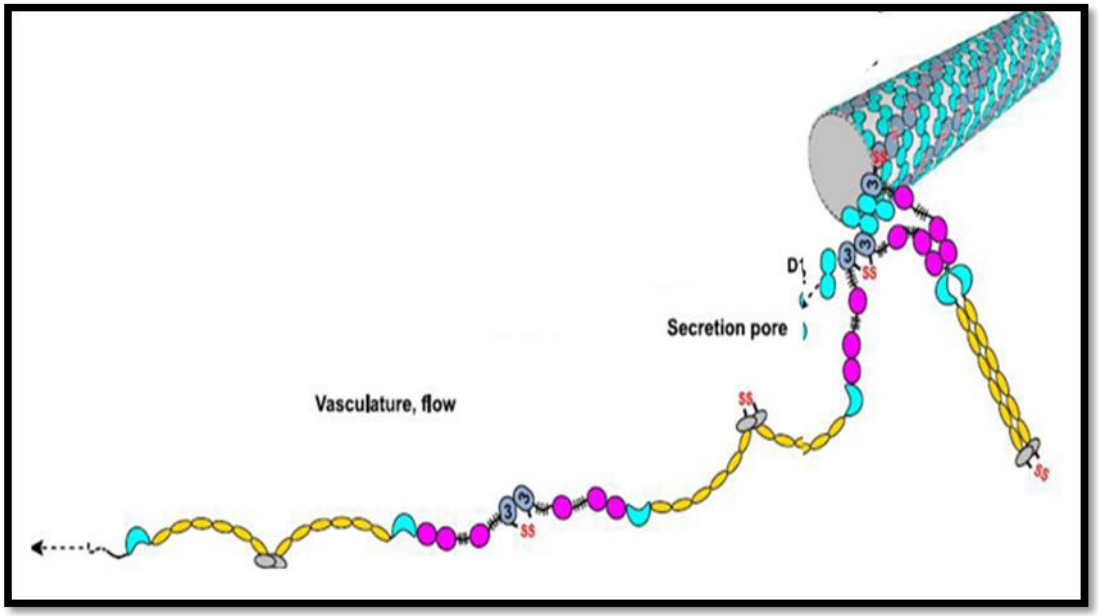


Figure 16: ***Schematic organization of domains during biosynthesis and secretion based on structural data.***

[Reference: Springer TA, Blood, 2014]

2.3.4 Functions of VWF

VWF plays a critical role in haemostasis by acting as a bridging molecule at the vicinity of injury between the platelets and subendothelial structures. It also acts as a chaperone for FVIII, thereby protecting it from proteolytic degradation (Ruggeri and Ware, 1993). The large multimers are involved in haemostasis, which mediates platelet endothelial interaction by providing multiple binding sites in the vicinity of injury. Ultra large multimers released from the cytoplasmic granules in response to agonists such as thrombin and fibrin, result in luminal and abluminal secretion of VWF (Chow et al., 1998). They were found to be hemostatically more efficient than larger multimers in plasma. Abluminal secretion was found to confer binding of platelets to the subendothelium in response to the hemostatic challenge, and luminal secretion may facilitate clot formation.

2.3.4.1 Binding to Platelets

Platelet adhesion and aggregation at high shear rate occurs as follows. The first step involves unfolding of VWF were A3 domain binds to subendothelial components (collagen). The second phase is preceded by binding of VWF to platelets mediated by A1 domain resulting in the formation of GP Ib-IX-V on the surface of the platelets (Miura et al., 2000). These two processes confer activation of VWF. Under high shear stress, vimentin a structural protein enhances the binding of VWF to platelets (Da et al., 2014). Under low shear stress conditions, aggregation of platelets occurs mediated by fibrinogen (Timmons et al., 1984).

2.3.4.2 Binding to subendothelium

VWF binds to various types of collagen including (types I, II, III, IV, V, and VI). Under high shear stress type VI collagen, binds to A1 domain which is critical for binding to platelets (Flood et al., 2015). Binding of collagen to VWF brings about conformational changes in VWF. VWF, on uncoiling, exposes the domain for the FVIII to bind, whereby binding occurs, at the D'-D3 domain, to release FVIII occurs locally. This process aids in the formation of a fibrin clot (Bendetowicz et al., 1999).

2.3.4.3 Carrier function of factor VIII

In plasma, VWF circulates as a non-covalent complex with FVIII. These interactions extend survival of FVIII. Plasmatic changes in VWF level results in a concordant decrease in FVIII levels. The key role of this complex formation was described in patients with undetectable levels of VWF. The binding site for FVIII on VWF localised within a tryptic fragment termed as SPIII-T4 composed of

3 distinct domains, namely TIL′ (residues 766-827), E′ (residues 829-863), and VWD3 (residues 867-1031), previously termed as the D' domain (Foster et al., 1987). On molecular analysis, mutations harboured at the FVIII binding site were found to have decreased FVIII survival (Shiltagh et al., 2014) which implicates that the VWF is essential for stabilization of FVIII in circulation. In response to a hemostatic challenge, VWF binds to platelets where interaction occurs between plasma proteases resulting in the subsequent conversion of FVIII into an active form. VWF controls survival of FVIII. The mean circulating half-lives of VWF ranges, from 12 to 18 hours and FVIII 12 hours.

2.3.4.4 Functions of VWF propeptide

VWF, initially synthesized as propeptide imparts fundamental role in the intracellular processing of VWF. Propeptide represents 26.3% of the primary translation pro-VWF product encompassing 741-aminoacids (Rosenberg et al., 2002). These propeptides act as "intramolecular chaperone" which aid proper folding of the proteins.

Cysteine-rich homologous D domains characterize propeptides. Studies suggest that propeptide also imparts vital role in the process of multimerization, and regulated storage of VWF (Haberichter, 2015). VWFpp binds to VWF via D' and D3 domain were the affinity increases at low pH. These interactions occur only in response to the haemostatic challenge.

2.3.5 Clearance of Von Willebrand Factor

In plasma, VWF circulates as multimers of different size. Ultra large multimers are involved in hemostatic functions. Multimer size is an important

determinant of the key functions, which is highly regulated by the enzyme ADAMTS-13 (Lenting et al., 2015). ADAMTS-13 serves as the natural substrate, for the proteolysis of the VWF, under shear stress conditions ranging between 3000 and 5000s. This process occurs by uncoiling of the VWF, were A2 domain is sandwiched, between A1 and A3 domains (Valentijn and Eikenboom, 2013). VWF, when cleaved by ADAMTS-13, adopts a globular conformation, which is resistant to further proteolysis. Four decades ago Sodetz and co-workers reported that presence of sialic acid is crucial for the survival of VWF protein in circulation (Sodetz et al., 1977). This phenomenon was confirmed in the clinical setting that, the half-life of VWF released on desmopressin treatment was found to correlate with the extent of sialylation (Sodetz et al., 1977). The removal of sialic acid results in exposure of penultimate galactose residues, which is recognized by asialoglycoprotein receptor (ashwell receptor).

Under normal physiological, conditions clearance of VWF, mediated by ashwell receptor is limited. However, under pathological conditions including pathogen infection, the clearance pathway is upregulated. As described earlier, there lies a strong correlation between VWF levels and ABO (Piagnerelli et al., 2005) locus. Individuals with O blood group were found to have ~25% low VWF levels compared to non-O (Piagnerelli et al., 2005) blood group, even more, reduced in rare Bombay blood group phenotype (O'Donnell et al., 2005), which may be due the inability to produce H antigen. However, the exact mechanism remains unclear. The Correlation between the amount of A antigenic determinants and level of expression of A transferase suggest that the effect is mediated by ABH antigenic

structure on the molecule rather than by indirect mechanisms (Morelli et al., 2007). The ratio of VWFpp: VWF released in circulation is 1:1 ratio, however, varies between blood groups with the higher ratio for O blood group in par with non-O blood groups. Hence, higher ratios may result in decreased half-life of VWF in blood group O persons compared to non-O blood group individuals, (Fischer et al., 2009). Hence, blood group on the VWF molecule could potentially affect the synthesis or secretion of VWF.

2.3.5.1 Receptors VWF clearance

Macrophages in the liver and spleen are involved in the uptake of circulatory VWF, as evidenced by the intravenous accumulation of VWF antigen in VWF-deficient mice (van Schooten et al., 2008). Under pathological conditions, asialoglyco protein receptor is involved in the clearance of VWF. Further, Siglec-5 expressed on different cell types including, macrophages was identified as the key receptor for recognizing the sialic acid structures on the protein, to facilitate removal of VWF from circulation [Figure 17] (Crocker et al., 2007). Genes encoding three distinct receptors namely stabilin-2, expressed in the sinusoids of the endothelial cells, CLEC4M, endothelial cells (C-type lectin domain family four-member M) and LRP1, ubiquitous receptor have been proposed as the determinants of plasmatic levels of VWF (Rastegarlari et al., 2012).

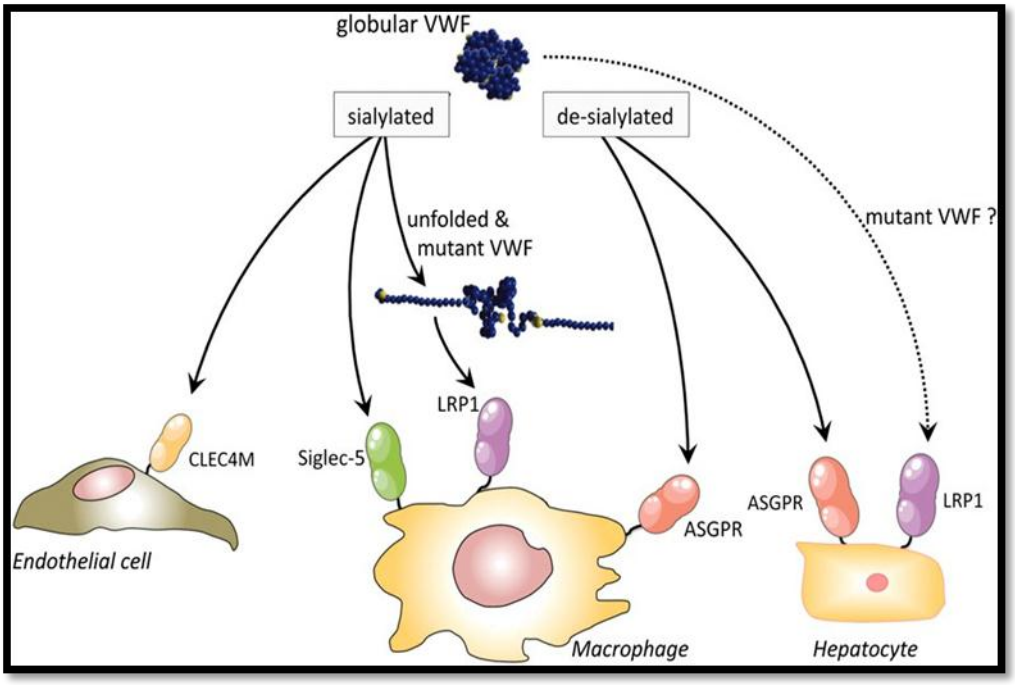


Figure 17: ***Pathways associated with clearance of VWF.*** *In circulation, VWF circulates as a globular protein with the majority of its glycan structures being sialylated. Two different receptors mediate the removal of VWF from the circulation: CLEC4M on endothelial cells and Siglec-5 on macrophages. Unfolding of the VWF under shear stress results in the exposure of interactive site(s) for LRP1. LRP1 plays a vital role in the uptake of VWF by the macrophages, supported by the in vitro and in vivo experiments. Accelerated clearance of VWF occurs in the absence of shear stress, due to certain mutations. Exposure of terminal galactose residues occurs resulting in desialylation, favouring efficient interaction with receptors on the hepatocytes and macrophages.*

[Reference: Casari C et al, J Thromb Haemost, 2013]

2.4 Molecular Basis of Von Willebrand Disease

2.4.1 Inheritance pattern of VWD

VWD is caused due to mutations in the VWF gene; displays marked heterogeneity in their clinical presentation. Based on the phenotype of VWF in the plasma, the disease is categorized into (a) quantitative deficiency of VWF (type1/3) (b) qualitative (type-2) deficiency of VWF (Goodeve, 2010). Type 1 and type 2A, B, M follows the autosomal dominant pattern of inheritance [Figure 18]. There is a 50% chance with each pregnancy that the child will inherit VWD or will not inherit VWD mutation, if only one of the parents has a dominant inheritance type of VWD. Type 3 and type 2N follows the autosomal recessive pattern of succession. If both the parents carry the defective recessive gene for type 3 or 2N VWD, there is 25% chance of having an unaffected child or child with VWD and 50% chance of being a carrier [Figure 19].

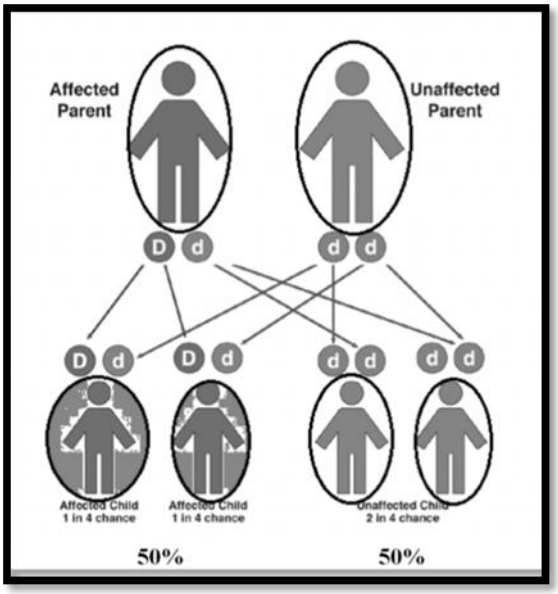


Figure 18: ***An autosomal dominant pattern of inheritance.*** *[Type 1, and type 2 VWD]. Dominant gene represented as capital D.*

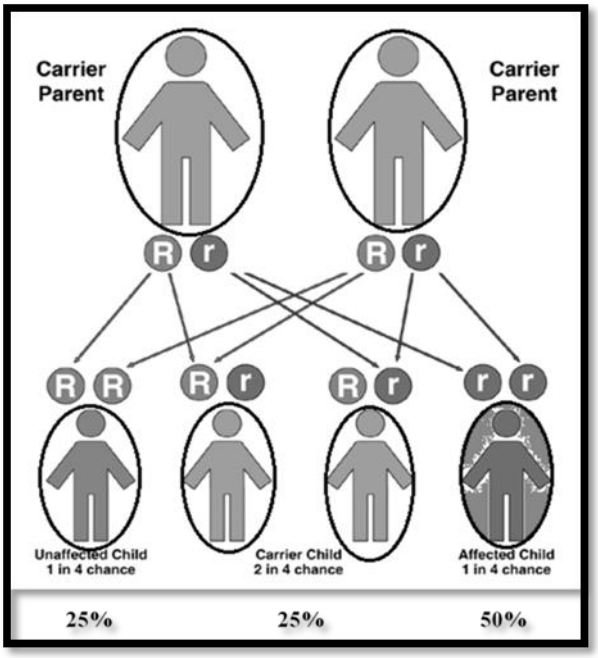


Figure 19: ***An autosomal recessive inheritance pattern of inheritance*** *– the possible gene combinations with types[2N and 3 VWD]. The recessive gene represented as small r.*

Globally in the last two decades, there has been growing appreciation of the clinical impact of VWD. Since the cloning and characterization of the gene in 1985, simultaneously by four groups, several studies have attempted to elucidate the molecular mechanisms contributing to pathogenicity (Goodeve, 2010). To understand the females at risk of being a carrier, it is important to comprehend their inheritance pattern. In sporadic cases, de novo mutations were observed. Apart from measuring the VWF: Ag levels, genetic analysis is required to determine the carrier status reliably. Germline and somatic mosaicism complicate the genetic diagnosis of VWD, however, reports on such cases is limited (Murray et al., 1991, Budde et al., 2008). In these cases, conventional mutation scanning techniques may fail to detect the underlying defect, if the proportion of the mutant allele is < 5% of the wild-type allele background

(Murray et al., 1991). Mutations are distributed throughout the VWF gene (Goodeve, 2010). Information on the molecular lesions underlying VWD is scarce due to the large size (178-kb) and the complexity associated with the mutation analysis of the VWF gene (Gadisseur et al., 2009). Many different types of mutations contribute to the pathogenesis of VWD [Table 3] (Goodeve, 2010). In general, knowledge on understanding the molecular basis of VWD will aid in clinical management of this condition (Lillicrap, 2013a).

2.4.2 Genetic background and Molecular basis of VWF subtypes

2.4.2.1 Type 1 VWD

Type 1 VWD accounts for about 75-80 % of cases, characterized by a partial quantitative deficiency of VWF (0.05-0.50 U/mL) in the presence of normal multimers (Sadler et al., 1995). Disease follows autosomal inheritance pattern, coupled with incomplete penetrance. A common feature, observed in kindreds with milder phenotype accounting to 60% (Miller et al., 1979). Before type 1 large cohort study, several lines of evidence suggest environmental factors (Liu et al., 1993), may also contribute to the variability of VWF in plasma. Results from the large cohort study suggest, relatively very few mutations were associated with the VWF gene. These keen observations indicate that loci outside VWF may also contribute to the pathogenesis underlying type-1VWD (James et al., 2006). Since 2005, four cohort studies has helped to understand the molecular basis of type 1 VWD comprising 500 index cases (James and Lillicrap, 2013). Summary of these studies are as follows: Multicentre studies conducted by three groups (European Union (EU), Canada, and the UK (Goodeve et al., 2007).

Mutation type	Process	Description	VWD type(s)
Transcription	Transcription of mRNA	mRNA impaired due to disruption of transcription factor binding sites (TFBS synthesis	1
Splice site	Removal of introns	Disruption of GT and AG dinucleotides at 5′ and 3′ end of each intron or flanking nucleotides can lead to exon skipping, intron retention or other mRNA abnormalities Exon skipping may result in in-frame deletion and an abnormally shortened protein	Null alleles result in 3, 2N, 2A and type 1 Dominant type 1 and 2A
Nonsense	Protein translation	Altered sequence results in a stop codon replacing an amino acid. Nonsense-mediated decay can eliminate aberrant mRNA limiting production of abnormal protein	Null alleles results in 3, 2N, 2A and type 1
Small deletion/ insertion/ duplication	mRNA production/ or protein translation	Loss/gain of one or small number of nucleotides. Often affect repeated sequence motif. Lack of protein production where amino acid coding is interrupted, similar to nonsense mutation. In-frame loss/gain of amino acid(s) where multiples of 3 nucleotides are affected	Null alleles contribute to recessive type 3, 2N, 2A and type 1 Effect similar to missense mutation; types 1, 2A, 2B, 2M
Gene conversion	mRNA production or protein translation	Replacement of VWF by VWFP sequence can result in nonsense or missense changes. Sequence altered ranges from 8–335 bp	1, 2B, 2M, 3
Large deletion	mRNA production or protein	Single exon to > entire gene deleted. Lack of protein production where amino acid coding is interrupted, similar to nonsense mutation In-frame loss of amino acid (s) where multiples of 3 nucleotides are affected	Null alleles contribute to recessive type 3 and 2A Mutation 1, 2 (unclassified), 3
Missense	Protein translation	Replacement of single amino acid with different residue. Effect dependent on amino acid position and nature of its' replacement	1, 2A, 2B, 2M, 2N, 3

Table 3: ***Mutation types that contribute to VWD.***

[Reference: Goodeve A, Blood reviews, 2010]

A total of 300 patients were analysed in the study MCMDM-type1 study. Criteria used for the recruitment of patients were as per ISTH SSC guidelines. However each centre differed in their recruitment criteria, as follows [Table 4]. Mutations were screened spanning 5' promoter region to cysteine knot domain using various approaches, including Conformation-Sensitive Gel Electrophoresis (CSGE), Single Strand Conformation Polymorphism (SSCP) analysis, Denaturing High-Pressure Liquid Chromatography (DHPLC) and direct sequencing approach. Candidate mutations were identified only in 60% of the index cases, were 18% of the patients were found to harbour more than one mutation. Types of mutations identified in these cohort studies include [missense: 70% : splice 9%: transcription 8%: small deletion 6%: nonsense 5%: small insertion/duplication 2%: gene conversion: 3%].

Most common type of mutation identified in the study was missense. These mutant residues were found to affect synthesis or secretion of VWF. Hence, these multicentre studies suggest that the likelihood of finding a mutation increases with decreasing VWF levels (Collins et al., 2008). The Canadian study was able to identify mutations in 75% of the cases were the VWF: Ag level was below 30 IU dL. In the European cohort, the chance of identifying the mutation was 20 fold greater, when the levels were spanning 0–15 IU dL in par with the Canadian cohort. When the antigen levels were >40 IU dL, the chance of identifying a candidate mutation was found to be 52%. Despite the approaches made to understand the pathogenesis type 1 VWD, the molecular mechanism underlying low plasma VWF remains unanswered.

	EU study	Canada study	UK study
Inclusion criteria			
Local diagnosis of type1 VWD	**Yes**	**Yes**	**Yes**
Central laboratory confirmation of diagnosis	**Not required**	**Required**	**Required**
Family history	**Required**	**Not required**	**Required**
Exclusion criteria			
VWF:Ag	**-**	**< 5IUdL^{-1} > 50IUdL^{-1}**	**-**
VWF:RCo	**-**	**< 5IUdL^{-1} > 50IUdL^{-1}**	**> 50IUdL^{-1}**
VWF:RCo/VWF:Ag	**-**	**< 0.6**	**< 0.7**
Abnormal multimer profile	**Not excluded**	**Excluded**	**Excluded**
Characteristics of patients recruited			
Families / index cases recruited	**154**	**194**	**40**
Families / index cases excluded	**4**	**71**	**8**
Family size (mean)	**4.8**	**3.2**	**6.7**
VWF:RCoIUdL^{-1}Mean (range)	**36.3 (< 3-170)**	**34.0 (7-50)**	**44.5 (< 10-87)**
VWF:Ag IUdL^{-1}Mean (range)	**39.2 (3-127)**	**36.0 (7-50)**	**47.4 (6-87)**
VWF:RCo/VWF:Ag ratio	**0.88 (0.07-2.58)**	**Not stated**	**0.89 (0.38-1.67)**
Normal multimers (%)	**62**	**100**	**100**
Blood group O (%)	**65**	**62**	**81**

Table 4: ***Summary of the enrolment criteria and cohort characteristics of the three multicentre studies on type 1 VWD.*** *[Reference: Good eve A, Blood, 2007]*

2.4.2.1.1 Disease mechanism

The pathological mechanism that contributes to type-1 VWD predominately includes accelerated clearance of VWF from circulation. Other factors associated with abnormal functions include defective transcriptional activation (Othman et al., 2010) resulting in aberrant splicing and process which impairs, subcellular targeting or storage of VWF (Lillicrap, 2013b). Molecular insight underlying type 1-VWD has been recently gained, through *in vitro* expression studies. Fourteen candidate missense mutations were evaluated, by transfecting the mutant plasmids in mammalian cell lines (Eikenboom et al., 2009). Results of this study inferred that intracellular retention and clearance of VWF as the mechanism contributing to the phenotype. The variant Y1584C and the Vicenza mutation R1205H were highly prevalent in the MCMDM type1-VWD study accounting to 6% and 13% respectively. The Vicenza mutation results in increased intracellular retention and clearance of VWF. Clearance of VWF in the plasma was determined, by the ratio of VWFpp to VWF: Ag or the recovery of VWF after desmopressin infusion. In circulation VWF propeptide and the mature VWF circulate in the ratio of 1:1, with a mean half–life of VWF in the plasma of about 8-12h. Mutations associated with clearance mechanism have shown to possess robust response to desmopressin, which represents a surrogate marker for reduced secretion of VWF (Castaman et al., 2008). Other mechanisms associated with the increased clearance of VWF, include the co-inherited blood groups with increased susceptibility to ADAMTS13 (Davies et al., 2008). Other missense substitutions which have shown to confer pathogenicity include W1144G (D3 domain), I1416N and S1279F (A1 and D4 domains). Intracellular retention of VWF and accelerated clearance of VWF was

proposed as a mechanism for these mutations, likely due to misfolding of the proteins (Millar et al., 2008). Results from these studies suggest although, intracellular retention of VWF, as an important mechanism contributing to type 1 phenotype, but these studies were not able to address the external factors underlying the phenotypic expression of a mutation. There was a trend towards incomplete penetrance with increasing VWF levels. Further linkage analysis carried by the cohort studies using polymorphic markers depicted that 50% of the families inherited the affected allele, displaying their penetrance. In the European cohort in about 13% of the patients candidate mutation was not identified and in the multicentre study 35%. These observations suggest incomplete co-segregation contributing to the bleeding phenotype. Astute observations from these studies conclude marked heterogeneity in patients with type 1 VWD. The molecular mechanism of other variants awaits elucidation.

2.4.2.1.2 ABO-blood group

Based on cohort studies O-blood group was found to be more prevalent in patients with type-1VWD (Gill et al., 1987). Individuals with blood group O, lack ABO glycosyl transferase, which protects VWF from clearance (Castaman et al., 2009). Hence, VWF plasma levels were 30% lower than AB. These observations were reproducible in cohort studies as follows: Canadian study: 66%: European study: 76%. Incomplete segregation with the VWF was observed accounting to 89%, represented blood group O, on subgroup analysis. Mechanism being postulated due to an altered susceptibility to ADAMTS13, resulting in the catabolism of VWF (Franchini et al., 2007). Although these studies suggest, in patients were a candidate mutations not identified, the cause of decreased antigen level, and the bleeding score

was found to be equivalent to that of those were mutations were identified (Goodeve et al., 2007). Hence, other factors associated with a decrease in antigen level, include polymorphic variants in VWF, platelet function defects (Daly et al., 2009).

2.4.2.2 Type 2 VWD-A Heterogeneous Disease Subgroup

Type 2-VWD encompasses qualitative disorders; that affects the structure or function of VWF (Goodeve, 2010). Current classification identifies type 2 VWD, as four subgroups type 2A, B, M, N (Sadler et al., 2006). Although subtyping of VWD remains straight forward, molecular characterization awaits (Tosetto and Castaman, 2015). The types of mutations which confer pathogenesis to type 2 VWD in the majority of the cases include missense, insertions or duplications and in-frame deletions (Goodeve, 2010). Studies have shown that mutations were highly penetrant, which in turn correlates with the bleeding phenotype within the family, with the exception being typed 2N follows recessive inheritance pattern. Most commonly Type 2-VWD pathogenesis was found to be associated with the epitopes involved in binding of the platelet to VWF (A2 domain), validated using *in vitro* approach using ristocetin (Tosetto and Castaman, 2015).

So far few large cohort studies have been carried out to elucidate the pathogenesis underlying type 2 VWD, of which two being notable. The first study by Meyer and colleagues comprising 150 patients including four subtypes (Meyer et al., 1997) and a second study from Frederic group comprising 67 patients with type 2N VWD (Federici et al., 2009).

2.4.2.2.1 Type 2A VWD

Type 2A VWD subtype represents 10-15% of all VWD cases, defined by autosomal dominant or recessive pattern of inheritance (Ginsburg and Bowie, 1992, Sadler et al., 2006). The disease is characterized preferential loss of high and intermediate molecular weight multimers paralleled with reduced ability of the VWF to bind to the platelets. More than one mechanism has been postulated to confer pathogenesis, as follows. (1) Impaired dimer formation (CK domain) or multimer assembly (D3 and D2 domain) (2) and increased susceptibility to ADAMTS 13 (A2 and A1 domains) and (3) intracellular retention, due to misfolding of the proteins (D3, A1, and A2 domains) (Goodeve, 2010). Mutations which confer type 2A VWD is divided into group 1 and group 2 (O'Brien et al., 2005). Most of the mutations were found to impair the key process, associated with synthesis /secretion of multimers and aberrant response to ADAMTS13. Both group1 and group 2 mutations, in absent of haemostatic challenge, expose ADAMTS 13 binding site Y1605-M1605 on VWF. This results in the synthesis of multimers in the absence of satellite bands, with limited endothelium and platelet binding ability. Based on the observed multimeric patterns they are categorized into type (IIA, IIC, IID, and IIE) (Sadler et al., 2006). Common mutations which affect D3 domain include C2771 and C2773. These mutations are found to impair the process associated with disulphide bond formation, a key process necessary for the multimerization. Type 2A(IIE) type, characterized by markedly reduced HMWM, related to aberrant triplet structure, results in reduced proteolytic cleavage and affinity for the platelets GPIbalpha (Meyer et al., 1997). Mutations identified onto D2 domain span exons

(exons 11–17). Subtype 2A (IIC), is characterized by absences of large multimers, with increased dimers, Mutations observed in these patients were homozygous or compound heterozygous, with a second null allele. Exon skipping, resulting in aberrant protein formation has been demonstrated at the 3' end of exon 26 (James et al., 2004). In a French study the relative proportion of mutations, identified with type 2A VWD were predominantly localised in A1 domain (82%), D2 and CK domain (8%) and D3 (1%) (Meyer et al., 1997). Cell expression studies were carried out in 293-EBNA cell line for missense mutations P1266Q, V1439M, and N1635I. Experimental results suggest these mutations brings about, a conformational change in the A1 domain mediated by Van der Waals interactions/ gain or loss of hydrogen bonds, while the gain of function for the mutant R1308C (Ahmad et al., 2013b). Although the *in vitro* expression studies aids in understanding the pathogenesis associated with type 2BVWD, the translational information derived from these mutants remains minimal (James and Lillicrap, 2013).

2.4.2.2.2 Type 2B VWD

Type 2B VWD, a unique gain of function variant, characterized by increased affinity of the VWF towards the platelets, resulting in thrombocytopenia accounting to 76% of all cases (Federici et al., 2009, Ruggeri et al., 1980). This subtype follows autosomal dominant inheritance (Rayes et al., 2010). In these patients, selective loss of HMWM and formation of platelet aggregates was observed as a key feature contributing to this subtype. The multimers were found to be preferentially cleared by ADAMTS, resulting in clearance of VWF from circulation. On molecular analysis mutations were found to be harboured in A1 domain of VWF (Meyer et al., 1997).

Most of the mutations were missense, localised at the 5' end of exon 28 which affects 16 amino acids of all cases. Studies from mouse models suggest, the BS correlates with the observed mutation (Rayes et al., 2010). Insight on genotype-phenotype correlation has been gained by the study from Federici group. The highest bleeding score was observed in patient with missense mutation V1316M, while patients with 1266Q/L and R1308L were found to have the lowest bleeding score. Recurrent mutations identified in this subtype include R1308L, R1306W, and R1341Q. This phenomenon relates to hypermutability at the CpG dinucleotides or through founder effect (James and Lillicrap, 2013). These mutants were found to stabilise the bond formation between A1 domain and increase the ß finger site of platelet GPIbα. As a result, thrombocytopenia coupled with increased clearance of VWF occurs mediated by ADAMTS13. In patients with type 2BVWD, were a causative mutation could not be defined, the presence of platelet-type pseudo-VWD (PT-VWD) is suspected, which also confers the same phenotype. Hence, mutation analysis of exons spanning GPIBA (exon 1 and 2), is sequenced to resolve the pathogenicity. Mutations frequently observed in this type include p.Gly249 and p.Met255. An in-frame deletion resulting in Pro449_Ser457del spanning 27 nucleotides has also been described (Othman, 2011).

2.4.2.2.3 Type 2M VWD

Type 2M VWD is characterized by decreased affinity of the VWF to bind to platelets, in the presence of a normal distribution of HMW multimers (Sadler et al., 2006). The disease follows autosomal dominant inheritance, with complete penetrance in a given family (Goodeve, 2010). In these series of patients, the response to

desmopressin is poor. Hence, adequate hemostasis is not restored on administration (James and Lillicrap, 2013). The most common mutations identified include missense mutations or in-frame deletions. The majority of the mutations were harboured on to A1 domain (exon 28) spanning codons 1266-1467. However exons 29-32, must also be scanned, to determine the mutation which interferes in binding to subendothelium (James and Lillicrap, 2013).

Isolated reports of mutations identified in D3 domain, spanning exon 24 and exon 52 spanning CK domain is documented. So far more than 25 missense mutations have been described. A1 domain binds to collagen including types I, III and 1V. So far four missense substitutions localised on A3 domain including S1731T, W1745C, S1783A, and H1786D has been documented (Flood et al., 2012). Mutations identified in collagen VI includes R1399H, S1387I and Q1402P. Interestingly, these variants were also observed in healthy individuals, in the absence of bleeding symptoms (James and Lillicrap, 2013) which in turn complicates the diagnosis of this condition. Hence, *in vitro* studies must be carried out to address the impact of these mutations resulting in bleeding phenotype. These mutations do not impair the assembly or the secretion of the multimers (Sadler et al., 2006). Hence the high molecular weight multimers though exist in the plasma, confer impaired binding to the platelets, since the larger multimers are shifted, resulting in decreased ADAMT13 cleavage. In some cases, these mutations were found to be associated with the bleeding symptom, however under certain conditions, these changes would act as secondary modifiers influencing the phenotype (James and Lillicrap, 2013)

2.4.2.2.4 Type 2N VWD

Type 2N VWD mimics mild haemophilia is characterized by the impaired, affinity of the VWF to bind to FVIII, termed as Normandy (Mazurier et al., 1994). This process occurs as follows (a) loss of electrostatic interaction between VWF and FVIII, which eliminates binding of FVIII (b) Mutations in cysteine residues localised on the D' domain can impair the secondary structure formation a, key process involved in FVIII binding to VWF (Casonato et al., 2003). The multimers were normal in the majority of the cases.

The disease follows autosomal recessive inheritance pattern, representing homozygosity or compound hertozygozity for a given mutation. The majority (85%) of the mutations identified were missense spanning exons 18-20 representing D' domain (Mazurier et al., 2001). In some cases, mutations were also identified in the regions spanning C-terminus, which interferes with the release of the mature subunit (Casonato et al., 2003). An extensive study carried out by Meyer et al., in 73-Type 2N patients, compiled 51 mutations, addressing the pathogenesis (Meyer et al., 1997). Homozygosity was observed in 49% of the patients, 12% being compound heterozygous for a given mutation, while in 39% of the patients, mutations were identified in a single allele (Meyer et al., 1997). The common mutation identified in this subtype include R854Q, with decreased FVIII levels (~25%) representing milder phenotype. Studies have shown that on desmopressin administration restores hemostasis in minor procedures, in contrast to missense mutation R816W and T791M, being poor responders (James and Lillicrap, 2013). Mutations in these series of patients were also found to be localized in exons 17, 24, 25 and 27 being infrequent (James and Lillicrap, 2013).

Characterization of these mutants using *in vitro* approach has shown that most of the mutants were found to impair the binding of VWF to FVIII, resulting in decreased expression of VWF. Characterization of the mutant residues involving C788Y, C788R, D879N, C858F and C1225G was carried to identify the molecular etiology contributing to this condition. Results from these studies suggest in addition to the impaired FVIII binding ability, decrease in the high molecular form of VWF was observed (Jorieux et al., 1998, Allen et al., 2000, Jorieux et al., 2000). This feature was in agreement to the phenotypic characteristics observed in the patient, harbouring the candidate mutation.

2.4.2.3 Type 3 VWD

Type 3 VWD, is the severe form the disease, were the mutations are distributed throughout the gene which makes the diagnosis time-consuming process. Studies suggest exon 28 as the hotspot in the majority of the patients (Ahmad et al., 2013b). Defects defined in this type include large deletions, transcriptional defect, frameshift and nonsense mutations comprise 83%, while 17% accounts to missense mutations (Sutherland et al., 2009a). Four cohort studies international and a large study from India carried out, suggest mutations could be identified in 92% out of 111 cases: 100% in a study comprising 40 patients and 77% out of 85 patients. The first gene defect identified in a patient with type 3 VWD includes a large deletion (Shelton-Inloes et al., 1987).

So far complete gene deletion including partial deletion in exons spanning 42, 33-38, 22-43, and 23-52 and 17-18 has been documented. Deletions were identified by gene dosage or multiplex ligation-dependent probe amplification

(MLPA) for detection of homozygous or the heterozygous condition. Coding regions in VWF comprise 11CGA codons including exons 9, 28, 32 and 45, were nucleotide change from to C to T, results in premature truncation of the protein. Combined study by different groups including Angelo Bianchi Bonomi Haemophillia and Thrombosis Center; IRCCS Maggiore Hospital was carried out to understand the molecular underlying type-3VWD. Various approaches used include RFLP (Restriction Fragment Length Polymorphism) SSCP (Single Strand Conformation Polymorphism) and direct sequencing. Twenty-four different gene alterations in 21 patients were identified (Baronciani et al., 2003). Stop codon mutations in 18 patients was identified. The recurrent mutation identified in these series includes R2535X (Eikenboom et al., 1992). This mutation was also recurrent in other populations including Dutch, German, Italian and Turkish origin. In Dutch, population the frequency of occurrence of this mutation is estimated to be 0.23%, suggesting founder effect based on haplotype analysis. The frame shift mutation 2680delC, accounts to about 50% of the patients in the Swedish population, were it suggests founder effect (Zhang et al., 1992a). Cell expression studies on this mutant revealed truncation of the proteins. Second recurrent mutation includes c.2908delC observed in 12 index cases, from Indian population. Although this approach narrows down the screening approach, however in the majority of the cases remains unanswered. An explanation for the low level of VWF level includes the nonsense, frameshift, splice site mutation and large deletions. In additions to these defects, many missense mutations have also been identified; however the role of these missense mutations in contributing to this phenotype remains unanswered.

Mutations in these series of patients are highly heterogeneous. These mutations were found to impair the process including synthesis, dimerization, multimerization, secretion, storage, binding to FVIII, platelets (Valentijn and Eikenboom, 2013). Missense mutations identified at the cysteine residues in C-terminus of the VWF includes C2671Y, C2739Y, C2754W, C2804Y and C2806R (Valentijn and Eikenboom, 2013). *In vitro* characterization of these mutants C2773S, C2754W, and C2806R, suggests impaired dimerization. *In vitro* expression studies for D141Y, C275S (Baronciani et al., 2008) suggests these mutations affect the survival and multimerization of VWF. The mutant C2739Y on *in vitro* expression studies impairs storage of VWF. Mutants identified in the A2 domain includes G1631D, and S1506L, L1503A, V1607D, and D1, D2 mutant M740I, were able to form high molecular weight multimers, however unable to drive WPB formation (Valentijn and Eikenboom, 2013).

Hence, these studies suggest that critical structural arrangements are crucial for proper folding of the protein in the endoplasmic reticulum, prior exit to Golgi. It is of great importance to note that, mutations within the same domain can have different molecular signatures. One classical example being Y87S and R273W were on characterization, impaired secretion in R273W, in contrast to Y887S (Valentijn and Eikenboom, 2013). This further illustrates the complexity in biosynthesis and secretion of VWF. Second interesting mutation being R854W, harboured on to the D, D3 domain, impairs WPB formation (Berber et al., 2009), while the same residue with R854Q promotes WPB formation.

2.5 Clinical Features Diagnosis Classification and Treatment of Von Willebrand Disease

2.*5.1 Historical aspects*

VWD is the most common inherited bleeding disorder. In Scandinavia where VWD, was first described its prevalence is estimated to be 100 per million. In 1926, Professor Erik Von Willebrand from Helsinki published his first paper on a heritable bleeding disorder, which he observed in several members of the family from Fogo in Aland Islands [Figure 20] (Lassila and Lindberg, 2013). Erik provided a splendid description of the clinical and genetic features observed in the family. This novel bleeding disorder was identified first in a five-year girl, named Hjördis S, who presented with several episodes of life-threatening mucosal bleeds and bleeding following tooth extraction. The index patient bled to death at the age of 14, during her fourth mensural period. Following which, Erik made an exhaustive observation of the 66 family members of the family and concluded that 23 of them exhibited symptoms as observed in the index patient. Mucocutaneous bleeding was the predominant clinical feature seen in the members of the family. The hemorrhagic diathesis in the family members was distinct from "hemophilia" (Berntorp et al., 2013). Normal platelet count coupled with prolonged bleeding time was the key observation. The cause of this novel bleeding was found to be in association with the blood vessel and platelets. Further extensive studies made by the two groups investigators in 1970s, paved way to identify that FVIII and VWF are distinct entities. Nyman and co-workers reinvestigated the family and concluded that mildly affected members were categorized as type 1-VWD and severely affected members were VWD type-3 in the year 1981.

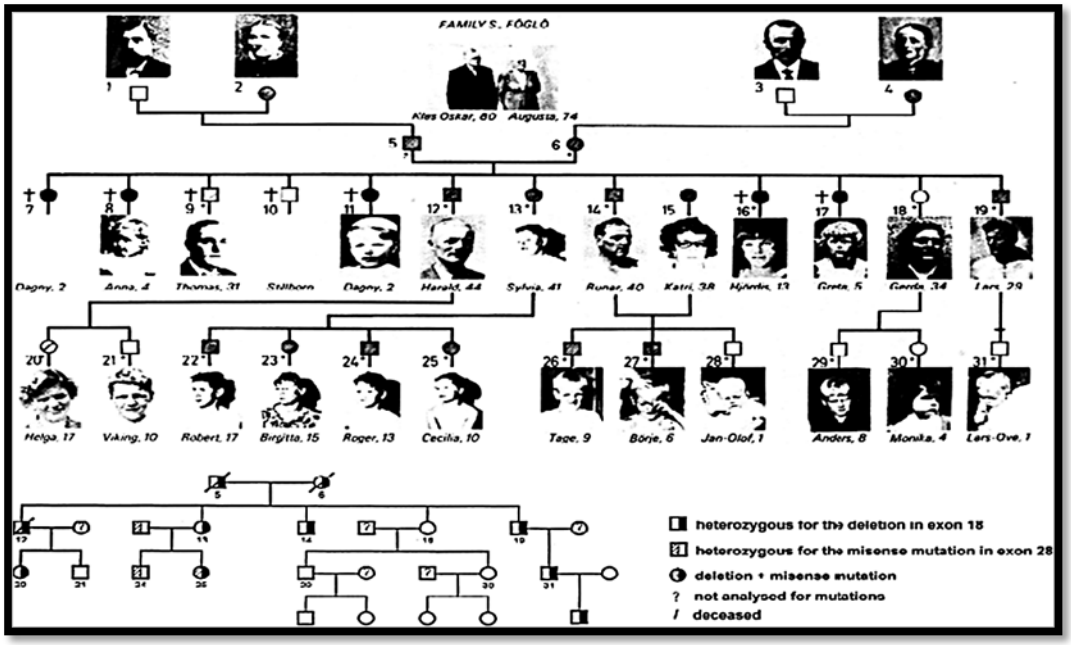


Figure 20: ***Pedigree of the family described by Erik Von Willebrand***

[Reference: Blomback M, Haemophilia, 1999]

The prominent feature observed in patients with VWD includes, recurrent epistaxis lasting for more than 10 minutes, which affects the quality of life 38-63% of the cases (Valente and Abramson, 2006). The second typical clinical presentation observed includes easy bruising with minimal or no apparent trauma and oral cavity bleeding (26-35%) (Valente and Abramson, 2006). Other clinical manifestations observed in a patient with VWD include bleeding following skin laceration, dental extractions (29-52%) or other forms of surgery. Bleeding from the gastrointestinal tract associated with angiodysplasia is rare, however, is a serious complication leading to life-threatening episodes (Hirri et al., 2006). Common symptoms experienced by women with VWD include fatal haemorrhage accounting to 74-92% (Lukes et al., 2005, James et al., 2011) when they reach menarche and postpartum

haemorrhage after delivery (Kadir et al., 1998). Type -3VWD patients were associated with moderate to life-threatening episodes.

2.5.2 Diagnostic approaches to VWD

2.5.2.1 Bleeding assessment tools for the diagnosis of VWD

Diagnosis of VWD [Table 5] begins by reviewing the patient's personal and family history of bleeding. Patients with VWD experience significant mucocutaneous bleeding including epistaxis, gum bleeding and easy bruisability (Metjian et al., 2009). In 2005 standardized guidelines on bleeding symptoms and family history, was put forward by International Society on Hemostasis and Thrombosis: VWF SSC Subcommittee on VWF (ISTH SSC: VWF) (Sadler et al., 2005). Bleeding symptoms in patients with VWD range from bleeding associated with trauma or surgical procedure to spontaneous bleeding. Since patients with VWD present with significant bleeding history, many attempts have been made to assess the potential of bleeding using questionnaire based surveys (Tosetto et al., 2006). Existing lines of evidence suggest the bleeding assessment tools (BAT) are universally not sensitive and specific (Tosetto et al., 2006, Tosetto et al., 2011). BAT scores define wide variability in their ability to determine the type of VWD, and the overall time spend represents an obstacle for the decision-making process in a clinical setting. Hence, a condensed version of the BAT (James et al., 2006), could be used to identify patients who are at risk in a clinical setting, however, the lack of hemostatic challenge, in early life can make these bleeding scores age dependent (Ng et al., 2015).

Symptom	Score					
	1	0	1	2	3	4
Epistaxis	–	No, or trivial (less than 5)	>5 or more than 10.'	Consultation only	Packing or cauterization or antifibrinolytic	Blood transfusion or replacement therapy or desmopressin
Cutaneous	–	No or trivial (<1 cm)	>1 cm and no trauma	Consultation only		
Bleeding from minor wounds	–	No, or trivial (less than 5)	>5 or more than 5.'	Consultation only	Surgical hemostasis	Blood transfusion alternatively, replacement therapy or desmopressin
Oral cavity	–	No	Referred at least one	Consultation only	Surgical hemostasis or antifibrinolytic	Blood transfusion or replacement therapy or desmopressin
Gastrointestinal bleeding	–	No	Associated with ulcer, portal hypertension, hemorrhoids, angiodysplasia	Spontaneous	Surgical hemostasis, blood transfusion, replacement therapy, desmopressin, antifibrinolytic	
Tooth extraction	No bleeding in at least two extraction	None done or no bleeding in one surgery	Referred to < 25% of all procedures	Referred to >25% of all procedures, no intervention	Resuturing or packing	Blood transfusion or replacement therapy or desmopressin
Surgery	No bleeding in at least two surgeries	None was done or no bleeding in one surgery	Referred to < 25% of all surgeries	Referred to >25% of all procedures, no intervention	Surgical hemostasis or antifibrinolytic	Blood transfusion or replacement therapy or desmopressin
Menorrhagia	–	No	Consultation only	Antifibrinolytics, pill use	Dilatation and curettage, iron therapy	Blood transfusion or replacement therapy or desmopressin or hysterectomy
Postpartum hemorrhage	No bleeding in at least two deliveries	No deliveries or no bleeding in one delivery	Consultation only	Dilatation and curettage iron therapy, antifibrinolytics	Blood transfusion or replacement therapy or desmopressin	Hysterectomy
Muscle hematomas	–	Never	Post-trauma no therapy	Spontaneous, no therapy	Spontaneous or traumatic, requiring desmopressin or replacement therapy	Spontaneous or traumatic, requiring surgical intervention or blood transfusion
Hemarthrosis	–	Never	Post-trauma no therapy	Spontaneous, no therapy	Spontaneous or traumatic, requiring desmopressin or replacement therapy	Spontaneous or traumatic, requiring Surgical intervention or blood transfusion
Central nervous system bleeding	–	Never	–	–	Subdural, any intervention	Intracerebral, any intervention

Table 5: ***Condensed MCMDM-1VWD Bleeding Questionnaire for Von Willebrand Disease, assigned score for each bleeding symptom***.

[Reference: Bowman M, J Thromb Haemost, 2008]

Standardized bleeding scores have been developed and tested on type-1 VWD patients retrospectively to quantitative the bleeding symptoms. These questionnaires were designed to decide whether the bleeding symptoms observed in the patient merit lab testing in patients with VWD. The most common symptom being epistaxis, cutaneous bleeding, tooth extraction, post-operative bleeding and bleeding from minor wounds. At least one bleeding symptoms were observed in 98% of the index cases, 89% of the affected relatives, 32% of the unaffected relatives and 12% of healthy controls [Table 5] (Tosetto et al., 2006).

2.5.2.2 Diagnosis of VWD

Initial screening for VWD is carried out using Prothrombin Time (PT) and Activated Partial Thromboplastin Time (aPTT). Although aPTT in patients with type 3 and type 2N VWD is prolonged, it cannot be classically used as a screening test for VWD, since it could be normal in most cases (Ng et al., 2015). The second criterion includes Complete Blood Count (CBC) which is not sensitive, nor aids in the specific diagnosis of VWD, however in patients with type 2B VWD, characterized by thrombocytopenia, this test remains informative (Ng et al., 2015). Bleeding Time (BT) is considered as a historically significant test for mucocutaneous specific bleeding (Cariappa et al., 2003). Since the sensitivity coupled with the specificity of the test has widely questioned from different centers, this test rarely exists in practice (Cariappa et al., 2003). Hence, Platelet Function Analyser (PFA 100), a test which measures both adhesion and platelet aggregation, has shown to be sensitive for VWF, however, lacks uniformity in sensitivity and specificity (Castaman et al., 2010). Hence given the inability of the test to distinguish clearly

the type of VWD, specific must be run in parallel with the screening test when associated with a significant bleeding history. The current standard of practice in the diagnosis of VWD relies on two specific tests (a) Quantification of VWF present in the plasma (b) Functional test to check the ability of the VWF to interact with the platelets in the presence of ristocetin [Figure 21]. Although several novel tests are being added up, these two criteria are essential to make a definitive diagnosis of type 3VWD (Gill et al., 1987).

2.5.2.2.1 VWF: Ag

This test aids in the quantification of VWF present in the plasma. This test solely relies on enzyme-linked immunosorbent assay (ELISA). The standard range for VWF: Ag varies between laboratories. Accepted value ranges from 50 and 200 IU/dL (Ng et al., 2015). Levels below 50 although considered to be low, however, remain as a controversy in certain populations. As a well-known fact that individuals with blood group O exhibit 25% decrease in the levels of VWF, in comparison with blood group A (Gill et al., 1987).

2.5.2.2.2 VWF: RCo

Ristocetin cofacto activity evaluates interaction mediated by GP1bα on the surface of the platelet to VWF in the presence of ristocetin (Flood et al., 2011). The normal value for VWF: RCo is 50 to 200 IU/dL.VWF: RCo activity has several limitations, including where the interactions can be affected by certain variations in the VWF sequence. The nucleotide change D1472H, localized on to the A1 domain can affect the interactions with the VWF, without altering the *in vivo* activity.

Although this feature has no clinical significance, in the diagnosis of VWD, but in some cases can lead to misdiagnosis of VWD (Flood et al., 2010).

2.5.2.2.3 Ratio of VWF: RCo/VWF: Ag

Differentiation of type 1 and type 2 VWD is calculated by dividing the ratio of VWF: RCo to VWF: Ag. The ratio for type 1 VWD is 1, conversely being for type 2 VWD, were a disproportionate decrease in the ratio of VWF: Ag:VWF: RCo<0.6 (Laffan et al., 2014) [Figure 21].

2.5.2.2.4 FVIII: C

Measurement of FVIII activity is a diagnostic criterion for the evaluation of VWD. In patients with type 3 VWD and type 2N, dramatic decrease in FVIII level is observed, conversely being in type 2 characterized by low levels of FVIII activity (Ng et al., 2015) [Figure 21].

2.5.2.2.5 VWF multimers

High molecular weight multimers are hemostatically active (Moake et al., 1986), which are involved in maintaining haemostasis. The normal pattern for the multimer is defined by the even proportion of the multimer size ranging from large to small. Some lab uses this test as first line rather than second line testing. This test helps to differentiate qualitative VWD. Limitations of this test include accuracy in the quantification of multimers and associated preanalytical variables. Studies have shown that test can assess the response to therapeutic interventions such as desmopressin (Ng et al., 2015) [Figure 21].

2.5.2.2.6 Specialized laboratory testing

In reference laboratories, after the diagnosis of VWD is made using a battery of tests, specialized tests are carried out to aid correct subtyping of VWD, for therapeutic interventions.

2.5.2.2.7 Collagen binding assay

Collagen binding assay uses collagen type 1 and 3, and collagen 6, to identify potential VWF binding defects (Flood et al., 2013). This test is sensitive in the presence of high molecular weight multimers. This test has been proposed as a replacement for VWF: RCo activity (Ng et al., 2015).

2.5.2.2.8 VWF propeptide

VWF circulates in complex with the propeptide in a ratio of 1:1. This test is of greater significance; in a situation where accelerated of VWF is observed leading to pathological effect. The ratio of VWF:Ag to the propeptide, will aid in identifying individuals with a high clearance of VWF (Haberichter et al., 2008).

2.5.2.2.9 Low-dose ristocetin-induced platelet aggregation

Low dose ristocetin-induced platelet aggregation test, is used to assess the hyperactivity of VWF as seen in type 2B (Miller et al., 1983) or platelet-type pseudo-VWD [Figure 21].

2.5.2.2.10 VWF: FVIII-binding Assay

VWF: FVIII-binding assay measures the ability of the VWF, to bind to the recombinant FVIII, which is measured using ELISA. This assay aids in the identification of type 2N VWD (Montgomery et al., 1982) [Figure 21].

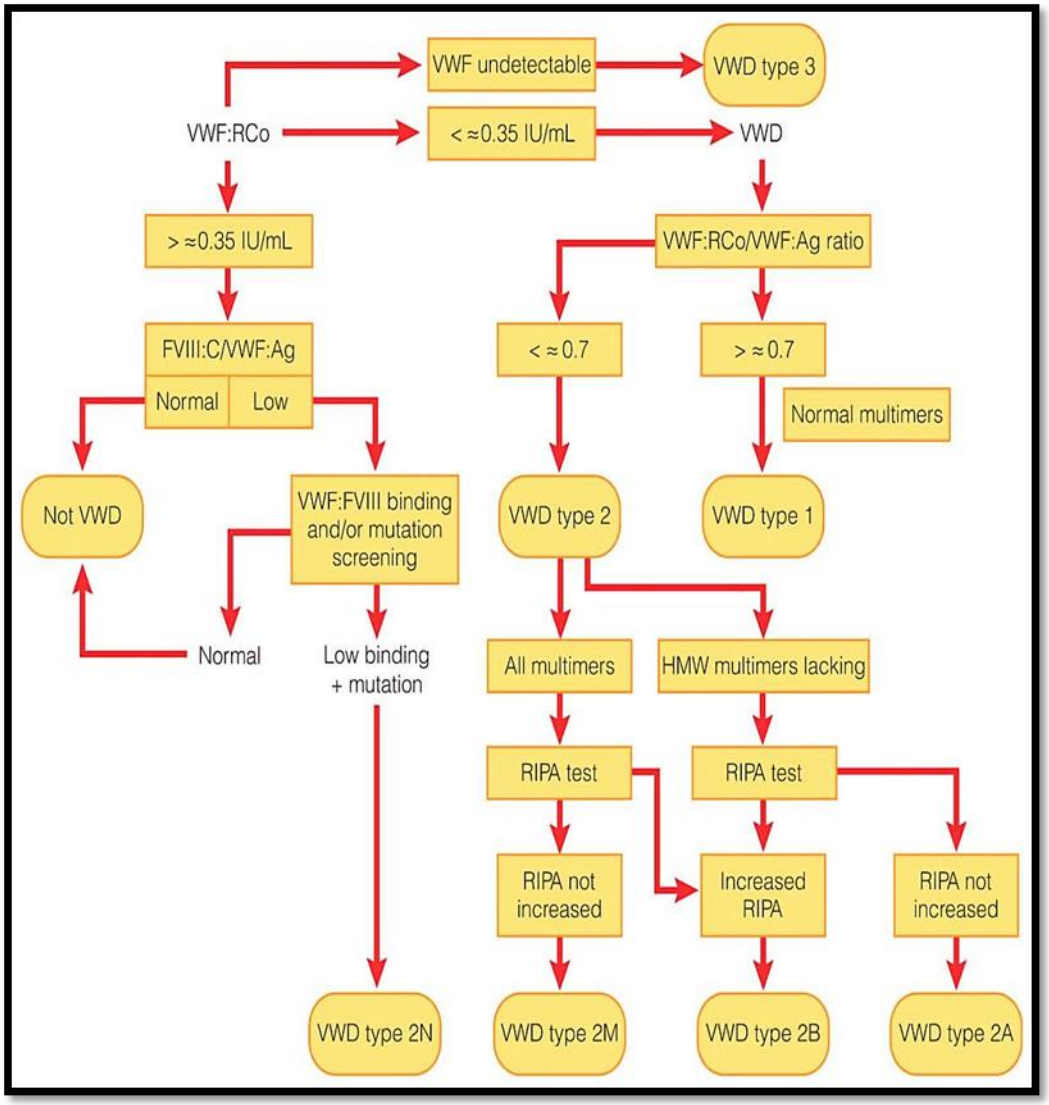


Figure 21: ***Algorithm of the diagnostic approach in patients suspected for VWD.*** *The diagnosis of VWF includes VWF:Ag, FVIII:C and VWF:RCo, however, specialized tests were used to diagnose different types of VWD. Specific groupings were by the underlying pathophysiology of various VWD types. RIPA, ristocetin-induced platelet aggregation. [Reference: Christopher NG, Blood, 2014]*

2.5.3 Alloantibodies to VWF

Alloantibodies against VWF is a rare phenomenon, which accounts for ~5% to 10% of type 3 VWD patients (James et al., 2013). One patient with type 2B

VWD has been documented with the development of antibodies (Baaij et al., 2015). Development of antibodies poses serious complications in treatment. Affected patients present with a broad range of symptoms including the absence of hemostatic response elicited during infusion of VWF concentrate and in rare cases resulting in an anaphylactic reaction resulting in adverse outcomes. The majority of the patients with alloantibodies harbor partial or complete VWF gene deletion; subsequently nonsense, frameshift mutations were also found to impart vital role in the development of antibodies (James et al., 2013). Antibodies were found to display polyclonal characteristics, including subclasses of IgG, 1-4. On laboratory analysis reveal the antibodies were found to directed against N-terminal part of the VWF (Tout et al., 2000) subunit.

2.5.4 Classification of VWD subtypes

Classification of VWD was put forward by Evan Sadler in the year 2006 [Table 6] (Sadler et al., 2006). The disease was classified into two categories (i) Quantitative VWD or qualitative VWD (Sadler, 1994).

Type	Description
1	Partial quantitative deficiency in VWF
2	Qualitative deficiency in VWF
2A	Decreased platelet-dependent function associated with the absence of VWF HMWM
2B	Increased affinity for platelet glycoprotein, associated with (usually) reduced high molecular weight multimers
2M	Decreased platelet-dependent function not caused by the absence of VWF HMWM
2N	Markedly decreased affinity for FVIII
3	Complete deficiency in VWF

Table 6: ***Classification of Von Willebrand Disease.***

[Reference: Sadler JE et al., J Thromb Haemost, 2006]

2.5.4.1 Type 1 VWD

Type 1 VWD is the most common autosomal dominant inherited disease; it accounts for about 80% of cases. The disease is characterized by, partial reduction of VWF in plasma and platelets with normal multimeric distribution (Rodeghiero and Castaman, 2001).

The disease follows incomplete penetrance with variable expressivity, which in turn complicates the diagnosis. Clinical manifestations including mucocutaneous bleeding directly correlate with the decrease in VWF levels. Commonly observed clinical feature include easy bruising, epistaxis, and women in reproductive age experience menorrhagia and post-partum hemorrhage. Laboratory diagnosis of VWD is based on the ratio of activity of VWF, and its antigen (VWF: RCo/VWF: Ag) is >0.7 (Eikenboom et al., 1996). When the levels of VWF are <15 IU/dL the clinical severity increases.

2.5.4.2 Qualitative Type 2 VWD Heterogeneous disease subgroup

Type 2 VWD, caused due to a qualitative defect in VWF. The disease accounts for about 20-30% of the cases (Ginsburg and Sadler, 1993). Bleeding symptoms at birth or early childhood including epistaxis, easy bruising, gum bleeding, and skin hematomas. Studies have shown that women with type 2 VWD experience heavy mensural bleeding requiring blood transfusion accounting to 50%. The decrease infers diagnosis of Type-2VWD in the ratio of VWF: RCo/VWF: Ag to 0.7. Type 2-VWD, affect the interactions with the ligands leading to functional defects.

2.5.4.3 Type 2A VWD

Type 2A VWD is defined by preferential loss of high and intermediate molecular weight multimers. Hence, the ability of the VWF to bind to platelets is decreased. The underlying pathophysiology can be heterogeneous associated (i) impaired synthesis of VWF (ii) increased susceptibility to ADAMTS, on exocytosis. Low dose ristocetin can be used to differentiate type 2B from type 2A-VWD. Functional activity of the multimers is predicted by collagen binding assay (VWF: CBA), which is highly sensitive to the loss of high molecular weight multimers (Riddell et al., 2009). Hence, type 2A is differentiated from type 2M VWD by reduced VWF: CBA/VWF: Ag ratio (<0.6) (Flood et al., 2012).

2.5.4.4 Type 2B VWD

Type 2B VWD disease comprises 5 to 10% of all VWD cases (Favaloro, 2011). The disease is characterized by the enhanced affinity of VWF (high molecular weight multimers) to platelet GpIbα, resulting in a gain of function, which is resolved by enhanced aggregation of the platelets to low dose ristocetin (RIPA) to plasmatic VWF. The resulting aggregates formed are cleared from the circulation, resulting in low VWF and thrombocytopenia. In about 40% of the patients mild to moderate thrombocytopenia has been reported (Federici et al., 2009) were *in vivo* clumping of the platelets has been observed. Using surrogate markers such as VWF: CB and VWF: RCo or multimer analysis can be used to detect the preferential loss of higher molecular weight multimers (Favaloro, 2009). It is critical to differentiate type 2B from platelet-typeVWD since the therapeutic

approach is different in two cases were in, type 2B do not respond well on the infusion of VWF concentrate (Hamilton et al., 2011).

2.5.4.5 Type 2M VWD

In Type 2M VWD decreased interaction of VWF with the platelets (GP1bα) occurs. Laboratory analysis reveals reduced VWF: RCo/VWF: Ag ratio (< 0.7). The multimers are normal, however dysfunctional, were the binding efficiency of the platelets to VWF is decreased. Hence, the diagnosis of VWD includes, low VWF: RCo disproportionately low in comparison with VWF: Ag in most of the patients in the presence of normal multimers (James and Goodeve, 2011).

2.5.4.6 Type 2N VWD

Type 2N VWD is caused due to the inability of VWF to bind to FVIII, were accelerated clearance of unbound FVIII occurs. Prevalence of this subtype is found to be 1.2-2% among all VWD patients (Bowen et al., 1998). Clinical features include hemarthrosis, hematuria and bleeding after invasive procedures (Gaucher et al., 1991). Laboratory diagnosis reveals reduced FVIII levels with decreased VWF: Ag levels. This subgroup mimics classical, mild haemophilia in their presentation. Both Type 2N and type 3 VWD markedly reduction in FVIII levels. Definitive diagnosis of type 2N-VWD was carried out by measuring the FVIII binding assay, which follows recessive inheritance pattern. Schematic representation of different VWF subtypes and the pathogenic mechanisms is illustrated in the [Figure 22]

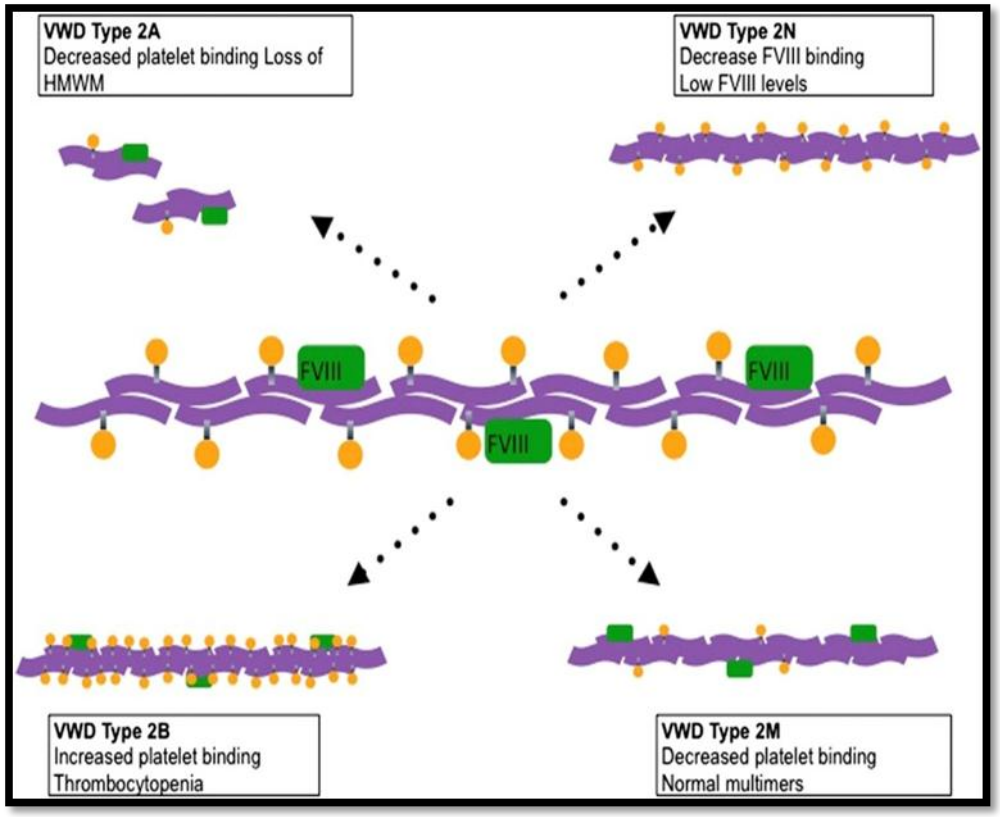


Figure 22: ***Mechanisms for type 2 VWD.*** *Representation of VWF and its interaction with platelets and FVIII, and how these interactions, when affected, cause VWF functional defects and subsequent clinical bleeding.*
[Reference: Christopher NG, Blood, 2014]

2.5.4.7 Type 3 VWD

Type-3 VWD is the severe form of the disease. Classically type 3 VWD, follows autosomal recessive inheritance pattern with an equal sex distribution (Mannucci et al., 1984). The disease is characterized by markedly decreased VWF and FVIII levels. Hence, type 3 VWD patients are predisposed to both primary and secondary haemostasis defect, leading to severe bleeding diathesis including hemarthrosis, muscle hematomas, etc. (Eikenboom, 2001). Clinical manifestations observed at infancy (e.g., with circumcision), or occurrence of bleeding due to

minor trauma when they become mobile at the age of 1, the onset of walking. Bleeding associated with oral cavity becomes apparent at the age of two resulting in gum bleeding and loss of deciduous teeth. Compared to the healthy controls the obligatory carriers of type 3 VWD were associated with bleeding symptoms, however, less symptomatic than carriers of type1 VWD (Castaman et al., 2006)

In a study summarizing the bleeding episodes were 385 patients were enrolled of which epistaxis accounted to 77%, oral cavity bleeding 70%, menorrhagia 69%, muscle hematoma 52%, post-operative bleeding 41%, and hemarthrosis 37% (Metjian et al., 2009). In comparison with patients with type-3VWD and severe haemophilia, post-operative bleeding was similar, but patients with severe haemophilia had twice the frequency of bleeding into joints and muscles, but rarely did they bleed from the mucosal surface. Clinical manifestations in women with type 3 VWD, is severe due to their association with mensural cycle and delivery (Foster, 1995). In at least two-third of women with type 3-VWD, require a transfusion during menorrhagia to overcome the deficiency of iron (Foster, 1995). Also, some patients undergo life-threatening hemoperitoneum, were women experience midcycle pain with ovulation resulting in significant bleeding in corpus luteum. In an international registry; studies on the health of women with VWD in their reproductive age carried out, reported that in women with type 3 VWD, replacement therapy was needed in one or more episodes of menorrhagia in 13 out of 14 women with VWD (Foster, 1995). Hysterectomy was performed secondary to menorrhagia in 5% of the patients secondary to menorrhagia. In patients lacking prophylactic treatment post-partum hemorrhage was observed in all the patients

after 24 hrs of delivery while in 25% of the patients were found to receive prophylactic treatment after cessation of replacement therapy (Foster, 1995). Surprisingly, the levels of VWF and FVIII levels are known to increase during pregnancy. Hence, patients with type-3 VWD were not associated with excessive bleeding or the requirement for prophylactic replacement therapy. However in about 15% of the patients were found to related to postpartum hemorrhage requiring transfusion (Foster, 1995).

2.5.5 Treatment of Von Willebrand Disease

Standard of care in patients with VWF deficiency involves, using plasma-derived factor VIII/VWF concentrates (Ofosu et al., 2012). The therapeutic choice is made based on the following observations (i) severity of the disease (ii) exposure to hemostatic challenge (iii) and the nature of actual or potential bleeding (Laffan et al., 2014). Strategies used to achieve therapeutic levels include one or either of the following. (i) Stimulation of endothelial cells to release endogenous VWF using non-concentrate therapies including desmopressin and tranexamic acid (ii) replace the deficient VWF by using plasma-derived VWF, inactivated against virus containing FVIIIVWF (iii) use of agents to maintain hemostasis to aid in wound healing, without altering the plasmatic concentration.Treatment products utilized for each type is as depicted in [Table 7]

VWD Type	Desmopressin	VWF/FVIII concentrate	Platelets
1	Treatment of choice if trial dose results in therapeutic hemostatic levels	If desmopressin is not effective or if higher levels are required	Not indicated
2A	May result in transient responses useful for minor bleeds and procedures	Required for major bleeding and surgery	Not indicated
2B	May cause further decrease in platelet count	Required for major bleeding and surgery	Indicated if thrombocytopenia remains severe after VWF replacement
Platelet-type	May cause further decrease in platelet count	May cause further decrease in platelet count	Initial treatment of choice
2M	May result in partial responses useful for minor bleeds and procedures	Usually required for major bleeding and surgery	Not indicated
2N	May result in partial, but transient, responses useful for minor bleeds and procedures	Usually required for major bleeding and surgery; highly purified FVIII concentrate not effective	Not indicated
3	Not indicated	Required for major bleeding and surgery	Not indicated unless bleeding persists despite adequate plasma VWF/FVIII levels

Table 7: ***Treatment Alternatives for Von Willebrand Disease.***

[Reference: Cox Gill J, Hematol Oncol Clin N Am, 2004]

2.5.5.1 Desmopressin (1-deamino-8-d-arginine vasopressin)

DDAVP synthetic analog of vasopressin is used to treat patients with VWD (Kaufmann and Vischer, 2003). This analog temporarily aims to elevate FVIII and VWF levels by releasing it from the endothelial stores. Desmopressin binds and activates V2 vasopressin receptors, leading to secretion of VWF. Based on two controlled prospective studies carried out in healthy volunteers, the dose recommended is 0.3 *m*g/kg, which is administered intravenously in 30 to 50ml of saline to achieve hemostasis (Lethagen et al., 1987). The peak increment is measured using VWF: RCo and FVIII assays after 30 to 90 minutes after infusion. (Nair et al., 2011). An adequate response was observed in patients with type -1 VWD, converse being typed 2B VWD and type 3 VWD. On exposure of the patients diagnosed with type 2B VWD to DDAVP, the formation of platelet aggregates accompanied with thrombocytopenia results, hence, these products can be avoided in these patients. (Nair et al., 2011).

2.5.5.2 Antifibrinolytic drugs

Antifibrinolytic drugs are used as an adjunct, to treat injuries associated with the mucous membrane (Federici et al., 2000). Two agents that exist in use include aminocaproic acid (EACA; Amicar) and tranexamic acid (Cyclokapron). These drugs are most commonly used to treat epistaxis or oral cavity bleeding in combination with clotting factor concentrates/desmopressin by topical application (Nair et al., 2011). For example, in patients undergoing dental extraction diagnosed with VWD, in the presence or absence of desmopressin avatine or fibrin glue could be used to gain adequate hemostasis (Federici et al., 2000).

2.5.5.3 Von Willebrand factor/FVIII concentrates

In patients with serious bleeding complications, and who do not respond to desmopressin, factor replacement is considered (Thompson et al., 2004). In developing countries, usually, it is done with the aid of cryoprecipitate if VWF-containing factor concentrates are not available (Nair et al., 2011). However, these concentrates are prone to increased risk of viral transmission, because of the reliance on screening for viral safety. Commercially, available plasma-derived concentrates include Humate-P, characterized by the presence of intact multimers (Thompson et al., 2004). In patients with life-threatening bleeding episodes and during surgery Humate-P is used. In these products four-fold difference exists in the ratio of FVIII:C per unit of VWF:R Co, generally varies from 2:1 to 0.5:1 (Cox Gill, 2004) These products are virally attenuated. Hence, the risk of transfusion-mediated transmission of viral infection with HIV and hepatitis is eliminated (Nair et al., 2011).

2.5.5.4 Recombinant Human VWF (rVWF)

Recombinant human VWF (vonincog alfa, Vonvendi) is characterized by the presence of a full spectrum of VWF multimers, including ultrahigh molecular weight multimers, with adequate levels of VWF activity. The half-life of rVWF was found to be higher when compared to the plasma-derived products. Hemostasis was achieved within 6 hours of administration and sustained for 72 hours. Thirty-seven patients with a median age of 37 years on exposure to rVWF were 22 patients experienced at least one bleeding episode. Bleeding episodes in these patients include (122 minor, 61 moderates, 7 (major, severe) and 2 of unknown severity (Gill et al., 2015) symptoms.

	Number of bleeding episodes	Total number of infusions	Median number of infusions / bleeds (range)	Median rvWF dose (IU/kg) per infusion	Median rFVIII dose (IU/kg) per infusion	Percentage of bleeds (n=192) ratings (excellent/good)
vWD Type						
Type 3	175	219	1(1-4)	48.2 (range, 23.8-184.9)	33.6 (range, 16.6-129.3)	100% (171/4)
Type 2A	16	18	1(1-2)	50.2 (range, 32.9-90.2)	32.5 (range, 23.7-38.6)	100% (14/2)
Type 2N	1	1	1(1-1)	54.3 (range, 54.3-54.3)	N/A	100% (1/0)
Bleed Severity						
Minor	122	132	1(1-3)	43.3 (range, 25.2-158.2)	33.5 (range, 17.6-86.2)	100% (119/3)
Moderate	61	89	1(1-4)	52.7 (range, 23.8-184.9)	36.9 (range, 16.6-129.3)	100% (59/2)
Major/ Severe	7	15	2 (1-3)	100 (range, 57.5-135.0)	39.0 (range, 25.0-42.3)	100% (6/1)
Unknown	2	2	1(1-1)	33.4 (range, 33.1-33.8)	23.3 (range, 23.1-23.6)	100% (2/0)

Table 8: ***Treatment Summary of Bleeding Episodes.***

[Reference: Gill, J. C et al, Blood, 2015]

A single infusion was sufficient to manage these bleeding episodes. For minor to moderate bleeding, the dose administered ranges from 40 to 60 international units/kg. Summary of the treatment of the bleeding episodes described in [Table 8] adverse effect associated with the product was found to be minor. However, one patient with a history of allergic reaction to VWF concentrate had tachycardia and chest discomfort lasting for 10 minutes. No thromboembolic or anaphylactic complications or VWF neutralizing antibodies were observed in using this product (Gill et al., 2015).

2.6 Von Willebrand Disease in India

One of the most common inherited coagulopathy in humans is considered to be VWD (Nichols et al., 2008). Epidemiological data on the prevalence of various subtypes of VWD from developing countries is limited (Srivastava and Rodeghiero, 2005). Over the several decades, attempts were made to understand the biology of the disease; the knowledge is not widely exploited in countries with low economic resources and a high number of affected patients (Nichols et al., 2008). In the developed world a good quality of life was assured in the form of prophylaxis, availability of factors and health insurance. Further to promote the cause of this disorder advocacy organisation, like world federation of hemophilia, does not exist for the VWD. Hence, in developing countries the awareness with reference to the diagnosis and therapeutic approaches is limited (Nair et al., 2011).

2.6.1 Potential and relevance of VWD

India with a population of about 1.3 billion has an estimated prevalence of 20000 patients with haemophilia A and 20000 patients with haemophilia B. In a

Caucasian population the frequency of VWD vary as the prevalence estimates. Although there exists no accurate epidemiological data on the incidence of VWD in developing countries, an estimate was derived by normalizing the reported numbers of patients diagnosed with haemophilia. Based on two presumptions that (i) patients with severe haemophilia are less likely to go undetected (Srivastava and Rodeghiero, 2005) (ii) and the ratio of patients with clinically significant VWD, could be similar to severe haemophilia. A ratio of 0.1-0.6 varied from patients with VWD to those with severe haemophilia against the norm of 1.0 thereby confirming the underdiagnosis of this condition (Nair et al., 2011). Among Caucasians the prevalence of VWD, has been reported to range from 30 per million to about 1% of the population (Nichols and Ginsburg, 1997). Iran reports the lower prevalence of VWD accounting to 6.5%, among 367 patients diagnosed with coagulation disorders (Karimi et al., 2002a). In Indian population, especially in southern India, the prevalence of VWD is expected to be higher due consanguineous marriages in certain communities (Mannucci et al., 2004).

Variable penetrance and heterogeneity complicate the diagnosis of this disorder. Hospital based surveys from India reports the lower prevalence of VWD compared to hemophilia (Karimi et al., 2002b, Srivastava and Rodeghiero, 2005) [Table 9] which, indicates the lack of awareness of the disease (Trasi et al., 2005). Therefore, VWD represents a significant clinical and social problem in India. Common clinical manifestation observed in women with VWD include menorrhagia, which accounts to about >70% of the affected female patients (Kadir et al., 1998). Reports from India suggest a prevalence of about 12% of the patients with menorrhagia (Trasi et al., 2005, Saxena et al., 2003). Hence, there arises a need to diagnose and therapeutically manage in these series of patients.

Reference	No. of Patients with Inherited Bleeding Disorders	No. of VWD Patients Diagnosed (%)	VWD: HA Ratio
Kumar *et al*	**230**	**40 (17.4)**	**–**
Trasi *et al*	**822**	**81 (9.8)**	**0.20**
Srivastava and Rodeghiero*	**200 x10^6**	**211 (0.000001)**	**0.23**
Ahmad *et al*	**1576**	**136 (8.6)**	**0.14**
Shanthala Devi *et al*	**430**	**22 (5.1)**	**–**
Mohanty *et al*	**178**	**12 (6.7)**	**0.1**
Kolkata (unpublished)	**3400**	**31 (0.91)**	**0.91**

Table 9: ***Prevalence of Von Willebrand Disease Reported from Different Parts of India.*** HA, haemophilia A *Population-based data derived from the regional registry.

[Reference: Ghosh K et al. Semin Thromb Hemost, 2011]

2.6.2 Diagnosis of Von Willebrand Disease in India

Diagnosis of patients with VWD in India begins with the evaluation of the patient's personal and family history of bleeding by experienced personnel preceded by documentation. When a positive bleeding history was documented, the initial screening test was carried out to pre-screen for the defects in intrinsic, extrinsic and common pathways of hemostasis. Screening tests include bleeding time, Complete blood count, prothrombin time, activated partial thromboplastin time and (iv) thrombin time. In hospitals with limited diagnostic facilities, the bleeding time can be established. In a total of 852, patients evaluated in our centre during 2004-2008, the sensitivity of the bleeding time was found to be 100% for type -3VWD

and 52% for type-1VWD (Nair et al., 2011). Laboratory diagnosis of VWD includes factor assays and agglutination assays which are available only in four to five centres in India (Ghosh and Shetty, 2011). The factor based assays include VWF antigen assay (VWF: Ag), ristocetin cofactor (VWF: RCo) and collagen binding (VWF: CB) assay. Other tests which are not done routinely in the laboratory include VWF: FVIII binding (VWF: FVIIIB) assay and multimer analysis. Since many of the labs in India do not afford ristocetin-induced platelet agglutination (RIPA), some patients are misclassified as hemophilia A. Considerable overlapping on different types of VWD on diagnosis suggested the misdiagnosis of the subtype. Hence, specific challenges do exist in the management of these patients, due to the limitations in the diagnosis of this condition. Although limitations, exists it was possible in our centre to afford a comprehensive health care for these patients as follows. Among 202 patients diagnosed with VWD in our centre, 107 patients were classified as type-3 while 62 being type-1; and 33 patients as type-2VWD (Nair et al., 2011). Cost-effective automated methods using advanced coagulometers for VWF antigen and ristocetin cofactor assay is being carried out in our centre. This novel approach has shown to reduce the turn around time coupled with a cost effective strategy. Discriminatory test including ristocetin-induced platelet agglutination (RIPA) assay and collagen binding assay are usually carried out in batches. In many labs though multimer analysis is not offered this test did not significantly to affect the diagnostic algorithm (Nair et al., 2011) as depicted by the distribution of the subtypes of VWD in our centre [Table 10].

Type	No. (%)
Mild or possible type 1	22 (10.9)
Moderate to severe type 1	40 (19.8)
3	107 (53.0)
2A	3 (1.5)
2M	3 (1.5)
2A/2M	5 (2.5)
2B	14 (6.9)
Platelet-type VWD	2 (1.0)
Unclassified	6 (3.0)
Total	202 (100)

Table 10: ***Distribution of Subtypes of VWD in Southern India.***

[Reference: Nair SC, Semin Thromb Hemost, 2011]

The foremost requirement in the accurate diagnosis of VWD includes training of the personnel. Various training programs including Twinning Program and International Haemophilia Training Centres is being conducted by World Federation of Haemophilia to educate the people involved in the diagnosis of bleeding disorders. However, these facilities are not adequately used in the developing countries including India (Giangrande and Black, 2005). By participating in international external quality assurance scheme, NEQAS, performance status of the labs can be updated.

Centre	Reference	No. of VWD Patients	Type 1, No (%)	Type 2				Type 3, No (%)	Tests Used for Diagnosis
				2A, No (%)	2B, No (%)	2M, No (%)	2N, No (%)		
BHU	Kumar *et al*	40	17 (42.5)	10 (25)	-	-	1 (2.5)	12 (30)	FVIII:C, RIPA, VWF:Ag
AIIMS	Gupta *et al*	94	20 (21.3)	38 (40.4)	-	4 (4.3)	-	32 (34)	FVIII:C, RIPA, VWF:Ag, VWF:RCo, multimer analysis
NIIH	Trasi *et al*	81	15 (18.5)	8 (9.9)	4 (4.9)	1 (1.2)	3 (3.7)	50 (61.7)	FVIII:C, RIPA, VWF:Ag, VWF:RCo, VWF:CB, VWF:FVIIIB, multimer analysis
CMC	Srivastava and Rodeghiero	183	-	-	-	-	-	95 (51.9)	FVIII:C, RIPA VWF:Ag

Table 11: ***Von Willebrand Disease Subtypes Reported from Different Parts of India.***

[Reference: Ghosh K et al. Semin Thromb Hemost, 2011]

2.6.3 Molecular diagnosis of VWD in India

Data on the molecular genetics of VWD has gained recently in India. Changes in VWF gene could alter process involved in biosynthesis, storage or the mechanisms associated with clearance. More than 500 mutants and polymorphisms associated with the VWF gene, is reported ISTH-SSC (http://www.vwf .group.shef.ac.uk/VWD.html). The allelic frequency of the polymorphisms identified in the Brazilian population was significantly distinct from the other ethnic groups comprising Euro-Brazilians, Afro-Brazilians, and Amerindian (Mazzini et al., 2000). These studies have paved way to identify the polymorphic markers for the diagnosis of VWD. Intron 40 VNTR markers (VWF1, VWF2, and VWF3), due to their high heterozygosity rates have been useful for carrier detection and prenatal diagnosis (Baronciani et al., 2003). Under conditions were the familial studies were not informative or non-availability of the index case phenotypic diagnosis was preferred using factor (F) VIII and VWF: Ag assays. Few labs from India have published their mutation profile of these patients predominantly by direct sequencing approach. In Indian and Iranian patients molecular genetics of VWD is described. Mutations were screened in 40 patients (14 Iranians, 14 Indians, 12 Italian) with type 3 VWD, were 45 mutations were novel from a total of 50 gene defects identified. The majority of the mutations defined in the study were nonsense, and frameshift mutations resulting in truncation of the protein, followed by missense and splice site mutations (Baronciani et al., 2003). The second large study included mutation screening in 85 unrelated patients diagnosed with type-3 VWD. The strategy used in this study to identify mutations includes RFLP i.e. screening for 11 CGA codons, arginine hotspot regions.

In patients where mutations could not be defined, direct sequencing approach was carried out. Multiple ligation probe amplification (MLPA) was used to determine the extent of deletion in patients were large deletions were suspected. Using these approaches mutations were identified in 77 index cases. Mutations numbering 59 were identified, these included missense 22%, splice site 6.8%, gene conversions 10.2%, insertions 3.4% duplication 1.7%, small deletions 17% and large deletions 5.1% were identified, of which 34 were novel. Founder effect accounted for two mutations including p.R1779* and p.L970del. Interestingly in this study a large deletion was identified, spanning exons 14-52 which correlated with the development of antibodies against VWF (Kasatkar et al., 2014). Another study from India were mutations screened in Type 2-VWD (n=56) patients by direct sequencing approach. Out of 23 mutations identified 16, were novel. Expression studies for these mutant's p.Pro1266Glu, p.Val1439Met, and p.Asn1635Ile represented a loss of function for the mutations. However, a gain of function for the mutation p.Arg1308Cys. The common cause of pathogenicity associated with type 2A VWD patients was found to be gene conversions (Ahmad et al., 2013b). Hence, development of simplistic approach could pave the way for other labs to afford genetic diagnosis to the affected families.

2.6.4 Management of VWD in India

The aim of the therapy is to achieve hemostatic levels of VWF during bleeding episodes, trauma, and surgery. The choice of the therapeutic product depends initially on the type of VWD diagnosed and severity of bleeding. In India, treatment products widely used includes desmopressin (DDAVP), direct infusion of VWF in the form of cryoprecipitate or FVIII derived concentrates (Nair et al., 2011).

Limited data exists mainly concerning the dose used to achieve hemostatic levels and their clinical outcomes in patients treated with these therapeutic products (Federici, 2005). Antifibrinolytic agents widely used as an adjuvant therapy include tranexamic acid and epsilon-aminocaproic acid (Nair et al., 2011). Oral contraceptives were preferred in women with menorrhagia as a supplementary treatment, to reduce the potential of bleeding in these patients. In few centres in India during minor or major surgeries hemostasis in these patients was maintained using FVIII or cryoprecipitate along with other therapeutic products. Studies from our centre have shown that during surgery, a modest dose of intermediate purity FVIII at 35 IU/kg and after surgery a dose of 10 to 20 IU/kg is sufficient to achieve hemostasis in these patients (Nair et al., 2011).

Although significant advances to understand the molecular basis of the disease was carried out, the landscape governing diagnosis and the therapeutic modality remain unchanged more than a decade in the developing countries including India. Limited published data exists on the patients diagnosed with VWD and its impact in the developing countries. The no of centres which can afford diagnosis to VWD is comparatively limited, in India. Lower prevalence of VWD in India, suggests the need to improve the diagnostic services and lack of awareness. Since the care for the bleeding disorder is not optimal, in developing countries preventive strategies could be adopted using genetic diagnosis. The VWF gene spans 178Kb. Hence, a simpler approach is required to facilitate the genetic diagnosis. As the data on the techniques and mutations gets accumulated from many labs, a simplistic diagnostic approach could be derived.

Type	Treatment	Product	Other Nonconventional Treatment Products
1	DDAVP	cryoprecipitate	Danazol, thalidomide, atorvastatin (gastrointestinal angiodysplasia), oral contraceptives, Ethinyl estradiol, epsilon-aminocaproic acid, tranexamic acid, thyroxine (hypothyroidism), atorvastatin, whole blood, fresh frozen plasma, packed cells, oral iron replacement (anemia). Local use: human thrombin or collagen, Bothrops atrox venom, oxidized cellulose soaked with human thrombin or platelet concentrates.
2A	DDAVP	cryoprecipitate	Local use: human thrombin or collagen, Bothrops atrox venom, oxidized cellulose soaked with human thrombin or platelet concentrates.
2B	Cryoprecipitate		
2M	Cryoprecipitate	DDAVP	
2N	Cryoprecipitate,	DDAVP	
3	Cryoprecipitate	FVIII-VWF more over, platelet concentrates	
Acquired	Intravenous immunoglobulin, cryoprecipitate,	FVIII-VWF concentrates, DDAVP	

Table 12: ***Treatment Products Used to Treat VWD Patients in India.***

[Reference: Ghosh K et al. Semin Thromb Hemost, 2011]

Therapeutic options in patients diagnosed with VWD vary depending on the type, severity, and ease of access to the therapeutic product (Nair et al., 2011). Developed countries able to afford on-demand therapy and factor replacement therapy, but in developing countries the amount allocated for bleeding disorders is

scarce. Therapeutic products including DDAVP, antifibrinolytic drugs, and clotting factor concentrates must be made accessible to the patients. Since 1990's the therapeutic approach used for the treatment has not changed. It could be of great interest to look at the molecules, which could enhance the half-life of VWF. Hence, this goal could be achieved by looking at the factors that contribute to the quantitative trait, especially the clearance mechanism. There lies no clear rational on therapeutic modality, although there exists some preclinical success on gene therapy (De Meyer et al., 2006). Hence, awareness coupled with the improved diagnostic and treatment facilities will probe for a better health care and management.

3. Patients, Materials and Methods

3.1 Patients

A total of one hundred and two patients from 90 families with Type 3-VWD seen at the Department of Hematology, Christian Medical College, were evaluated. These patients were diagnosed based on the clinical and haematological parameters. Patients recruited in the present study were predominantly from southern states of India. They were classified as type 3 based on the concentration of VWF antigen in the plasma, FVIII:C and collagen binding assay (Nair et al., 2011). Patients included in this doctoral work were recruited during 2012-2015. Since type 3-VWD follows a recessive pattern of inheritance, a positive family history of bleeding was not required for enrollment. The study was approved by our institutional review board (IRB min A13-25-04-2012).

3.1.1 Inclusion criteria

Inclusion criteria in the present study included an index case (IC) with a documented history of mucocutaneous bleeding and plasma (Nair et al., 2011) levels of VWF antigen (VWF: Ag), < 0.001 IU/mL.

3.1.2 Definitions

Type 3 VWD: Patients with VWF: Ag <0.001 IU/mL with a severely decreased FVIII:C of the normal, were considered as having type 3-VWD respectively (Nair et al., 2011). The disease is characterized by mucocutaneous bleeding occurring spontaneously or after surgery and minimal trauma.

3.1.3 Clinical evaluation

On enrolment, patients were evaluated for the frequency and site of haemorrhage through a prospective data collection questionnaire. Demographic profile and information related to patients bleeding history were documented using ISTH BAT questionnaire (Rodeghiero et al., 2010, Bidlingmaier et al., 2012).

Bleeding symptoms assessed using this questionnaire includes mucocutaneous bleeding including epistaxis, hemarthrosis, muscle hematomas, oral cavity bleeding, hematemesis, hematuria, surgical bleeding, menorrhagia, post-partum bleeding, CNS bleeding and surgical bleeding (Rodeghiero et al., 2010, Bidlingmaier et al., 2012). Bleeding questionnaires were administered by experienced personnel and scored. Score for a given subject was derived by summing the scores obtained for all the bleeding symptoms. The bleeding score is defined as the sum of grades of severity of bleeding. The pedigree of the family members for at least two generations was obtained in each index case.

3.1.4 Frequency and site of Bleeding

The frequency and the locationof mucocutaneous bleeding was documented as detailed in (Appendix 9.1).

3.2 Laboratory evaluation-Sample Processing

On enrolment, these patients were given a unique patient number (UPN), based on the VWD patient registry maintained in our department since 2001. Subsequently, a 9ml venous blood sample was drawn, and four aliquots were made for various investigations. These include (i) EDTA anticoagulated sample for

baseline haematological parameters Complete Blood Count (CBC) (BD Vacutainer, BD Medical Systems, Plymouth UK), (ii) an aliquot of citrated blood for measuring the coagulation parameters, (iii) aliquot of EDTA anti-coagulated blood for genomic DNA extraction and the final aliquot in the tube for (iv) serum fractionation. The blood samples were centrifuged at 3600rpm for 10-15min and plasma, or serum was stored at -80^{O}C till further analysis. For measuring coagulation parameters, the blood was centrifuged twice at 3000 rpm for 20 minutes, and the platelet poor plasma was stored at -80^{O}C.

Baseline haematological parameters of each patient were documented. The sample collected was processed for various coagulation assays and molecular studies. The scheme of evaluation of patients enrolled in the study is detailed in [Figure 23].

3.3 Coagulation studies

3.3.1 Baseline Investigations

Complete blood counts of the patients were estimated in a cell counter (Coulter LH755; BeckmanCoulter, Brea, CA, USA)

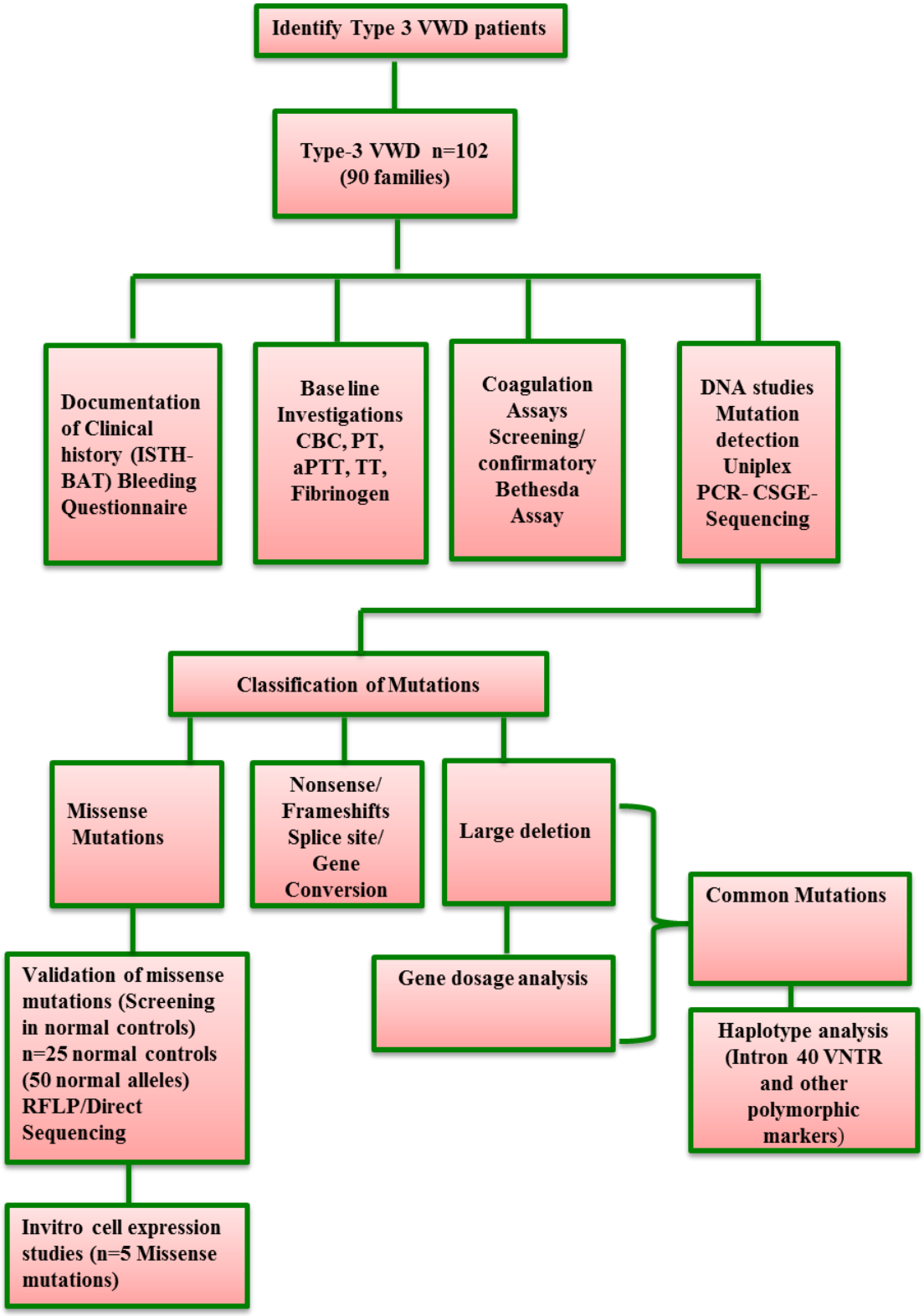


Figure 23: ***Scheme for evaluation of patients in the present stu***

3.3.2 Screening test for Von Willebrand Disease

These test were carried out in, Departments of Immunohematology & Transfusion Medicine.

Screening tests for the diagnosis of VWD include prothrombin time (PT), activated partial thromboplast in time (aPTT), and thrombin time (TT). These tests were carried out on a CS 2000i automated analyser (Sysmex, Kobe, Japan) with reagents Synthasil for APTT (IL, Milan, Italy) and Innovin for PT (Siemens, Marburg, Germany) reagents as per standard protocols. In patients were aPTT was prolonged, mixing studies with pooled normal plasma was carried out to assess the presence of a factor deficiency or inhibitory effect. Normal value ranges from (aPTT: 25.1-36.7 secs). The second line of tests includes (i) CBC analysed using an automated cell counter (DXH 800, Beckman Coulter, USA), preceded by the bleeding time. Bleeding time (BT) was performed based on the modified Ivy method. This test is considered as an important test for mucocutaneous specific bleeding (Mielke, 1984). It refers to the time taken for the standardised skin cut of 3mm depth & 1.5mm width to stop bleeding (Mielke, 1984).

Fibrinogen levels in the plasma were measured by modified Clauss assay (Jennings et al., 2009). This assay was carried out in an automated coagulometer ACL Top CS 2000i (Sysmex, Kobe, Japan).The reagents and the calibrators used was procured from the instrumental Laboratory (IL, Milan, Italy). The normal value ranges from 150-450mg/dL. This test was followed by the measurement of FVIII coagulant activity (FVIII:C) assay by One-stage aPTT-based clotting assay. FVIII:C was measured in automated coagulometer, in the presence of immune depleted

FVIII-deficient plasma (Siemens, Marburg, Germany) and IL calibrator (IL, Milan, Italy), and a standard curve was generated. The normal value for FVIII:C ranges from 50-150%. In patients with type 3 VWD, a concordant decrease in FVIII was observed. Further, these samples were subjected to specific tests for VWD.

3.3.3 Tests for Von Willebrand Factor

3.3.3.1 VWF Ristocetin Cofactor Assay (VWF: RCo)

Ristocetin cofactor activity defines the interaction of the platelets with the VWF (Evans and Austen, 1977). Initially, this assay was carried out in an automated centrifugal coagulometer (ACL 10000; IL, Milan, Italy) using commercially available platelets (Von Willebrand Reagent; Dade-Behring, Marburg, Germany). In this method the VWF: RCo activity was measured by the change in optical density in a reaction mixture at 405nm (chromogenic channel) in a centrifugal analyser at 600 rpm. The results were then plotted on a log-in graph. The test values corresponding to the decrease in OD was read directly from the graph expressed in percentage. Normal value ranges from 50-175% (Nair et al., 2011).

Recently with the availability of automated coagulometer, CS 2000i (Sysmex, Kobe, Japan), the RCo values were automatically calculated, unlike ACL 1000, where the measurement has to be done manually. In the presence of ristocetin, the VWF (ristocetin cofactor) in the sample causes agglutination of the stabilised platelets in the VWF reagent (Dade-Behring, Marburg, Germany). The agglutination process reduces the turbidity at the onset of the reaction, were the change in optical density is measured. The level of detection limit in ACL is found to be 14 IU/dL CS200i is 8 IU/dL

3.3.3.2 VWF Antigen Assay(VWF: Ag)

VWF: Antigen assay measures the amount of VWF present in the citrated human plasma. This test is based on a latex-enhanced immunoassay, carried out in an automated coagulometer. HemosIL VWF: Ag (IL, Milan, Italy) is a suspension of polystyrene polyclonal rabbit anti-human VWF antibody direct against human VWF. The latex particles agglutinate in the presence of VWF: Ag, by inducing a change in absorption measured at 540 nm (Nair et al., 2011). The linearity of this assay was found to range from 6 to150 IU/dL. Normal value ranges from (61.3-157.8 u/dL). For O blood group patients the value ranges from (41.1-125.9 u/dL) (Nair et al., 2011).

3.3.3.3 Ristocetin-Induced Platelet Agglutination (RIPA)

RIPA involves the assessment of aggregation of platelets, in platelet rich plasma (PRP) in the presence of ristocetin (normal dose: 1.5 mg mL^{-1}), (Sigma-Aldrich, Bangalore, India) in an aggregometer (560VS; Chrono-Log Corp, Havertown, PA, USA). The chrono-log is designed in a way that, when a beam of infrared light passes through the cuvettes one contains PRP (the sample) and other containing platelet poor plasma (PPP) (the reference), platelets tend to aggregate. The difference in light transmission outputs from the photodiodes was documented. PPP is considered to be 100 % light transmission or 100% aggregation (Nair et al., 2011).

3.3.3.4 VWF FVIII Binding Assay (VWF: FVIIIB)

FVIII binding assay assesses the ability of the patients VWF to bind to FVIII. The assay is based on the immobilisation VWF:FVIII:C complex on a microtiter plate, coated with the MAb raised against the VWF (Dako, Glostrup, Denmark).

Incubation removed endogenous FVIII with a high ionic strength buffer. The VWF bound to the MAb, was incubated with a recombinant preparation FVIII (rFVIII) at a physiological concentration in the presence of Ca^{2+}. The amount of rFVIII bound to the immobilised VWF was measured using primary antibody sheep anti-FVIII:C polyclonal Ab (RD1) (Recombinant, Baxter, Vienna, Austria), in the presence of tagged antibody (Donkey anti-sheep IgG peroxidase conjugated (Jackson, Immuno research), which defines the FVIII activity in a given subject (Nair et al., 2011).

3.3.3.5 VWF Collagen Binding Assay

VWF: Collagen binding (CB) assay detects the ability of VWF to bind to collagen. In this method, an in-house ELISA plate was coated with collagen (ICN Biomedicals Inc, Aurora, OH, USA). Pre-dilution of the test sample was made in a ratio of 1:10. The calibration curve runs from 400% to 0%. A pre-diluted peroxidase conjugated antibody was added to each well followed by the addition of the substrate O-phenylenediamine *(*OPD). The colour developed was measured at 492nm. The normal value ranges from 50 - 400 % (Nair et al., 2011)

3.4 Genotypic analysis

3.4.1 DNA extraction

DNA was extracted from the 9ml peripheral blood from the patients and available family members by standard phenol-chloroform method and stored at 4˚C until use.

3.4.2 RNA extraction

RNA was extracted from the plasmid transfected HEK-293 cells using TRIzol reagent (Invitrogen, CA, USA) as per the standard protocol and stored until use at -80°C.

3.4.3 DNA and RNA quantification

DNA and RNA extracted from these patients was assessed for the quality and quantity using Nanodrop 1000 spectrophotometer (Thermo scientific DE, USA). The ratio of absorbance at 260/280 was used to evaluate the purity of DNA/RNA. A ratio of 1.8 was acceptable for pure DNA and 2.0 for pure RNA. All the samples were diluted to equal concentration and used for analysis.

3.5 Uniplex PCR and Conformation Sensitive Gel Electrophoresis (CSGE) for VWF gene

3.5.1 PCR amplification of *VWF* gene

The *VWF* gene encompassing the coding regions, intron/exon boundaries and 5' and 3' regions were amplified by 52 pairs of primers designed in-house [Table 13]. The following conditions were used for standardization. The PCR tubes were labelled (n= number of test samples + negative control without DNA). The PCR reaction was set for different exons as follows. PCR was performed with 10 picomoles of each primer [Table 13] in a 2X concentration of a ready reaction mix (GeNei, Bangalore, India). Genomic DNA (200 ng) was used for amplification reactions. Thirty cycles of PCR amplification, denaturation at 94°C for 5 min, annealing at 58°C for 40 sec following extension at 72°C for 40 sec and final extension was at 72°C for 5 min. For exon

13 and 52, the PCR was performed in a 2X concentration of a ready reaction mix (GeNei, Bangalore, India) at 56°C. For exon 14 PCR reactions were carried out using Hotstar master mix (QIAGEN, GmBH, Germany) containing 10X Buffer, 1.5Mm MgCl2, and Hot start Taq DNA polymerase. PCR was carried in a 25 µl reaction containing 12.5µl master mix, 3 µl of Q solution, 200ng of genomic DNA, and 6.5µl of water. Samples were then amplified in a thermocycler Gene Amp 9700 (PE, Applied Biosystems, Foster City, CA). Thirty cycles of PCR was carried out with denaturation at 94°C for 40 seconds, annealing temperature at 58°C: 40 seconds and extension at 72°C: 40 seconds. The final extension was at 72°C for 5 minutes. Exon 28 was amplified using ready reaction mix HotStar Taq Master Mix (QIAGEN, GmBH, Germany), in the presence of Q solution in a PCR thermocycler Gene Amp 9700 (PE, Applied Biosystems, Foster City, CA) with the following conditions. Initial denaturation at 96°C for 15 minutes, 30 cycles of PCR amplification was performed with 98°C for 45 seconds denaturation, annealing at 58°C for 1:30 seconds and extension at 72°C for 2:40 seconds. The final extension at 72°C for 7 minutes. After amplification, internal primers were used for sequencing specific fragments in exon 28 [Table 14]. Mutations identified in the present study were validated by using a set of primers received on request from *Hampshire DJ et al* (Hampshire et al., 2010).

3.5.1.1 Amplification check

Agarose gel electrophoresis resolved the specificity of the PCR products. 5µl of the PCR product was loaded in 2% agarose gel containing SafeView (NBS Biologicals, UK) electrophoresed at 120V for 30 minutes in 0.5X TBE buffer.

PCR products were visualized by UV using BIORAD Gel Doc XR system (Bio-Rad, Hercules, and CA). Uniplex PCR could reliably detect gross deletions due to the failure of amplification of the successive fragments in VWF gene relative to a normal internal control.

Oligo name	Primer sequence (5'to3')	Product Size (bp)	Volume of 2X Master Mix (µl)	Concen-tration of forward and reverse primers (µM)	Annealing temperature (˚C)
VWF - Exon1F	GTCCATGTTCAAAGGGGAAA	455	12.5	10	58
VWF - Exon1R	TGTGCCACCTTTATGCTTCTT				
VWF - Exon2F	CCACAGCCCAGTTTCTATCA	400	12.5	10	58
VWF - Exon2R	TTCTCAGCAGTGACCTTTCC				
VWF - Exon3F	AAGACTTTTTGGGGCGTTTT	329	12.5	10	58
VWF - Exon3R	TTGGAACATTTGCTTCCATTC				
VWF - Exon4F	CACTTTGCACCATGTTCTGA	409	12.5	10	58
VWF - Exon4R	CAGGGAAGGCATGTTAGTGA				
VWF - Exon5F	AACACACAAAACCACCAGCA	333	12.5	10	58
VWF - Exon5R	CCTGCGTAAGTCCATTCCTC				
VWF - Exon6F	AGGGAGCTGCCTTGGTTT	399	12.5	10	58
VWF - Exon6R	AGCCCCGAAGCACCCTAA				
VWF - Exon7F	AAGACAGAGGGGAGCAGTCA	321	12.5	10	58
VWF - Exon7R	CACAAGTGGCCTTCATCTCA				
VWF - Exon8F	CAGAACAAGTTCTTTGAGCTTCC	375	12.5	10	58
VWF - Exon8R	CCACCAGTAGCCTCCAACTC				

Oligo name	Primer sequence (5'to3')	Product Size (bp)	Volume of 2X Master Mix (µl)	Concen-tration of forward and reverse primers (µM)	Annealing temperature (˚C)
VWF - Exon9F	TTCTTTTCCCACATCCCTTC	350	12.5	10	58
VWF - Exon9R	GCCTGTGAATGGGTTAGCAT				
VWF - Exon10F	TAGACTGGTTTGGGCAAAGG	434	12.5	10	58
VWF - Exon10R	GCTGGGTTTCTGGATGAATG				
VWF - Exon11F	TAGGTTATGAGAAGGCCAGAGG	400	12.5	10	58
VWF - Exon11R	GCCCTCCAAAAATAACTCTCC				
VWF - Exon12F	TGCCCCTAAGTCATTGCTCT	398	12.5	10	58
VWF - Exon12R	CAAACACCAGCCTCATAAACAA				
VWF - Exon13F	TCACCCGGGGAACTTTTT	449	12.5	10	56
VWF - Exon13R	TGCTGACGGTAAAAACAAAGC				
VWF - Exon14F	TTAGCAGCACTGGGCTATTT	403	12.5	10	58
VWF - Exon14R	GGAAACAACGCAGAGAAAGG				
VWF - Exon15F	ATTGGGGGTCACAGCTACAA	436	12.5	10	58
VWF - Exon15R	CCCAGTTTACCCATCCATGA				
VWF - Exon16F	TGTGCTTCAGGAGGGGTTAT	390	12.5	10	58
VWF - Exon16R	TCACTCACACAAACCCAGAAA				
VWF - Exon17F	TGGGCAACTCTGAGTCTCTT	400	12.5	10	58
VWF - Exon17R	CCTCCATTGCTATCCGTGTT				
VWF - Exon18F	TTACCCGTAGGCTCAAGTCTCA	329	12.5	10	58
VWF - Exon18R	AGAGAGTAACCAGGTTCCCACA				

Oligo name	Primer sequence (5'to3')	Product Size (bp)	Volume of 2X Master Mix (µl)	Concen-tration of forward and reverse primers (µM)	Annealing temperature (˚C)
VWF - Exon19F	TGTTCCTTCATTGCCTCCAT	377	12.5	10	58
VWF - Exon19R	TCAGGCACTTCTGTGAAGGA				
VWF - Exon20F	GCAAGATCCTGTGACACGTA	397	12.5	10	58
VWF - Exon20R	ACGGTCAGTTGCAATAGCTC				
VWF - Exon21F	AGAGTGGAGGGAGGATCTGG	296	12.5	10	58
VWF - Exon21R	TAGAGACCTACGATCAGGGA				
VWF - Exon22F	AGGAATGGGTCTTGGCAAT	450	12.5	10	58
VWF - Exon22R	CAGCCACCTGGAGGTCATAC				
VWF - Exon23F	GTGGGTGGTATGACCTCC	281	12.5	10	58
VWF - Exon23R	GCCAAGCCTTGGGACCGT				
VWF - Exon24F	CCTGGACCCCCTTATCCTTA	429	12.5	10	58
VWF - Exon24R	GCCAATGTCTTAACCCTCCTT				
VWF - Exon25F	CAA CAT TAT CTC CAG ATG GC	356	12.5	10	58
VWF - Exon25R	TTG CAG GTC AGA GAT AGG AC				
VWF - Exon26F	AAAAATGAGGCTTCCTCGTG	453	12.5	10	58
VWF - Exon26R	AGCAAAAACACTGTGGAGGAA				
VWF- Exon27AF	TGTCCACAGGTTCTTCCTGA	512	12.5	10	52
VWF - Exon27AR	TACTTCACCTGGCTGGCAAT				
VWF - Exon27BF	CCCAGAAGTGGGTCCGCGTGGCC	447	12.5	10	58
VWF - Exon27BR	CACAGAGGTAGCTAACGATCTCG				

Oligo name	Primer sequence (5'to3')	Product Size (bp)	Volume of 2X Master Mix (µl)	Concen-tration of forward and reverse primers (µM)	Annealing temperature (˚C)
VWF - Exon27CF	AGATCCGCCTCATCGAGAA	387	12.5	10	58
VWF - Exon27CR	TGAAGGGGTACTCCACAGTCA				
VWF - Exon27DF	AGGATCGGACAAAATTGGTG	321	12.5	10	58
VWF - Exon27DR	GGATTTCCGGTGACCATGTA				
VWF - Exon27EF	TGCGGTACCTCTCTGACCAC	397	12.5	10	58
VWF - Exon27ER	CGAGTCGTATCTTGGCAGATG				
VWF - Exon28F	GGCTATGTGTGTGTTTTGATGG	1.6Kb	12.5	10	58
VWF - Exon28R	CTTGGCAGATGCATGTAGCAG				
VWF - Exon29F	GAGGCTCTTTTTGTGGCTCT	387	12.5	10	58
VWF - Exon29R	TCAGAAACTCCAAGGAACACC				
VWF - Exon30F	TGGGTGTTCCTTGGAGTTTC	349	12.5	10	58
VWF - Exon31F	CCAGTCCATTTTGAGCCTTC	396	12.5	10	58
VWF - Exon31R	CACCTCCCATGAACAGAAACT				
VWF - Exon32F	TGCAGAGCATGTCTGAAGAA	384	12.5	10	58
VWF - Exon32R	GTACAGATGGACCCGCAAAA				
VWF - Exon33F	CCT CCT TGC TGT GTA GGC CT	560	12.5	10	58
VWF - Exon33R	GAAAAGCAATTCTTCCTTCCA				
VWF - Exon34F	AAGGGCCTGTTCCATTCTCT	429	12.5	10	58
VWF - Exon34R	AACTAAAAGCAACTGCCACCA				

Oligo name	Primer sequence (5'to3')	Product Size (bp)	Volume of 2X Master Mix (µl)	Concen-tration of forward and reverse primers (µM)	Annealing temperature (˚C)
VWF - Exon35F	GAGCTGCCGACAAATATCAA	442	12.5	10	58
VWF - Exon35R	CCTCGCTACTAGACCCTGAAAT				
VWF - Exon36F	TGGTAAGAACATTTTCTCAACTCCT	399	12.5	10	58
VWF - Exon36R	CAAACAGTGGTAAGAGGAGGAC				
VWF - Exon37F	TGTTCAGGAATGGACTGTGC	426	12.5	10	58
VWF - Exon37R	ATCTTCCATCTTATTTGATCCTAACT				
VWF - Exon38F	TGGTGGAAAATAGCCGTCTC	418	12.5	10	58
VWF - Exon38R	CACAGTTGGAAGAGGCCAAT				
VWF - Exon39F	TCCTTTCAGCCTGCTTTTGT	390	12.5	10	58
VWF - Exon39R	AAGCCCACCCACTCTAGGAC				
VWF - Exon40F	TGTGGGATGACCGTACAGAA	358	12.5	10	58
VWF - Exon40R	TCCAGAGGTAACCCTTTCCA				
VWF - Exon41F	AACCCATGTAATCTCTGTCTCCA	381	12.5	10	58
VWF - Exon41R	TCCCAACCCAGATTCAGCTA				
VWF - Exon42F	CACATTTCTGACTTAATCTTCTGAGT	459	12.5	10	58
VWF - Exon42R	TGAGGAGCACATGTTGCTTAG				
VWF - Exon43F	TTGCCACATTCTCAGCACTT	449	12.5	10	58
VWF - Exon43R	TTTCTTGCCCTTCCTCGT				

Oligo name	Primer sequence (5'to3')	Product Size (bp)	Volume of 2X Master Mix (µl)	Concen-tration of forward and reverse primers (µM)	Annealing temperature (˚C)
VWF Exon44F	AAATGCCCAGACCAGTGAT	450	12.5	10	58
VWF - Exon44R	GGCTTCTCCCAAAATCTACAAA				
VWF - Exon45F	ACGTCTAGAAACCACCTTCCTG	397	12.5	10	58
VWF - Exon45R	CGGTCCTATCCATTTCCCTA				
VWF - Exon46F	AGGAGGAGCCCCAAAGAGAG	320	12.5	10	58
VWF - Exon46R	CGGAGAGTCAGGAAGATGGT				
VWF - Exon47F	ATGGCTCATGGGAGCTATGG	392	12.5	10	58
VWF - Exon47R	CAGTTTGGGTGGGTGATTTT				
VWF - Exon48F	AGAGATGAGAGGCCAGCAAA	300	12.5	10	58
VWF - Exon48R	AAAGAAGCCAATACTGAACCAAA				
VWF - Exon49F	GGGTGTGGGGGCTTTATTAT	370	12.5	10	58
VWF - Exon49R	GAGAAATTATGCCGGAGCTG				
VWF - Exon50F	CATCTGGGCACTTAAGCACA	331	12.5	10	58
VWF - Exon50R	TGCAAGACTGAACATAATGACTGA				
VWF - Exon51F	CTG AAG AGT GTT CTC TAG AA	375	12.5	10	58
VWF Exon51R	GTA ACT AAG GTA AAG TAT CC				
VWF Exon52F	AGA TCA GAC CTGCCT TGC TT	311	12.5	10	56
VWF - Exon52R	TTG CCA TCT CAG CCC TAC GA	311	12.5	10	

Table 13: ***PCR primers used for the amplification of VWF gene***

Primer	Primer sequence 5'-3'
VWF -Exon 28 AF	CTTGGATGTGGAATGGTCCA
VWF -Exon 28 AR	CTTCAGCAAGATCGACCGCC
VWF -Exon 28 BF	CAGCAGGCTACTGGACCTGG
VWF -Exon 28 BR	CGAGATCGTTAGCTACCTCTG
VWF -Exon 28 CF	CAAGCAGATCCGCCTCATCG
VWF -Exon 28 CR	GAGATCAAGAGGCTGCCTGG
VWF -Exon 28 DF **VWF -Exon 28 DR**	AACAGGACCAACACTGGGC CGACTCGGGTTCTAATCCTG

Table 14: ***Internal primers used for exon 28 sequencing.***

[Reference: Daniel J. Hampshire, haematologica, 2010]

3.5.1.2 Hetroduplexing the samples

Heteroduplexing was carried out by mixing equal proportion of the patient PCR product and control DNA in a 0.5ml Eppendorf. These PCR products were then subjected to denaturation at 95˚C for 5 minutes and annealed at 55˚C for 30 minutes in a thermocycler Gene Amp 9700 (PE, Applied Biosystems, Foster City, CA).

3.6 Screening for mutations using CSGE

3.6.1 Principle

CSGE was first described in the year 1993 by Ganguly *et al* (Ganguly, 2002). This technique is based on non-radioactive heteroduplex based detection method for scanning mutations. This method relies on two working principles. First, single base mismatches can produce conformation changes in the double-stranded DNA, leading to differential migration of heteroduplex and homoduplex in polyacrylamide

gel electrophoresis. Second, mild denaturing solvents in an appropriate buffer can intensify the conformational changes produced by single-base matches, resulting in increased differential migration of heteroduplexes and homoduplexes. Ethidium bromide staining and visualization with a hand–held ultraviolet light torch, aids to determine the samples with aberrant banding patterns resulting from heteroduplexes. These samples were then subsequently subjected to DNA sequencing to determine the nature of nucleotide alteration.

3.6.2 Method

Prior to electrophoresis, 4-5µl of the heteroduplexed samples were mixed with 1.5µl of loading buffer (70% glycerol, 0.1% xylene cyanol, 0.1% bromophenol blue, 0.01% 1M EDTA) and electrophoresed in a mildly denaturing gel (400x330x1mm in size) containing 10% acrylamide (>99 % acrylamide (Sigma, St.Louis, USA) : bisacroyl piperazine (Fluka Chemie, Buchs, Switzerland), 10% ethylene glycol (Sigma, St. Louis, USA) 15% formamide (Sigma, St.Louis, USA) and 0.5X TTE buffer [20XTTE-1.78M Tris (USB, Ohio, USA), 570Mm Taurine (USB, Ohio, USA), 4Mm EDTA (USB, Ohio, USA)] as described previously (Sumitha et al., 2011). Polymerization was achieved by the addition of 0.1% ammonium persulphate (USB, Ohio, USA), and 0.07% N, N, N', N-tetramethylene diamine (TEMED) (Sigma, St.Louis, USA). The PCR products were electrophoresed at 400V for 18 hours in 20X TTE buffer. The bands were visualized by ethidium bromide staining.

3.6.2.1 Interpretation of Results

The aberrant patterns visualized on CSGE can reflect the nature of nucleotide change. These variations enhance or retard the migration of DNA to form heteroduplexes from homoduplexes, depend on the type or nature of nucleotide change. The degree of band separation during polyacrylamide gel electrophoresis depends on the fragment size, sequence composition, and flanking nucleotide sequence at the site of mutation. Large band separations were observed due to small insertions or deletion of few base pairs, due to an increased conformational change mediated by wild-type DNA. The banding patterns observed as a result of single nucleotide substitution can be identified easily in comparison to that of homoduplex wild type-DNA. In some cases, the heteroduplexes were found to induce a slight retardation of migration during polyacrylamide gel electrophoresis. Other conditions were a nucleotide substitution is overlooked being the “fatter” bands which can mimic an overloaded well. Hence, a substantial visual examination of CSGE is essential to compare all the samples with the normal or wild-type DNA. After documentation of the results, the altered banding patterns were investigated by two approaches (1) possibly with loading less DNA, or direct sequencing of the sample [Figure 24 and 25]

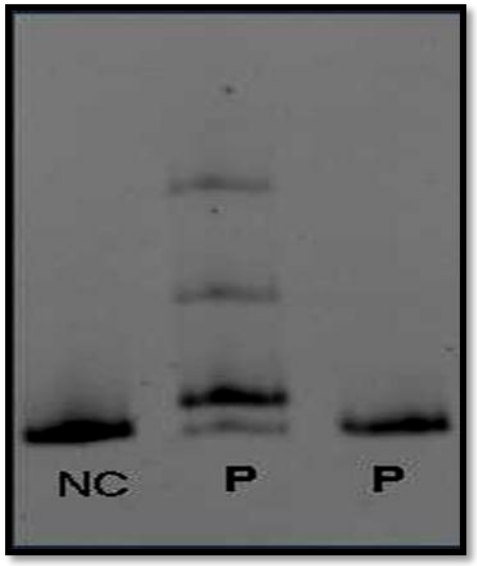


Figure 24: ***Representative gel photograph for uniplex PCR-CSGE analysis for mutation detection in VWF gene.***

The picture depicts the abnormal CSGE pattern relative to a normal control (NC) PCR product loaded on to the gel. Sequencing of these abnormal fragments identified the mutations. The picture shows the abnormal CSGE pattern for patient V-450, in exon 25 of the VWF gene. This mutation predicts a change c.3412_3413dupAA at codon 1138 resulting in premature termination of the protein at position 78, to cause severe type 3 VWD in this patient.

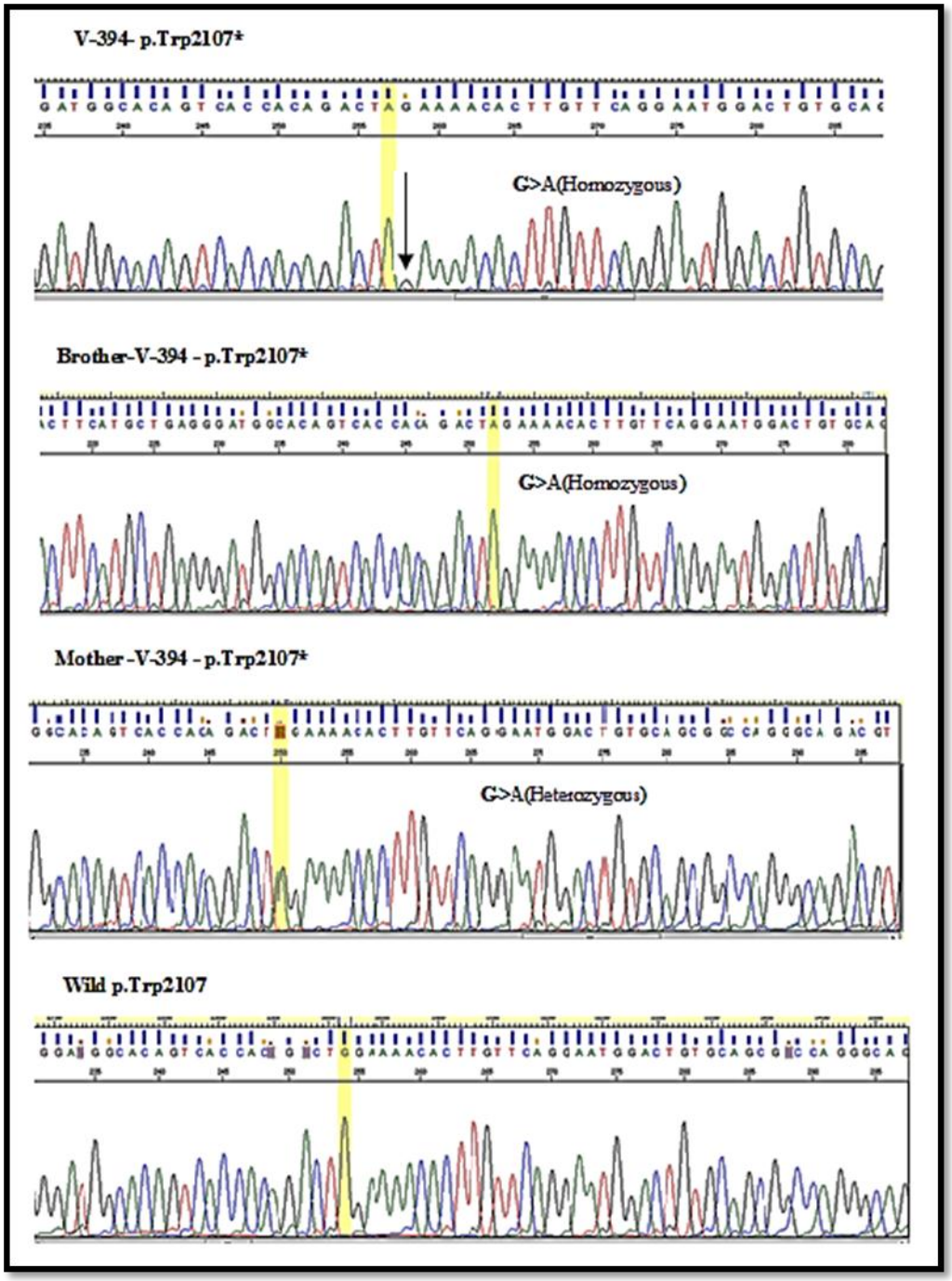


Figure 25: ***Chromatogram representing the forward sequence (5'→3') of VWF gene showing the mutation p.Trp2107 in the family.***

3.7 DNA sequencing

DNA fragments were sequenced using fluorescently labelled dideoxy nucleotide triphosphates using the Big Dye Terminator cycle sequencing kit Version(V) 1.1 or 3.1 (Applied Bio systems, Foster City, CA) in a genetic analyzer Applied Biosystems 3130 (Life Technologies, Warrington, UK). The PCR products were purified by enzymatic digestion to remove unincorporated primers and dNTPs using five units of ExoSAP (USB Corporation, Cleveland, Ohio) at 37˚C for 45 minutes, followed by heat inactivation at 85°C for 15 min. The purified PCR products were then diluted according to the intensity. Bidirectional sequencing reaction was carried out using primers, specific for the PCR product followed by cycling conditions at 95˚C for 15 Sec, 50 ˚C - 15 sec 60˚C - 4min for 25 cycles in a thermocycler Gene Amp 9700 (PE, Applied Biosystems, Foster City, CA). After the cycling reactions the PCR products were again purified using HighPrep^{TM} Magnetic beads or using either SEQ filter plates (EMD Millipore, Germany).

3.7.1 Principle of the beads method (HighPrep^{TM} Magnetic beads)

The HighPrep^{TM} Magnetic beads reagent is a paramagnetic based system used to remove unincorporated terminators from sangers sequencing reactions. The protocol comprises of selective binding of DNA to HighPrep^{TM} DTR (Dye Terminator Removal Clean Up) particles, followed by washing of the nucleotides, primers, and non-targeted amplicons. HighPrep^{TM} DTR involves three steps, bind-wash-elute. The mixture when applied to the magnetic plate (magnet stand), removal of unincorporated dyes, nucleotides, salts, and other contaminants occur. The pure

DNA was eluted and analysed by capillary Applied Biosystems 3130 genetic analyzer (Life Technologies, Warrington, UK).

3.7.1.1 Method

The High Prep DTR was initially thawed to room temperature, followed by vortexing to resuspend the magnetic beads. Ten microliters of High Prep DTR reagent and 40µl of freshly prepared 85% ethanol was added to the PCR product, followed by mixing thoroughly for 7-10 times. The sample plate was placed on the magnetic separation device for 4-5 minutes or until a clear solution resolves. The beads were adsorbed onto the side of the well. The supernatant was discarded followed by the addition of the 100µl of 85% ethanol to each well. The samples were incubated with ethanol for 1-2 minutes twice. The beads were dried for 10 minutes at room temperature with the plate still bound to the magnetic separation device after discarding the supernatant. The plate was dismantled from the magnetic separation device. Forty microlitres of water was added to individual incubated at room temperature for 5 minutes. The sample plate was further well followed by pipetting 20 times. The samples were then transferred to the magnetic separation device, and incubation was carried out for 5-7 minutes or until the magnetic beads clear from solution. The cleared supernatant (30-35 µl) was transferred to a new plate to, be loaded on a sequencer.

The chromatograms obtained in these patients were reviewed by at least two experienced technologists. Once a mutation was identified in an IC, the available family members were sequenced to confirm familial transmission. Novel nucleotide

changes identified in the study were confirmed by direct sequencing/ RFLP in 50 normal alleles to rule out the possibility of a common polymorphism

3.8 Confirmation of deletions

Deletion status in the patient was confirmed initially by co-amplifying a control gene (*FGB, FGG)* at the same annealing temperature. The following conditions were used to confirm the deletion status. The PCR tubes were labelled (n= number of test samples + control gene + negative control without DNA). The PCR reaction was set for different exons as follows. PCR was performed with 10 picomoles of each primer [Table 15] in a 2X concentration of a ready reaction mix (GeNei, Bangalore, India). Approximately two hundred nanogram of genomic DNA was used for amplification reactions. Following denaturation at for 5 min at 94°C, 30 cycles of PCR amplification was performed, with denaturation at 94°C for 40sec, annealing at 58°C for 40sec and extension at 72°C for 40 sec. The final extension:72°C for 5 min. The PCR products were resolved by agarose gel electrophoresis [Figure 26].

Exons 1-4 did not amplify consistently in the patient V-590, however, amplification of control gene FGG, FGA was observed. This phenomenon confirmed the deletion status in the patient. Since the normal PCR approach can not confirm the carrier status in the parents, gene dosage analysis was performed.

Oligo Name	Primer sequence (5'to3')	Product size (bp)	Volume of 2X Master Mix (µl)	Concentration of forward and reverse (Primers) (µM)	Annealing temperature (°C)
FGG-EXON 3F	AATCACTGTTATATTTTCAGGGTAGTT	543	12.5	10	58
FGG-EXON 3R	CAGGCATAATGTCACTGGGATA				
FGB-EXON 6F	AATGGAATGGACAGGGGATT	345	12.5	10	58
FGB-EXON 6R	GCTTCCACATTTTTGTCAGGA				

Table 15: Primers used to confirm deletion status by control gene PCR.

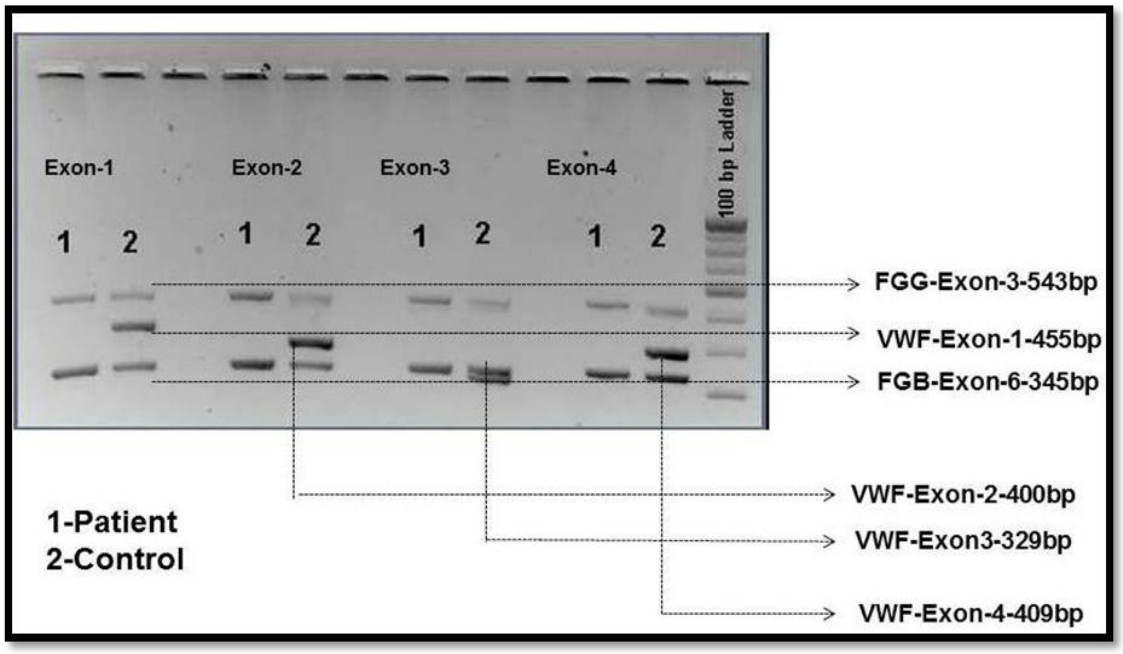


Figure 26: ***Electropherogram representing a normal (Control) and deletion pattern in V-590.***

3.9 Gene dosage analysis

3.9.1 Principle of the method

For gene dosage analysis, PCR was performed, for the exons deleted in VWF gene, using fluorescent labelled primers and albumin gene. Equal quantity of DNA was used. Capillary electrophoresis separated amplified product yielding 150-200bp (VWF gene) and 230bp (albumin gene) fragments in a Genetic analyser Applied Biosystems 3130 genetic analyzers (Life Technologies, Warrington, UK). The results were analysed by Gene Mapper (4.0) (Applied Biosystems). The ratio obtained between the peak heights of the exon in the VWF gene and albumin control gene in the parent samples were normalised to a normal control.

S.No	Oligo name	Sequences	Product size (bp)
1	VWF-Exon2F	5'fam GAGCTGATGGTCCCAGTTGT3'	181
	VWF -Exon2R	3'AACTGCAGTATCCCGCAAAG5'	
2	VWF -Exon13F	5'fam GTGGGTGGTATGACCTCCAG3'	269
	VWF -Exon13R	3'GACCGTCTGCTTCCCACTAC5'	
3	Albumin-F	5’fam CCAGAGATTTCCCAAAGCTGAG3.'	236
	Albumin-R	5’ GGACAGACGAAAGCACAGAAG3.'	

Table 16: ***Primers used for gene Dosage Analysis.***

3.9.1.1 Method

Gene dosage PCR was carried out using 200 ng/µl of the patient sample and normal control sample. Master mix was prepared using a 2X concentration of a ready reaction mix (GeNei, Bangalore, India) in the presence of 10 picomole primer [Table 16] containing VWF and albumin gene [Table 16].

DNA was amplified in a thermocycler Gene Amp 9700 (PE, Applied Biosystems, Foster City, CA). Initial denaturation for 5 min, at 94°C 20 cycles of PCR amplification was performed, with denaturation at 94°C for 40sec, annealing: 58°C for 40sec, extension at 72°C for 40 sec. Final extension:72°C for 5 min. The PCR products were resolved by agarose gel electrophoresis [Figure 27]

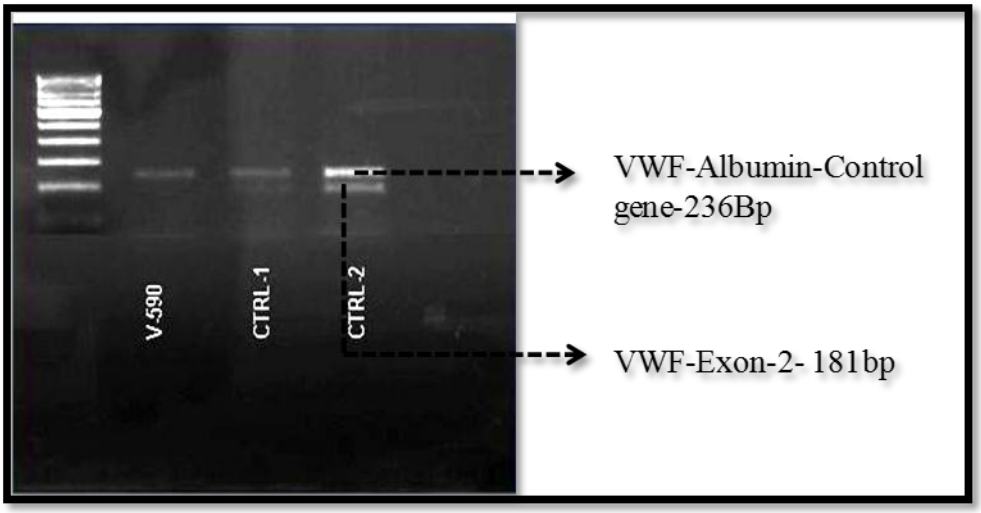


Figure 27: ***Electropherogram representing a normal (Control) and deletion pattern V-590 by gene dosage PCR.***

3.9.1.2 Gene scan analysis

The PCR products (1-2µl) was added to a mixture containing de-ionized formamide (14µl) and LIZ500 (0.25 µl) (PE Applied Bio systems, Cheshire, UK). The PCR products: Denatured at 94°C for 10 minutes in a PCR machine Gene Amp 9700 (PE, Applied Biosystems, Foster City, CA) followed by rapid cooling in ice for 5 minutes. The samples were then run on an ABI 3130/3500 (Applied Biosystems, Foster City, CA) and quantified using Gene Mapper V4 software.

3.9.1.3 Interpretation of results

The ratio obtained between the peak heights of the exon in the VWF gene and albumin control gene in the parent samples was normalized with a normal control. The ratio was calculated using the formula

$$\text{Ratio} = \frac{\text{[Peak height of Test gene/control gene] of patient}}{\text{[Peak height of Test gene/control gene] of normal control}}$$

The absence of expected product size [Figure 28] in turn infers the presence of a deletion, and a ratio of 0.5 confirms the carrier status in the parents.

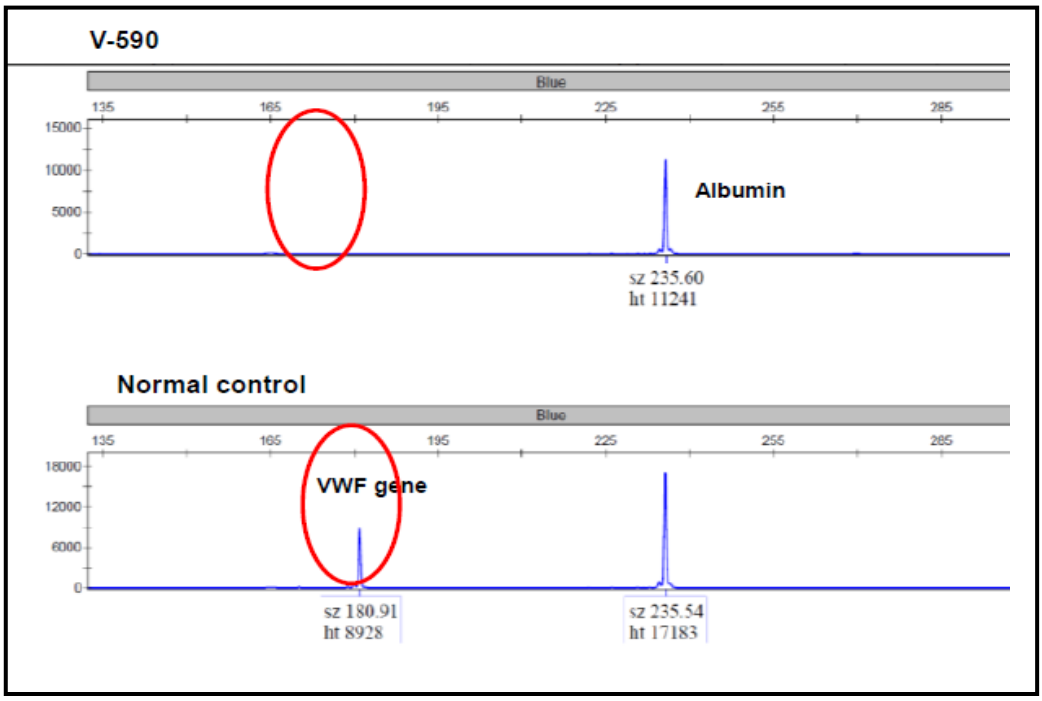


Figure 28: ***Electropherogram representing a normal and deletion pattern by gene dosage analysis***

Uniplex PCR was designed to amplify exons 2 of the *VWF* gene, along with the albumin gene. The VWF peak was absent in the index patient (V-590)

3.10 Restriction fragment length polymorphism (RFLP)

For the screening of novel nucleotide changes identified in the study, we screened in 25 healthy controls (50 normal alleles) by PCR amplification followed by restriction digestion with BsaAI (New England Bio Labs, Ipswich, MA, USA), to rule out the possibility of a common polymorphism.

3.10.1 Principle

Restriction endonucleases digest DNA at specific recognition sequences (restriction sites) resulting in the corresponding restriction fragments. A nucleotide substitution creates or abolishes the site for particular endonucleases resulting in varying length of the restriction fragments when compared with the wild-type DNA.

3.10.1.1 Method

PCR for the exon 39 displayed an aberrant pattern in CSGE in patient V-540. Following sequencing a nucleotide change c.6798G>C was observed. This mutation predicts a change from glutamine to histidine at codon 2266. The sequences when analysed by NEB cutter [Figure 29], to identify the restriction sites in the vicinity of mutation, where the enzyme BsaA1 (New England Bio Labs, Beverly, MA) was identified in the presence of nucleotide change compared to the controls. PCR products were subjected to digested with the appropriate restriction endonuclease and buffer, incubated at 37^{O}C for (12 hours). The digested products were electrophoresed in 2% agarose gel at 120 volts for 15-30 minutes in the presence of 100bp ladder.

3.10.1.1.1 Interpretation

The results were interpreted based on the product size. In the control a product size of 418bp size was obtained whereas in the patient (V-540), a product size of 324bp and 94 bp was observed on electrophoresis.

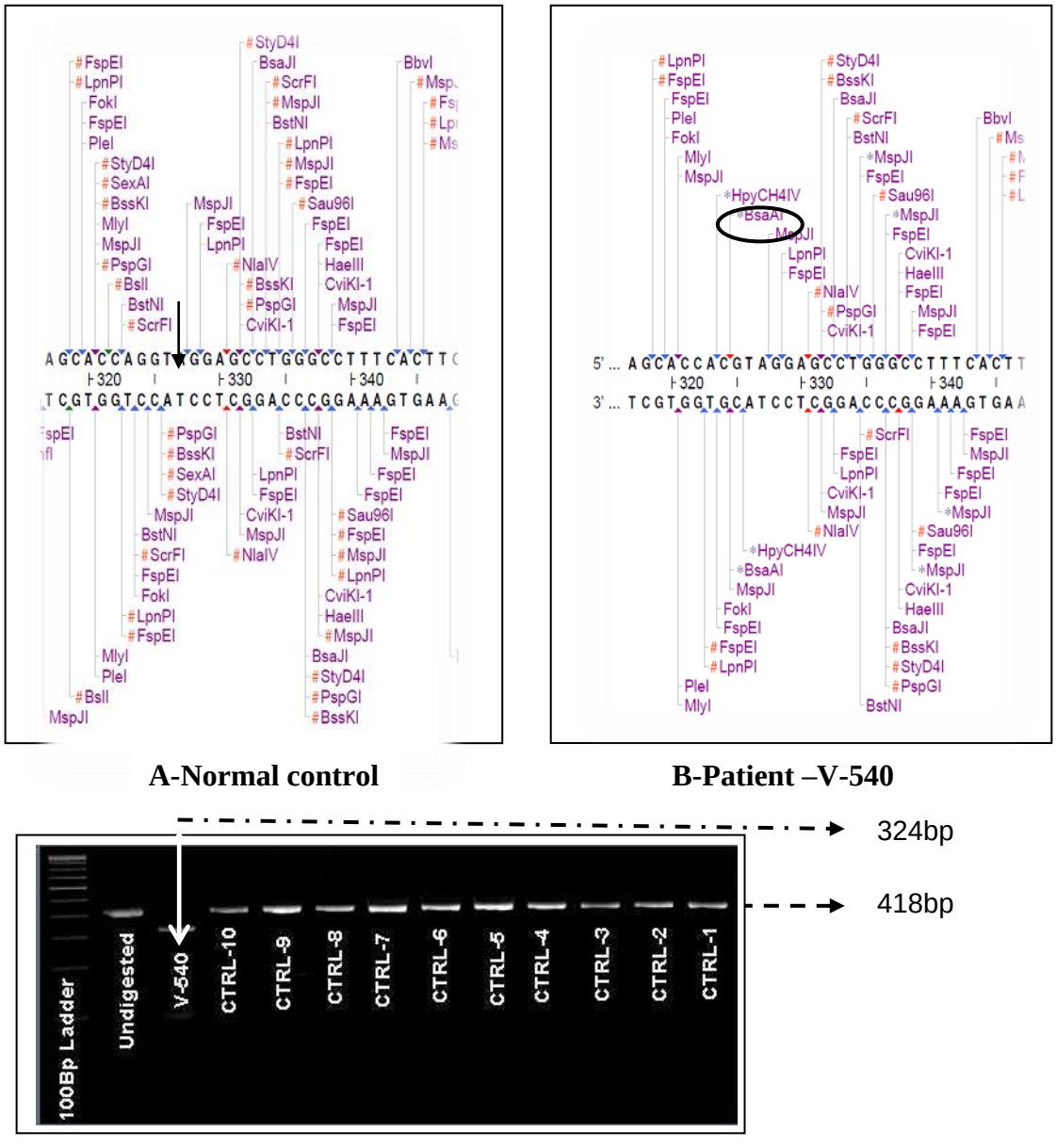


Figure 29: ***Schematic representation of Restriction sites in the normal control & patient -****(A) samples and normal control (NC-1) sample followed by digestion using BSaA1 enzyme displaying in normal control (NC-1)-418 bp and in patient (V-540)-(B) 324b and 94 bp.*

3.11 In silico analysis

For the novel mutations/variants identified in the study *in silico* analysis was carried out to classify them as causative or benign depending on the scores obtained on predicting the nucleotide substitution. The pathogenicity of these variants was classified based on the structure and sequence properties in association with the physical properties of the amino acids.

3.11.1 Evolutionary conservation studies

Evolutionary conservation studies were carried out to investigate the conservation of an amino acid mutated by missense change. VWF amino acid sequences from 10 different species (Accession numbers: *Mus musculus* -Q8CIZ8, *Canis familiaris*- Q28295, *Sus scrofa*- Q28833, *Homo sapiens*- P04275, *Macaca mulatta*- F5XVB6, *Oryctolagus cuniculus*- F5XVB8, *Xenopus tropicalis*- F5XVC3, *Danio rerio*- E7F9Z1, *Bos taurus*- F1N0R5, *Gallus gallus*- F5XVB5) were obtained from SwissProt and Trembl databases using PSI-BLAST. Multiple sequence alignment was performed with PRALINE (http://www.ibi.vu.nl/programs/praline) (Simossis and Heringa, 2005, Bawono and Heringa, 2014) [Figure 30]. Position specific conservation score for the novel missense mutations was also analysed using ConSurf server (http://bioinfo.tau.ac.il/ConSurf/) (Ashkenazy et al., 2016) which works on the principle of Bayesian inference.

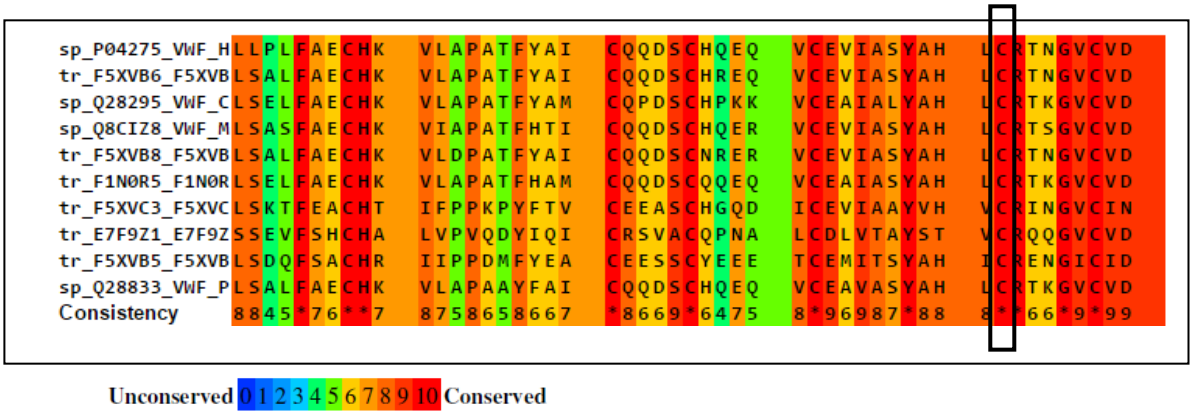


Figure 30: ***Schematic representation of Alignment of the mutated region in VWF of different species: p.Cys2184Tyr***

Impact of the novel missense mutations on the structure and function was studied using *SIFT* (http://blocks.fhcrc.org/sift/SIFT.html) *PolyPhen-2* (http://genetics.bwh.harvard.edu/pph2/) (Ramensky et al., 2002, Sunyaev et al., 2001) Mupro (http://mupro.proteomics.ics.uci.edu) SVM profile (PhDSNP SVM Profile [13] (Ramensky et al., 2002, Sunyaev et al., 2000, Sunyaev et al., 2001), SNAP2 (http://snps.biofold.org/snps-and-go/) [Figure 31] and hope server (http://www.cmbi.ru.nl/hope/input/) (Venselaar et al., 2010) and PROVEAN (http://provean.jcvi.org/index.php) (Choi and Chan, 2015) These machine learning methods utilize neural networks (SNAP2), for the classification of the variants, whereas other methods utilize Bayesian method (PolyPhen2) or other mathematical operations (SIFT) [Table 17]. Hope server helps to predict the properties of amino acids associated with size, charge, and hydrophobicity in association with the native and mutant.

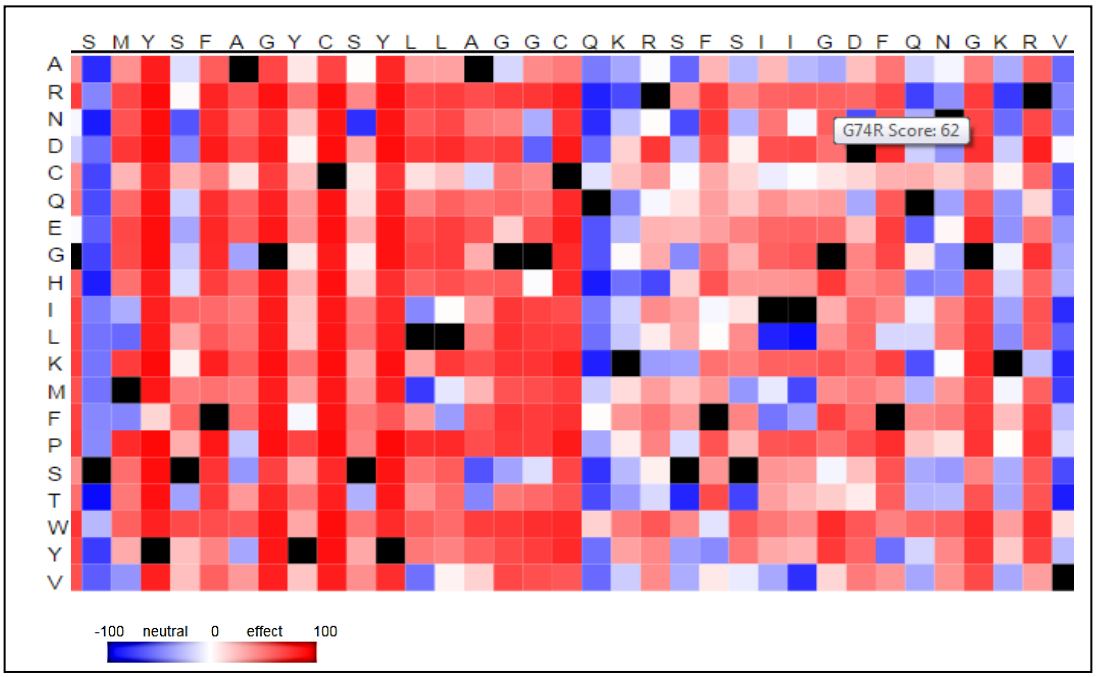


Figure 31: ***Schematic representation of the functional effect of missense mutation p.Gly74Arg on VWF is shown in a heat map (SNAP2)***

3.11.2 Splice site prediction

For the splice site mutations identified in the study *in silico* analysis tools including "The Splice-Site Analyser Tool" (http:// ibis.tau.ac.il /ssat/ Splice SiteFrame .htm) and GENSCAN (http://genes.mit.edu/ GENSCAN.html) was used to predict the impact of the nucleotide change at the consensus region spanning 5' or 3.' Further to classify the splice sites machine based -learning techniques such as artificial neural networks including NetGene2 (http://www.cbs.dtu.dk/services/NetGene2/) and Splice Site Prediction programs (http: //www.fruitfly. org/seq_tools/splice.html) was used to assesses the correlation of the neighbouring regions were the exon signal controls the threshold. Other tools used include Splice Port (http://spliceport.cbcb.umd.edu/) Splice Predictor (http://bioservices.usd.edu/splicepredictor/) which works based on

the Bayesian principle. To assess the affinity of the potential splice and regulatory regions CRYP-SKIP (http://cryp-skip.img.cas.cz/) and alternative splice site predictor **(**http://wangcomputing.com/assp/cgi-bin/assp.cgi**)** were used to identify the exons which are skipped [Table 17]. For the splice site nucleotide change observed beyond 5^{th} position from the exon upstream or downstream Splice man analysis (http://fairbrother biomed.brown.edu/splice man/index.cgi) was used.

Further to predict the branch points at the splice site Human Splicing Finder (http:// www.umd.be/HSF/) and SROOGLE (http://sroogle.tau.ac.il/) was used. In all these methods the scores are calculated based percentile differences between wild-type (WT) and mutant.

Position (bp)	**Putative splice site**	**Sequence**	**Score***	**Alt./ Cryptic**	**Confidence****
136	Alt. isoform/cryptic donor	CAATCTTCTGgtctggtgag	5.410	0.778	0.783
141	Constitutive donor	TTCTGGTCTGgtgagagcca	12.103	0.191	0.745
193	Constitutive acceptor	ctttccccagGATTACTGCG	12.933	0.203	0.743
233	Alt. isoform/cryptic acceptor	cggatcctagTGGGGAATAA	4.373	0.935	0.934
279	Alt. isoform/cryptic donor	CAAGAAACGGgtcaccatcc	4.982	0.913	0.931
327	Constitutive donor	TGACGGGGAGgtaagtgcag	13.662	0.180	0.760
386	Constitutive acceptor	cacttaatagGAACATTTTC	5.065	0.446	0.160

Representative image for - Alternative Splice Site Predictor (ASSP) –Wild type

Position (bp)	Putative splice site	Sequence	Score*	Alt./Cryptic	Confidence**
136	Alt. isoform/cryptic donor	CAATCTTCTGgtctggtgag	0.557	0.800	0.812
141	Constitutive donor	TTCTGGTCTGgtgagagcca	0.543	0.215	0.701
193	Alt. isoform/cryptic acceptor	ctttccccaaGATTACTGCG	0.529	0.752	0.685
194	Alt. isoform/cryptic acceptor	tttccccaagATTACTGCGG	0.543	0.559	0.228
233	Alt. isoform/cryptic acceptor	cggatcctagTGGGGAATAA	0.557	0.935	0.934
279	Alt. isoform/cryptic donor	CAAGAAACGGgtcaccatcc	0.571	0.913	0.931
327	Constitutive donor	TGACGGGGAGgtaagtgcag	0.486	0.180	0.760
386	Constitutive acceptor	cacttaatagGAACATTTTC	0.529	0.446	0.160

Representative image for - Alternative Splice Site Predictor (ASSP) -V-474 (c.2685-1G>A)

Table 17: ***In silico*** **analysis of splice site mutation** ***by using the program alternative splice site predictor*** *to predict changes in RNA splicing for splice site mutation identified (c.2685-1G>A) in a patient with type 3 VWD. Note that the creation of new cryptic splice site occurs in the patient (bottom panel). Scores of the pre-processing models reflecting splice site strength, i.e. a PSSM for putative acceptor sites, and an MDD model for putative donor sites. Intron GC values correspond to 70 nt of the neighbouring intron. ** Activations are output values of the back propagation networks used for classification. High values for one class with low values of the other class imply a good classification. Confidence is a simple measure expressing the differences between output activations. Confidence ranges between zero (undecided) to one (perfect classification).*

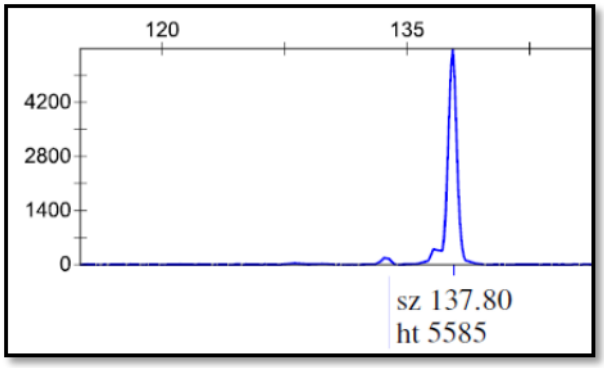
3.11.3 Molecular Modelling

The models for the D1, D4, C1 and C4 domains were generated by submitting their sequence to the ITASSER threading server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/; accessed between 02nd March-05th April 2016) (Yang et al., 2015). In addition a distance restraint of 2.0 Å was imposed on the C-α backbone of cysteine residues that are known or predicted to participate in disulphide bonds. The highest scoring model (according to the C-score) was chosen and subjected to a round of model refinement simulation lasting 500 ps in order to remove steric clashes and improve rotamer geometry. The final refined model were picked from the refinement simulation trajectory as ones having the lowest total energy and were further checked for stereochemical quality on the Molprobity server (http://molprobity.biochem.duke.edu/; accessed on 21st April 2016) (Chen et al., 2010). All models were also dimerized by blind docking them symmetrically on the MZ-docking server (http://zdock.umassmed.edu/m-zdock/; accessed between 01st-21st May 2016) (Pierce et al., 2014). The local molecular environment of the mutated residues was inspected in their corresponding final monomeric and dimeric models and the likely structure functional correlations were predicted. All structural analysis and graphical rendering were done on the YASARA version 16 platform (Krieger and Vriend, 2014).

3.12 Haplotype analysis

For the common mutations identified in the study haplotype analysis was carried out using the following polymorphic markers [*rs 216902 C>T; rs 216311 A>G, rs 1063857 T>C, rs 1063857 A>G, r s216867 T>C, rs 216321G>A,*

rs 216868-7C>T and intron 40 VNTR (Kasatkar et al., 2014)] by direct sequencing of the PCR amplicons or by using fluorescently labelled primers.The PCR products were further resolved by capillary electrophoresis and the data was analysed [Figure 32]



V-475 –Exon 37 –p.Trp2107*

Figure 32: ***Electropherogram representing a gene scan analysis of Intron 40 VNTR in a patient with p.Trp2107*.***

3.13 In vitro cell expression studies

3.13.1 Patients

Five missense mutations were chosen for further investigation.The novel missense mutations identified in the homozygous condition included p.Gly74Arg, p.Cys2184Tyr, p.Gln2266His, p.Cys2557Arg. One mutation that was previously reported (p.Cys370Tyr), but not characterized was also included for the analysis (Corrales et al., 2012). The location of these missense mutations spans domains D1, D4, and C2 domains [Figure 33].

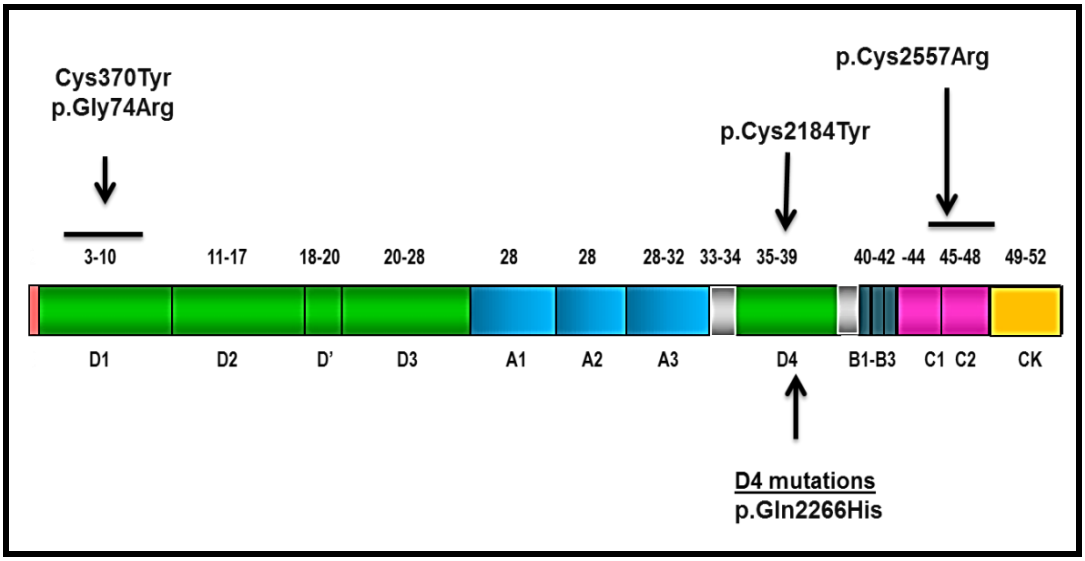


Figure 33: ***Schematic representation of the location of the missense mutations selected for in vitro expression studies.***

3.13.2 VWF expression plasmid

The human VWF expression vector pSVHVWF1, having the full-length VWF cDNA cloned into the pSV7d plasmid was received from Prof.J.E. Sadler (Washington University, St Louis, MO) (Jorieux et al., 1992) [Figure 34]. The plasmid was confirmed by restriction digestion using MfeI, HindIII and EcoRI (New England Biolabs, Hitchin, UK).

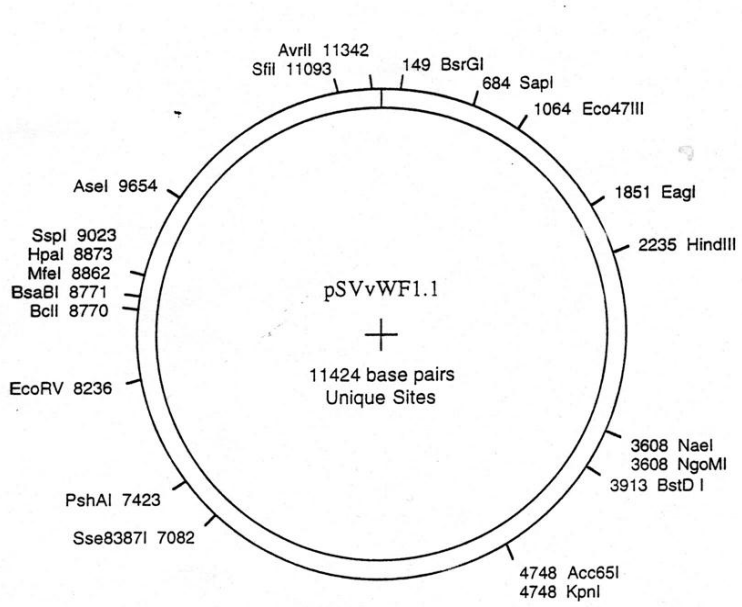


Figure 34: ***Map of the plasmids used for Invitro Cell Expression Studies***

3.13.3 Site-Directed Mutagenesis

Missense mutations corresponding to the specific amino acid change were introduced into the pSVHVWF1 plasmid using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. This method involves primer directed PCR amplification of the dsDNA using specific primers in the presence of PfuUltra High Fidelity DNA polymerase. Approximately 125ng of each primer (forward and reverse) was added to a reaction mixture containing 10ng of the template (ds plasmid), dNTPs and 1µl of Pfu polymerase in a total volume of 50µl. The oligonucleotide primers containing the desired mutation (e.g. c.1109G>A) was designed using, QuikChange® Primer Design: http://www.genomics.agilent.com/primerDesign Program.jsp. The primer sequences are detailed in the [Table 18]. The conditions used for PCR amplification include 95˚C for 1 min; 18 cycles of 95˚C for 50sec, 60˚C for 50sec and 68˚C for

I min, 68˚C for 7 min. Finally, a mutated plasmid with staggered nicks is generated after extension using oligonucleotide primers. The PCR product was then treated with *Dpn1* endonuclease, which specifically digests the methylated parental DNA template, thereby aids in the selection of the desired mutation in the synthesized DNA. One microliter of the *Dpn* I restriction enzyme (10 U/µl) was added directly to each amplification reaction preceded by incubation at 37˚C for 1 hour. Finally, 2µl of the *Dpn* I-treated DNA was transformed in to the XL10-Gold ultracompetent E.coli cells. To transform these plasmids, containing the desired nucleotide change 45µl of the competent cells and 2µl of DNA was gently mixed and incubated on ice for 30 minutes. Immediately, the reaction tubes were heat pulsed at 42˚C for 30 seconds followed by cooling on ice for 2 minutes. 500µl of pre-warmed NZY+ broth was added to the tube and incubated for 1hour at 37˚C with shaking at 200rpm. These transformed cells were then on spread LB agar plates containing ampicillin (100mg/ml) and incubated at 37˚C overnight. A single colony was picked from the Petri plate and inoculated in LB broth containing the appropriate selective antibiotic in a 10ml tube. These tubes were incubated for approximately eight hr at 37˚C with vigorous shaking (approx. 300 rpm).

Mutation	Primer Name	Sequence
p.Gln2266His	g6798c_antisense	5'-CAGGCTTCCAGGAAGTGGTGCTGGACTCC-3'
		5'-GGAGTCCAGCACCACTTCCTGGAAGCCTG-3'
p.Cys370Tyr	g479a_antisense	5'-GCTGTTTCGGCAAATGTAGGTGTTGCAGTCTCG-3'
		5'-CGAGACTGCAACACCTACATTTGCCGAAACAGC-3'
p.Cys2557Arg	t389c_antisense	5'GCCCGAGGGGCGGACAGGGACCT3'
		5'AGGTCCCTGTCCGCCCCTCGGGC3'
p.Gly74Arg	g220a_antisense	5'TCTGGAAGTCCCTAATAATCGAGAAGGAGCGTTTCT3'
		5'AGAAACGCTCCTTCTCGATTATTAGGGACTTCCAGA3'
p.Cys2184Tyr	g461a_antisense	5'GTTGGTCCGATAGAGGTGGGCATAAGAGGC 3'
		5'GCCTCTTATGCCCACCTCTATCGGACCAAC 3'

Table 18: Primers designed for site Directed Mutagenesis

3.13.2.1 Small-scale isolation of plasmid DNA

Plasmid DNA was extracted from the transformed cells using miniprep kit (QIAGEN, UK) according to the manufacturer's protocol. Briefly, a single isolated bacterial colony was incubated in 5ml sterile LB broth containing the required antibiotics (100µg/ml ampicillin) at 37℃ over night. The culture was centrifuged at 20,000g for 5 minutes, and the supernatant was discarded. The pellet was resuspended in Buffer P1 followed by addition of Buffer P2 and mixed by inverting the tubes 4-5 times. Buffer N3 was then added to the mixture and mixed. The tubes were centrifuged for 10min at 20,000g and the supernatant was applied to the QIAprep spin column. The column was centrifuged and washed by Buffer PB and Buffer PE. Finally, plasmid DNA was eluted into 20µl of buffer EB. Large scale isolation of plasmid DNA using an Endo-free maxiprep. Miniprep plasmid extraction was carried out preceded by direct sequencing to confirm the desired nucleotide change and the absence of undesired changes [Figure 35].

```
EMBOSS_001      142 CACTTCCTGGAAGCCTGGGTCCCGGACCACCAGCCCTGTCAGATCTGCNC    191
                    ||.||||||||||||||||||||||||||||||||||||||||||| |
EMBOSS_001      203 CAGTTCCTGGAAGCCTGGGTCCCGGACCACCAGCCCTGTCAGATCTGCAC    252

EMBOSS_001      192 ATGCCTCAGCGGGCGGAAGGTCAACTGCACAACGCAGCCCTGCCCCACGG    241
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001      253 ATGCCTCAGCGGGCGGAAGGTCAACTGCACAACGCAGCCCTGCCCCACGG    302
```

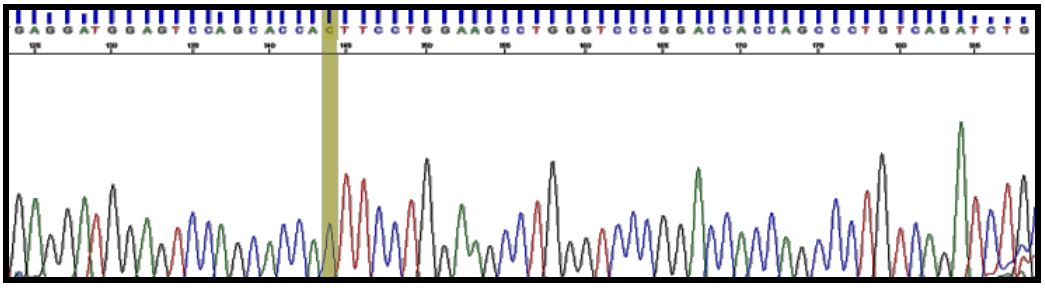


Figure 35: ***Validation of the nucleotide change by direct sequencing after site directed mutagenesis***

3.13.2.2 Large scale isolation of plasmid DNA using an Endo-free maxiprep

Plasmid DNA was extracted from the competent cells using Endo-free maxiprep (QIAGEN, UK) according to the manufacturer's protocol. Briefly, 250ml LB broth which contained a single colony was incubated in 37℃ overnight. The culture was centrifuged at 6000 x g for 15 minutes at 4℃. Buffer P1 resuspended the cell pellets and Buffer P2 was added to the mixture and incubated in 5min. Buffer P3 was added and applied in QIAfilter Cartridge and incubated in 10min at room temperature. The mixture was filtered, and Buffer ER was added to the filtered mixture. Then, the mixture was incubated 30 min on ice. The mixture was applied to a QIAGENtip 500 and was washed with Buffer QC twice. The plasmid DNA was eluted by Buffer QN and the eluted DNA followed by precipitation using isopropanol. The DNA pellet was washed using 70% ethanol and redissolved by 1ml Buffer TE.

3.13.4 Expression of recombinant VWF in eukaryotic cells

Human embryonic kidney cell line 293 (HEK293) was procured from American Type Culture Collection (ATCC, Rockville, USA). These cells were cultured in a Minimum Essential Medium Eagle alpha modified (MEM-α, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% Fetal Calf Serum,2 mM L-Glutamine and 50 μg/mL. For transfection, cells were seeded in the six-well plate (Corning, NY, USA) at the confluence of 60-70%. After 12 hours, plasmid transfection was performed in these cells by making transfection mix of X-treme Gene Transfection Reagent (4ul) (Roche, Mannheim, Germany), highly pure plasmids (4ug quantity) in a relative molar proportion and Opti-MEM serum-free medium (Invitrogen, Carlsbad, USA). Twenty four hours after post-transfection, media was changed with fresh medium containing 10% Fetal Calf Serum. Forty-eight hours after transfection the cells were then fixed for immunofluorescence.

3.14 Antibodies

Anti-Polyclonal Rabbit anti-human VWF (Ab6994) was purchased from Abcam (Cambridge, UK). Polyclonal Rabbit anti-Human VWF was purchased from Cell Signalling (Cell Signalling, Beverly, MA). Antibody PDI (ER marker) (Ab2792) was purchased from Abcam (Cambridge, UK). Anti-mouse IgG1 antibody (Cell Signalling Technology, Boston, MA) was used as secondary antibody for the endosomal marker. Alexa 488 and Alexa 594 conjugated secondary antibodies used for these experiments were purchased from Invitrogen.

3.14.1 Immunofluorescence Analysis

Forty-eight hours after transfection, the cells were fixed with 4% paraformaldehyde and incubated at room temperature (RT) for 20 minutes. After incubation, the cells were washed gently with 1X PBS. Then cells were permeabilized using a blocking buffer containing 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) heat inactivated BSA (5% bovine serum) and incubated at room temperature for 45 minutes to eliminate non-specific binding. After incubation, the cells gently washed with IXPBS (pH 7.4). Cells were incubated with the primary VWF antibody (Ab6994) in blocking buffer (without Triton X-100) to visualize VWF. Cells were incubated at 4ºC for 3 hours followed by 1hour incubation at RT. Cells were then washed with 1XPBS (pH 7.4) twice, and fluorescence-conjugated secondary antibody [polyclonal Rabbit anti-Human VWF/HRP (Cell Signalling, Beverly, MA)] in conjugation with Alexa 594 (Invitrogen) was added followed by incubation at RT for 1hour 30 minutes. After incubation, the cells were washed with 1xPBS (pH 7.4) twice. Monoclonal mouse anti-Human Protein-Disulphide Isomerase (PDI) antibody RL90 (Ab2792) used to visualize endoplasmic reticulum was added to the cells, preceded by incubation overnight at 4°C. The cell was then washed with 1XPBS, followed by addition of secondary antibody conjugated with anti-Mouse-488 (Invitrogen) and incubation at RT for 1hour 30 minutes. The cells were then rinsed with PBS and incubated at 4°C. Images were then acquired on a fluorescent microscope. Finally, the cells were rinsed with PBS and mounted with Vecta shield (Peterborough, UK) containing DAPI (Molecular Probes, Eugene, Orlando). Images were analysed using Olympus FluoView™ FV1000 Confocal Microscope with a 60X/1.40 NA oil objective.

4. Results

4.1 Enrol a large series of patients with Type 3 VWD and evaluate the clinical and haematological data.

4.1.1 General characteristics of the study group

The study population includes 102 subjects from 90 families diagnosed with type-3VWD characterised by severely reduced to absent antigen levels, and markedly decreased FVIII levels. The male to female ratio was 50:52 Sixty-two patients (61%) were born of the consanguineous marriage, and forty-seven (46%) of them had a family history of bleeding. These patients were predominantly from southern India. Characteristics of the study participants are detailed in [Table 19].

4.1.2 Bleeding profile

Bleeding scores (BS) of the patients recruited in the study is summarised in the table [Table 19]. Bleeding scores defines the severity of bleeding symptoms in patients with VWD in association with the overall correlation of the plasmatic levels of antigen, and FVIII levels. The median bleeding score was 9 (range 2-19). The median age of clinical presentation in these patients was two years range (range: 0-60). The most common clinical manifestation in our patients include bleeding from minor wounds (86.2%) and cutaneous bleeding (81.3%), menorrhagia (96%), oral cavity bleeding (75%), epistaxis (52.9%), muscle hematomas (26.4%), surgery (14.7%), hemarthrosis (14%), gastrointestinal bleeding (12.7%), post dental extraction (10.7%), postpartum haemorrhage (8%)[Table 20]. Sixty patients (59%) have received

cryoprecipitate or red cell transfusion to control the spontaneous or posttraumatic bleeding. The median age of receiving the first transfusion was 11yrs.

No of Males (%)	50[49.01%]
No of Females (%)	52[50.98%]
Median age at presentation, years (range)	2 years[0-60]
Median bleeding score (range)	9 [2-19]
Consanguinity	62[61%]
Median VWF: Ag, u/dL	0 [<1-1%]
Median FVIII:C, IU/ml (range)	2.8[<1-10.9]

Table 19: ***Clinical features and haematological parameters of the study subjects***

The majority of the families included in the present study comprised of two generations.

Clinical Manifestations	
Bleeding from minor wounds	86%
Cutaneous bleeding	81%
Oral cavity bleeding	75%
Menorrhagia (25/26)	96%
Epistaxis	54%
Muscle hematoma	26%
Surgery	15%
Hemarthrosis	14%
Gastrointestinal bleeding	13%
Post dental extraction	11%
Central nervous system bleeding	1%

Table 20: ***Clinical manifestations in patients with type 3-VWD***

4.1.3 Phenotypic analyses

Phenotypic data of the study participants is summarised in the [Table 19]. The plasmatic levels of VWF (VWF:Ag) and FVIII levels (FVIII:C) was documented. Antigenic levels of VWF in all these patients were severely reduced, with a marked decreased FVIII levels.

4.2 *Mutation analysis in the VWF gene*

4.2.1 Genotypic analysis

Mutations were screened in 102 patients from 90 families. The regions scanned for mutations include 52 exons and their adjacent intronic regions by CSGE strategy followed by sequencing. In patients (n=19) were a causative mutation could not be established direct sequencing of the exons was carried out.

Mutations were identified in 93 (91%) patients from 81 (79%) families. Ninety patients had the mutations identified in both alleles: 83 homozygous and 7 compound heterozygous for the mutations. In three patients mutations were identified only in a single allele. In 9 (9%) patients, the causative mutation could not be established. A total of fifty-five different disease-causing mutations were identified, of which 35 (64%) were novel [Figure 36]. These included frame shift (n=15, 27.27%) missense (n=13, 23.64%), non-sense (n=12, 21.82%), large deletion (n=2, 3.64%) gene conversion (n=3, 5.45%) and splice site mutation (n=10, 18%). Mutations were localised throughout the VWF gene. Maximum number of the mutations were identified were localised in the propeptide (27%) region of VWF. Novel mutations identified in the present study was not reported in the databases including

[(*https://grenada.lumc.nl/LOVD2/VWF/home.php?select_db=VWF), Hemobase (http://vwf.hemobase.com/), HGMD, http://www.hgmd.cf.ac.uk/ac/all.php: vWF and ISTH, (http://www.ragtimedesign.com/vwf/mutation/mutationtableresults.php*)].

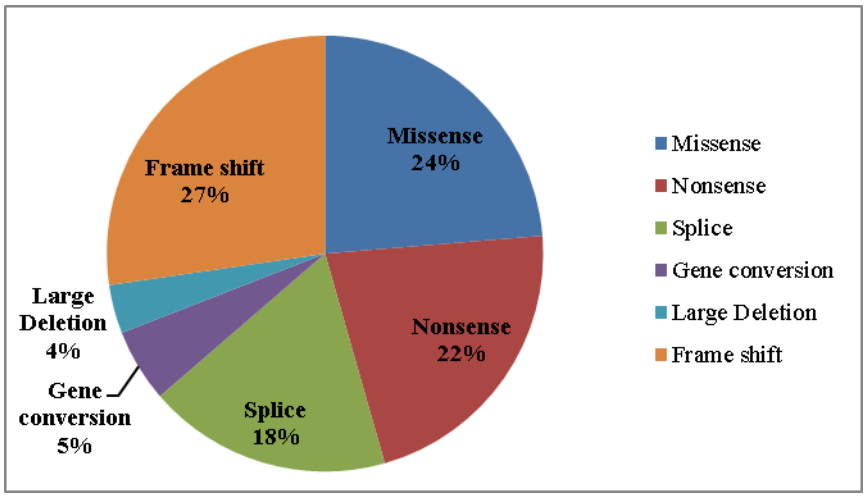


Figure 36: ***Schematic representation of type of mutations identified in the present study (n=55)***

In the present study, eight mutations were identified in more than one family. Mutations include four nonsense mutations including [(c.6321G>A; p.Trp2107*), (c.1117C>T; p.Arg373*), (c.3931C>T; p.Gln1311*), (c.2683C>T; p.Gln895*)], two splice site mutations [(c.2443-1G>C, c.3675+1G>C)], two missense mutations [(c.8411G>A; p.Cys2804Tyr), (c.3835G>A, p.Val1279Ile)]. The most frequent mutation identified in this cohort of patients include c.2443-1G>C and p.Arg373*accounting to 21% of the patients. Twelve patients were homozygous for c.2443-1G>C while two were heterozygous for same, and 9 patients were homozygous for p.Arg373*.

4.2.1.1 Frameshift mutations (n = 15)

Fifteen novel frameshift mutations were identified in a total of 24 patients. These included duplication c.3412_3413dupAA (V-450), c.6090dupT (V-319), c.6767_6770 dupAGTG (V-546), and c.2749_2752dup (V-557, V-558, and V-316) and deletions c.3656delC (V-270), c.757_770del (V-136), c.6483delC (V-353), c.2417_2418delCT(V-424, V-619, V-616, V-309), c.3560del T (V-312, V-598) c.5782_5783delCC (V-559, V-611), c.311_312delAG(V-289), c.3479delC (V-603, V-614), c.1486delG (V-605). Deletion and insertion c.289_292delinsACA (V-588, V-589) and insertion c.7652_7653insT (V-607). These mutations possibly disrupt the reading frame of the protein resulting in-frame loss/or gain of amino acids (Goodeve, 2010).

4.2.1.2 Nonsense mutation (n=12)

A total of 12 nonsense mutations were identified in 32 patients of which c.6321G>A (V-394, V-475, V-544, V-283, V-294, V-115, V-612), c.1117C>T (V-369, V-144, V-370, V-473, V-541, V-331, V-545, V-225, V-407), c.7650C>T (V-591) c.4387G>T (V-596, V-597), c.1812C>G(V-599), c.4697C>T (V-281), c.2683C>T (V-553, V-282), c.5335C>T(V-554, V-563), c.3931C>T(V-604, V-493, V-606,V-606, V-282, V-585, V-419), c.3359G>A(V-585), c.7603C>T (V-579), c.665G>A (V-260) of which 4 were novel. These mutations possibly result in premature termination of translation of the respective proteins leading to the lower levels of circulating VWF levels (Goodeve, 2010).

4.2.1.3 Splice site mutation (n=10)

Ten splice mutations were identified in the present study including c.2443-1G>C (V-471, V-499, V-481, V-539, V-554, V-563, V-576, V-307, V-600, V-608, V-415, V-529, c.3675+1G>C(V-259, V-488, V-561, V-613), c.2685-1G>A(V-474), c.7437+5G>T (V-574), c.6598-2A>T(V-578),c.1729-5C>T(V-609), C.7729+27T>C (V-615), c.6798+1G>C(V-290), c.3379+1G>A(V-138), c.6977-1G>C(V-348) of which 7 were novel. These mutations can disrupt the highly conserved GT and AG dinucleotides at the 5′ and 3′ end of each intron. This may result in exon skipping or exon retention or creation of new splice site.

Point mutations away from splice sites can introduce a novel splicing site recognized in preference to the WT site leading to cryptic splice siteactivation or creation of a novel exon within the intronic sequence. *In silico* analysis of the novel splice site mutations are depicted in the [Table 21]. In our splice prediction analysis, these mutations abolished or altered the physiological donor splice as evident by reduced splice site scores, however, mRNA studies are required to elucidate the pathogenicity of these splice site mutations.

S.No	Nucleotide Change	Program (Score :Native------- Mutated)				
		NetGene2 score	MAxEnt scan	HSF score native	ASSP	Analyzer splice site tool
1	c.2685-1G>A	0.94----0.17	0.3-4.94	95.34-------66.4	9.0---- splice site destroyed	16.37----21.33
2	c.3675+1G>C	Splice site normal/ splice site destroyed	0.6--2.33	86.12----57.22	4.9----splice site destroyed	39.14----41.67
3	c.7437+5G>T	1.0-0.24	4.1--2.12	68.4-----39.45	13------8	39.12 ---45.78
4	c.6598 -2A>T	0.0-0.0 --No difference	12-2.73	87.61----58.66	10.5------ splice site destroyed	41.79----31.64
5	c.1729 -5C>T	1.0-0.97	2---2.87	88.49----89.38	No difference	11.15----15.56
6	c.3379+1 G>C	Splice site normal/ splice site destroyed	11--3.74	86.16----57.22	12.9--splice site destroyed	49.10----52.67

Table 21: ***In silico analysis of splice site mutations identified in the present study.***

Different scoring models used in MAXENTSCAN

MAXENT- maximum entropy model: HSF- Human splicing finder.

ASSP-Alternative Splice Site Prediction Tool: Analyzer-Splice site tool

4.2.1.4 Missense mutation (n=13)

Thirteen missense mutations (23.64%) were identified in 16 patients, of which 7 were novel including c.220G>A (p.Gly74Arg), c.3164T>A (p.Met1055Lys), and c.3448G>C (p.Ala1150Pro) c.6551G>A (p.Cys2184Tyr), c.6798G>C (p.Gln2266His), c.7669T>C (p.Cys2557Arg) were identified in homozygous condition in 6 patients. Missense mutations identified in the heterozygous condition in two patients includes c.140A>T (p.Asp47Val) and c.7604G>A (p.Arg2535Gln).

UPN	Amino acid change	SIFT Comparision score	Poly phen Comparision score	MUpro		Provean Score
				SVM Score	Protein structure Stability	
V-548	p.Asp47Val*	Affect function (0.0)	Probably damaging (1.00)	-0.3	Decrease protein stability	Deleterious (-9.528)
V-534	p.Gly74Arg*	Tolerated (0.63)	Probably damaging (1.00)	-0.7	Decrease protein stability	Neutral (0.150)
V-501	p.Ala141Asn	Tolerated (0.38)	Probably damaging (1.00)	-0.2	Decrease stability of protein	Neutral (-0.483)
V-403	p.Cys370Tyr	Affect function (0.0)	Probably damaging (1.00)	-0.4	Decrease protein stability	Deleterious (-9.528)
V-400	p.Met771Ile	Affect function (0.0)	Probably damaging (0.4)	-0.8	Decrease protein stability	Deleterious (-3.044)
V-553	p.Met1055Lys*	Affect function (0.0)	Probably damaging (0.52)	-0.65	Decrease protein stability	Deleterious (-2.617)
V-610	p.Ala1150Pro*	Affect function (0.0)	Probably damaging (0.96)	-0.6	Decrease stability of protein	Deleterious (-2.503)
V-518	p.Arg1315Cys	Affect function (0.0)	Probably damaging (1.00	-0.7	Decrease stability of protein	Neutral (-1.348)
V-340	p.Cys2184Tyr*	Affect function (0.0)	Probably damaging (1.00)	-0.7	Decrease protein stability	Deleterious (-6.161)
V-540	p.Gln2266His*	Damaging (0.0)	Probably damaging (0.96)	0.5	Increase protein stability	Neutral (-1.596)
V-348	p.Arg2535Gln	Tolerated (0.28)	Tolerated (0.03)	-0.8	Decrease protein stability	Neutral (-0.548)
V-564	p.Cys2557Arg*	Affect function (0.0)	Probably damaging (1.00)	-0.5	Decrease protein stability	Deleterious (-4.930)
V-422, 491, 538, 605	p.Cys2804Tyr	Affect function (0.0)	Probably damaging (0.99)	-0.9	Decrease stability of protein	Deleterious (-4.703)

Table 22: ***In silico analysis of the missense mutations identified in the present study (n=13)*** *Polyphen-2 - score 0 to1 shows low to high confidence for the probability of protein damaging. SIFT- mutations considered as pathogenic showing score, 0.05. MUpro - higher the negativity in SVM score higher the probability of a decrease in protein structure stability. SVM- Support Vector Machine (SVM score – negative score for novel missense mutations showed the pathogenic probability). PROVEAN- variants with a score equal or below -2.5 deleterious, above -2.5- neutral. Novel missense mutations identified in the present study (*)*

In one patient mutation in another allele was identified in exon 41; while inpatient (V-548) mutation could not be identified inother allele. Previously reported mutations include c.421G>A (p.Asp141Asn), c.2313G>A (p.Met771Ile), c.1109G>A (p.Cys370Tyr), c.3943C>T(p.Arg1315Cys), were identified in four patients in homozygous condition. The mutation c.8411G>A (p.Cys2804Tyr) displayed homozygosity in 3 patients and heterozygous in one patient. Mutation in other allele was observed in exon 13 (V-605). These mutations when analysed by when analysed by SIFT (http://blocks.fhcrc.org/sift/SIFT.html) and Polyphen (http://www.bork.embl-heidelberg.de/PolyPhen), PROVEAN (http://provean.jcvi.org/seq_submit.php) had a damaging effect on VWF proteins [Table 22]

4.2.1.4.1 Structural modelling of VWF protein

The structural data of VWF for the domains D1, D4, C1 and C4 was obtained fromI TASSER threading server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/. These domains are characterised by a large number of disordered flexible regions. Functionally these domains are necessary, during tubular assembly, since these domains undergo significant conformational changes on both intra and inter-domain structures. Another characteristic of these domains is that they are cysteine-rich, and a relatively large number of them exist in oxidised disulphide bonded forms. A majority of these disulphide bonds belong to the allosteric category, and they serve as functional switches for the conformational changes taking place in the VWD multimeric assembly. Although being disordered, order is brought to these domains in two ways: A) a small region usually in the core of these domains is comprised of ordered forms i.e. in this case beta sheets, most of the disordered

regions extend out as fringes of this ordered core and B) in regions which are lengthy as well as disordered, a core comprised of more than one disulphide bonds maintains its specific shape. Although such still showed a disordered coil like flexible structure, the flexibility is limited by the rigidity induced by a combination of disulphide bonds. Such regions are observed primarily in D4 domain which is larger than the C4 domain. Also, it is very likely that such regions participate in dimeric interactions because they provide the flexibility to do so and also contain disulphide bonds which can take part in disulphide exchange during the process bringing about changes in shapes that can then symmetrically fit into each other during the process.

4.2.1.4.2 Functional implications for mutations occurring on propeptide D1, D4, C1 andC4 domain

The Gly74Arg mutation occurs on the propeptide (D1 domain) of VWF. Substitution of the wild type residue (neutral) with a mutant (positive) residue can lead to non-native interactions with the adjacent residues [Figure 37]. These interactions can introduce rigidity thereby resulting in incorrectly associated or completely disassociated VWF. This would have the eventual effect of either accumulation within the cell and eventual degradation of the mutated variants or even if the incorrectly associated or completely disassociated VWF are secreted they may be non-functional. The mutation Cys2184Tyr lies on the disordered coil area of the D4 domain. The residue Cys2184 bonded to Cys2150 and provides stability also is characterised by the presence of an unbound cysteine residue (Cys2174) [Figure 38A]. This mutation could, therefore, disrupt the disulphide bond

formation, the thereby orientation of the region Cys2174 and interferes with dimerization of D4 domain. Steric clashes might be introduced with the neighbouring residues Trp2107, Tyr2160 and His2182 resulting in instability of the domain [Figure 38B]. The mutation Gln2266His occurs in the C1 domain, which is disulphide bond bonded to Cys2257-Cys2283 of VWF [Figure 39A]. An unbound cysteine in this model, which is disulphide bonded to (Cys2254-Cys2220) at the D4 domain [Figure 39B]. The residue Gln2266 is surrounded by aromatic residues His2265 and Phe2267. Substitution by histidine residue might introduce rigidity; thereby prevent the process associated dimerization of the domains needed to produce functional VWF. The mutation Cys2557Arg occurs on the disulphide bridge at the C4 domain of VWF [Figure 40]. Substitution of the positively charged residue may lead to native interaction thereby impairs the process associated with multimerization.

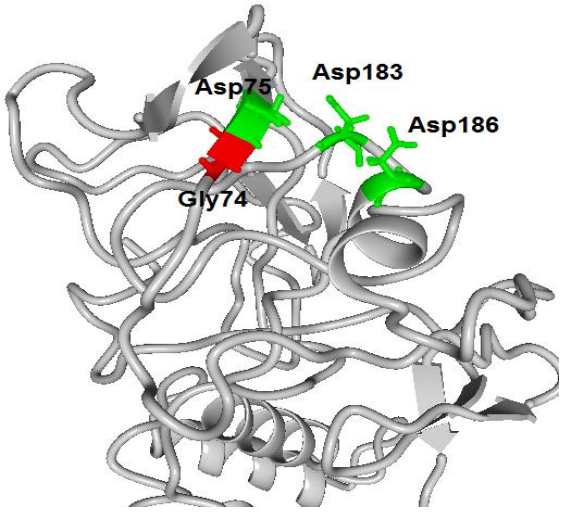


Figure 37 **:** ***This figure displays a close-up view of the D1 domain region on which the mutation has been reported.*** *The protein backbone is depicted in grey coloured ribbon format. The residue on which a mutation has been reported as well as neighbouring residues of interest have been depicted in coloured stick forms.*

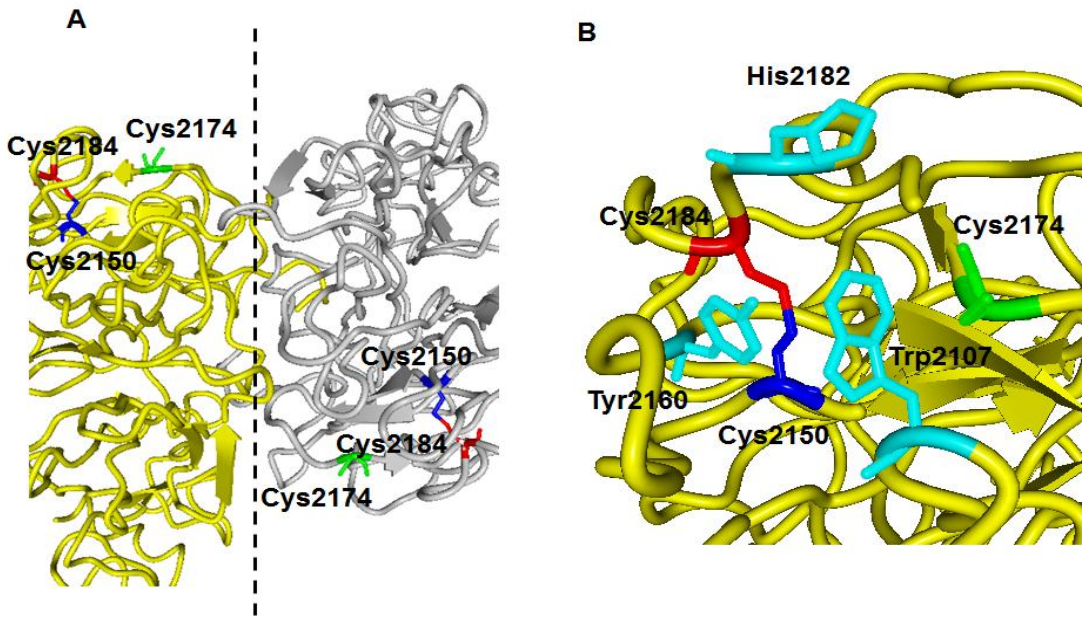


Figure 38A: ***The above figure is split into two panels***. *Panel A illustrates the D4 dimeric interface with the respective monomer protein backbones depicted in grey and yellow coloured ribbon format. Dimeric symmetry is shown by a dotted line running along the interface.*

Figure 38B: ***The figure shows a close-up view of the D4 domain (monomer)*** *region on which the mutation has been reported. The residue on which a mutation has been reported as well as neighbouring residues of interest have been depicted in coloured stick forms.*

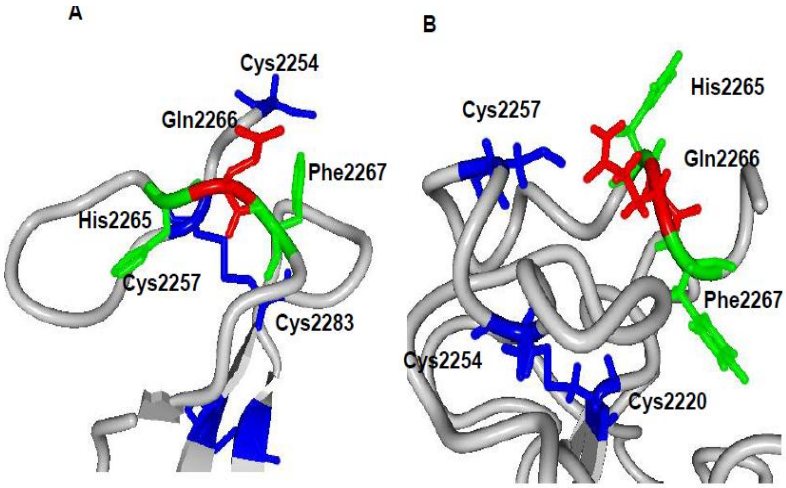


Figure 39 A ***The above figure is split into two panels.*** Panel *A illustrates the C1-D4 connector region as viewed in a model of the C1 domain.*

Figure 39 B ***Illustrates the same area as seen in a model of domain D4****. The protein backbone is represented in grey coloured ribbon format. The residue on which a mutation has been reported as well as neighbouring residues of interest have been depicted in coloured stick forms.*

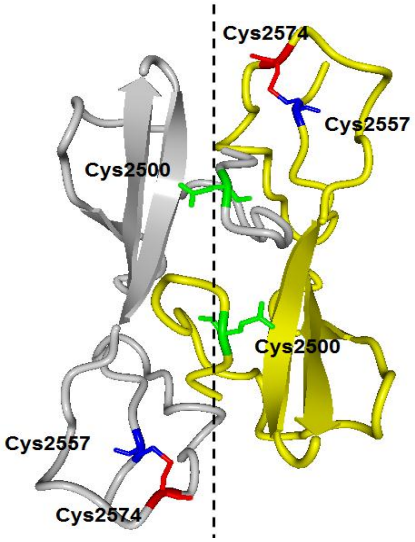


Figure 40: ***The above figure is split into two panels.*** *Panel A illustrates the C4 dimeric interface with the respective monomer protein backbones depicted in grey and yellow coloured ribbon format. Dimeric symmetry is illustrated by a dotted line running along the interface. The residue on which a mutation has been reported as well as neighbouring residues of interest have been depicted in coloured stick forms.*

4.2.1.5 Large Deletions

In the present study we identified two partial deletions in two index cases (V-155, V-590). The presence of a partial deletion in the affected patient was identified by the failure of amplification for VWF gene, but satisfactory amplification of the normal fibrinogen gene used as an endogenous control for DNA amplification described earlier (Sumitha et al., 2013). To further confirm these deletions in the affected patient and to offer the carrier status in the parents, gene dosage analysis was carried out. This method relies on the amplification of the target gene by fluorescently labelled primers and subsequent analysis by capillary electrophoresis. The ratio of the peak amplification between the test (VWF gene) and the control (albumin gene) was normalised to control. VWF specific peak was absent in the proband but not in the control gene albumin gene thereby confirming the deletion status.

4.2.1.6 Gene conversion

In our patient series gene conversion was identified in 5 patients. Gene conversion is defined as the as one of the mechanisms of homologous recombination, were a unidirectional transfer of genetic material from a 'donor' sequence to a highly homologous 'acceptor' (Chen et al., 2007) occurs. Gene conversion between the pseudogene and VWF is reported earlier (Gupta et al., 2005). In two index case (IC) gene conversion (V-419, V-606)was identified in homozygous condition and one IC (V-282) in a heterozygous condition involving the nucleotide changes a nonsense mutation p.Gln1311* (c.3931C>T), and the missense substitution p.Val1279Ile (c.3835G>A). The possible length of gene

conversion was found to range from 117-229bp respectively. The extent of gene conversion is depicted in the [Figure 41]. In index case (V-585) the extent of gene conversion was found to be 165 and 291bp. Gene conversions observed include p.Pro1266Leucine, p.Valine1279Ile, p.Gln1311*. Two silent mutations were also observed at codon 1263 and codon1317, resulting in nucleotide changes c.3789G>A and c.3951C>T. In IC (V-493), gene conversion was to occur resulting in nucleotide changes p.Val1279Ile, p.Gln1311 and p.Arg1315His.

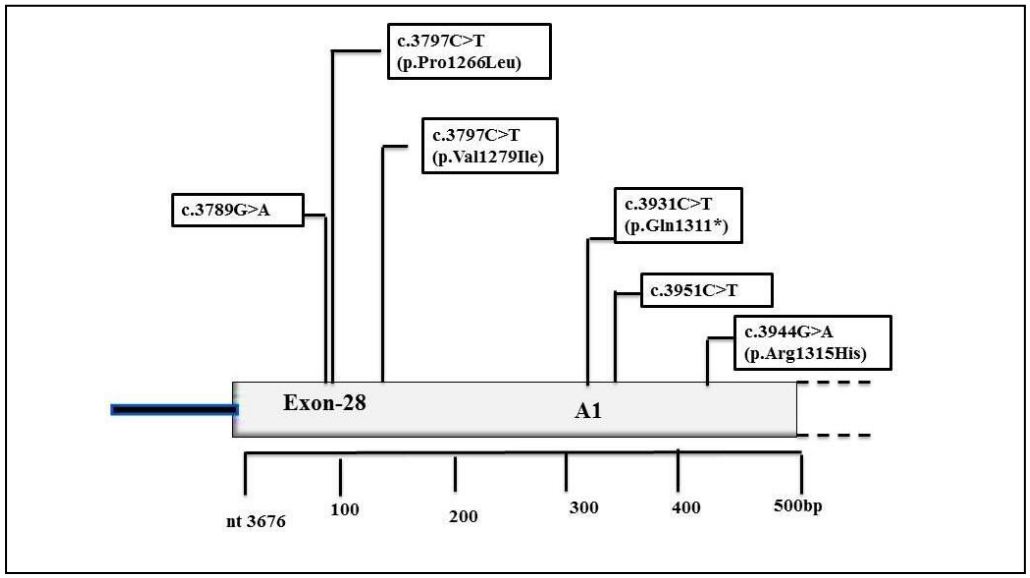


Figure 41: ***Schematic representation of the Gene Conversion mutations identified in the present study***

Gene conversion occurs between VWF gene and pseudogene. In boxes represents the mutations defined in the present study. Summary of the mutations identified in the present study is depicted in the [Figure 42].

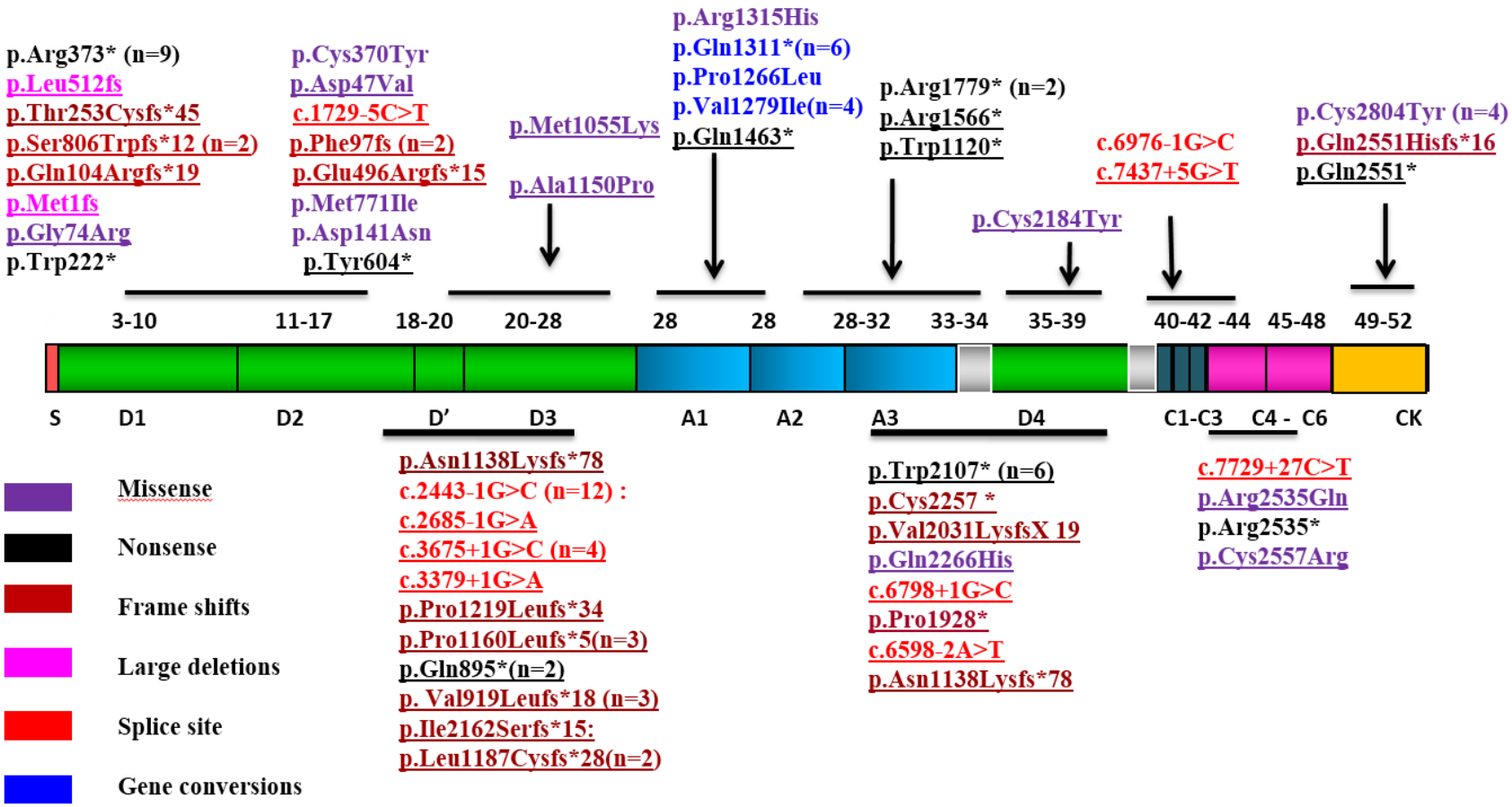


Figure 42: ***Location of 55 mutations identified in the present study***

*The diagramis based on historically annotated domains of VWF. Human Genome Variation Society (HGVS) nomenclature; * represents a nonsense mutation. Underlined represents novel mutations identified in the study*

4.3 Haplotype analysis

Interestingly, in the present study, five common mutations were identified including (c.2443-1G>C, n=12; p.Arg373*, n=9; 4 p.Gln1311*, n=6; c.3675+1G>C, n=4) accounting to 36%. To investigate the possibility of a founder effect in our population a haplotype panel was constructed using eight SNPs (*rs 216902 C>T; rs 216311 A>G, rs 1063857 T>C, rs 1063857 A>G, r s216867 T>C, rs 216321G>A, rs 216868-7C>T and intron 40 VNTR).* Based on the highly polymorphic nature these SNPs were chosen (http://exac.broadinstitute.org/) [Table 23].

S.No.	Nucleotide No	Codon No	rs number	Allele frequency [http://exac.broadinstitute.org/]
1	c.5844 C>T	p.Cys1948Cys	rs 216902	0.4577
2	c.4141A>G	p.Thr1318Ala	rs 216311	0.6798
3	c.2385T>C	p.Tyr795Tyr	rs 1063857	0.3231
4	c.2365A>G	p.Thr789Thr	rs 1063857	0.3232
5	c.7239T>C	p.Thr2413Thr	r s216867	0.8687
6	c.2535G>A	p.Gln852Arg	rs 216321	0.8992
7	c.7082-7C>T		rs 216868	0.3354

Table 23: ***The haplotype panel studied in patients with common mutations*** **[c.2443-1G>C, n=12; p.Arg373*, n=9; 4 p.Gln1311*, n=6; c.3675+1G>C, n=4]**

A common haplotype (TGCGCGC) was observed for p.Trp2107* [Table 24, 25].

S.NO	UPN	cDNA,HGVS	EXON 35	EXON 28	EXON 18	EXON 18	EXON 42	EXON 20	EXON 42
			c.5844 C>T	c.4391A>C	c.2385T>C	c.2365A>G	c.7239T>C	c.2535G>A	c.7082-7C>T
			p.Cys1948Cys	P.Thr13181Ala	p.Tyr795Tyr	p.Thr789Thr	p.Thr2413Thr	p.Gln852Arg	c.7082-7C>T
			r s 216902 C>T	r s 216311 A>G	r s 1063857 T>C	r s 1063857 A>G	r s216867 T>C	r s 216321G>A	r s 216868-7C>T
1	V-554	2443(-1) G-->C	T	G	C	G	C	G	C
2	V-415	2443(-1) G-->C	T	G	C	G	C	G	C
3	V-563	2443(-1) G-->C	T	G	C	G	C	G	T
4	V-481	2443(-1) G-->C	T	G	C	G	C	G	C
5	V-576	2443(-1) G-->C	T	G	C	G	C	G	T
6	V-600	2443(-1) G-->C	T	G	C	G	C	G	C
7	V-539	2443(-1) G-->C	T	G	C	G	C	G	T
8	V-307	2443(-1) G-->C	T	G	C	G	C	G	C
9	V-608	2443(-1) G-->C	T	G	C	G	C	G	C
10	V-499	2443(-1) G-->C	C	G	C	G	C	G	C
11	V-529	2443(-1) G-->C	T	G	C	G	C	G	C
12	V-471	2443(-1) G-->C	T	G	C	G	C	G	C
1	V-488	c.3675+1G>C	C	G	T	A	T	G	C
2	v-259	c.3675+1G>C	T	G	T	A	T	G	C
3	v-561	c.3675+1G>C	T	G	T	A	T	G	C
4	v-613	c.3675+1G>C	C	G	T	A	T	G	C
1	V-370	c.1117C>T	T	G	T	A	C	G	T
2	V-369	c.1117C>T	T	G	T	A	C	G	T
3	V-144	c.1117C>T	C	G	T	A	C	G	T
4	V-473	c.1117C>T	T	G	T	A	C	G	T

	UPN	**cDNA,**HGVS	**EXON 35**	**EXON 28**	**EXON 18**	**EXON 18**	**EXON 42**	**EXON 20**	**EXON 42**
			c.5844 C>T	c.4391A>C	c.2385T>C	c.2365A>G	c.7239T>C	c.2535G>A	c.7082-7C>T
			p.Cys1948Cys	P.Thr13181Ala	p.Tyr795Tyr	p.Thr789Thr	p.Thr2413Thr	p.Gln852Arg	c.7082-7C>T
			r s 216902 C>T	r s 216311 A>G	r s 1063857 T>C	r s 1063857 A>G	r s216867 T>C	r s 216321G>A	r s 216868-7C>T
5	V-541	c.1117C>T	T	G	T	A	C	G	T
6	V-331	c.1117C>T	T	G	T	A	C	G	T
7	V-545	c.1117C>T	T	G	C	G	C	G	T
8	V-225	c.1117C>T	T	G	T	A	C	G	T
9	V-407	c.1117C>T	T	G	T	A	T	G	C
1	V-493	c.3931C>T: c.3944G>A	C	G	T	A	C	G	C
2	V-419	c.3931C>T: c.3835G>A	C	G	C	G	C	G	C
3	V-604	c.3931C>T	T	G	C	G	C	G	C
4	V-585	c.3931C>T:c.3835G>A c.3931C>T	C	G	T	A	C	G	T
5	V-606	c.3797C>T:c.3835G>A	T	G	T	A	C	G	T
1	v-544	c.6321G>A	T	G	C	G	C	G	C
2	V-115	c.6321G>A	T	G	C	G	C	G	C
3	V-475	c.6321G>A	T	G	C	G	C	G	C
4	v-612	c.6321G>A	T	G	C	G	C	G	C
5	V-394	c.6321G>A	T	G	C	G	C	G	C
6	V-294	c.6321G>A	T	G	C	G	C	G	C
7	V-283	c.6321G>A	T	G	C	G	C	G	C

Table 24: ***Haplotype analysis of the common mutations identified in the present study***

Mutation	Haplotype	%
c.6321 G>A N=7	TGCGCGC	100%
c.2443 -1 G>C N=12	TGCGCGC	67%
	TGCGCGT	25%
	CGCGCGC	8.30%
c.3675 +1G>C N=4	CGTATGC	75%
	TGTATGC	28%
c.1117 C>T N=9	TGTACGT	66%
	TGTACGC	5%

Table 25: ***Haplotype analysis common mutations***

4.4 Elucidate the relationship between genetic defects and clinical/ haematological parameters.

4.4.1 Genotype-phenotype correlation

To understand the relationship between the clinical and haematological parameters, we attempted to study genotype-phenotype correlation as there is a paucity of data in the literature. In a total of 50 females, 25 females presented with a history of menorrhagia with a median bleeding score 11(range, 6-19). To negate the effects of menorrhagia on phenotype, we recalculated the bleeding scores excluding menorrhagia. The bleeding scores were segregated into 4 quartiles (group 1= ≤5; group 2= 5.1-8; group 3= 8.1-11 and group 4= >11). We then compared these quartiles with the location of mutations in the VWF gene, where the majority of the

patients were found to have mutations clustered on to the D domain of VWF. When mutation subtypes were compared with these quartiles, no correlation was observed. Further on comparision of the FVIII:C with the bleeding score, no correlation was observed.

Based on the report of BS of 10 being the possible divider of severity (Federici et al., 2014), we also evaluated mutation subtypes with BS >10 and BS ≤ 10. No statistical significant difference was observed between two groups (p value =0.960). We then compared the location of mutations in patients with BS >10 and BS ≤ 10. No significant difference was observed (p value = 0.560).

4.5 Evaluate the functional impact of selected mutations through expression studies

4.5.1 Mutations - *in vitro* cell expression studies

Four novel missense mutations localised in the D1, D4 and C domain (p.Gly74Arg, p.Cys2184Tyr, p.Cys2557Arg, and Arg2266His) and a previously reported mutation in the D1 domain were chosen for *in vitro* cell expression studies based on *in silico* and structural analysis. Results from *In silico* suggest suggest that these mutations were found to affect the structure or function of VWF. The plasmids expressing desired nucleotide change were generated by site directed mutagenesis in the plasmid (pSVHVWF1) as per the protocol. The nucleotide change was validated by direct sequencing. The plasmids were then expressed in HEK 293 cells to analyse the effect of these mutations.

4.5.2 Intracellular Distribution of VWF in Transfected HEK293 Cells

We analyzed the expression of the mutant-VWF along with the wild type [Figure 43]. Intracellular localization of VWF in transfected HEK293 cells was analysed by confocal microscopy by co-staining with ER marker Protein Disulfide Isomerase (PDI). Forty eight hours after transfection, VWF storage vesicles, that resembling weibel palade bodies (WPB) in endothelial cells were observed in HEK-293 cells [Figure 44]. There seems to be an increase in expression of the VWF in the mutants (Gly74Arg, Cys370Tyr, Cys2184Tyr, Cys2557Arg, and Arg2266His) when compared to the wild type VWF (pSVHVWF1). This probably can impair the secretion of VWF. Further studies including effect of these variants on secretion, shape of WPB's and multimer analysis must be carried out to validate the true nature of these mutations.

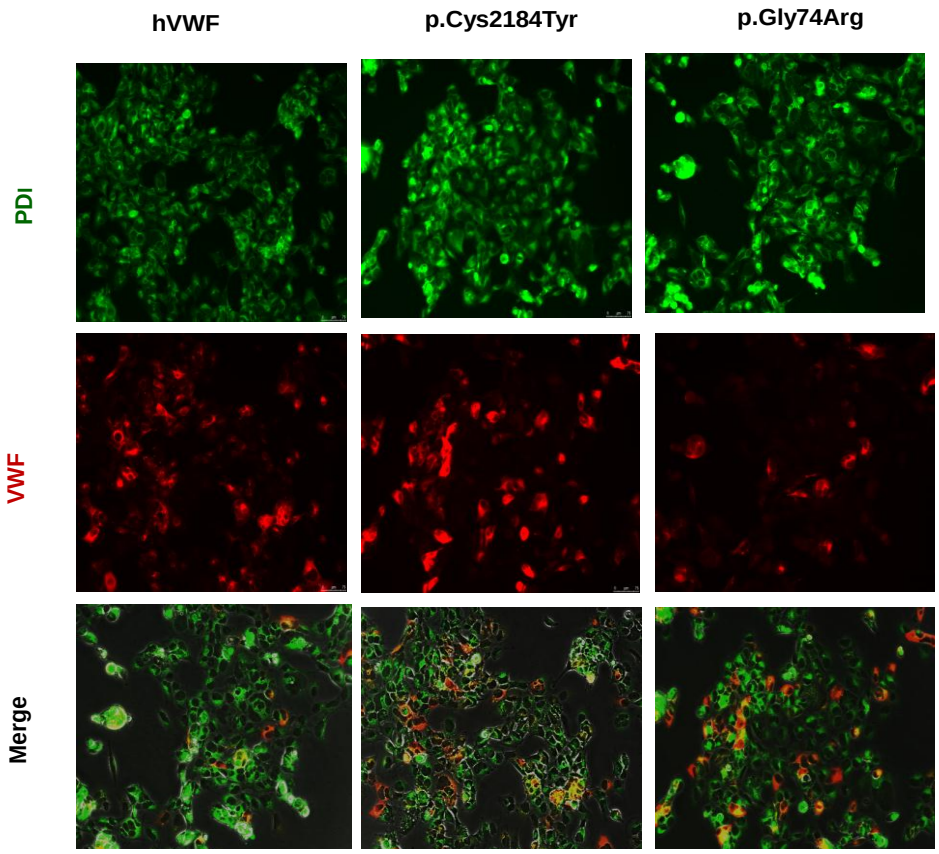


Figure 43: ***Intracellular distribution of WT-VWF and variants*** (at 20X Magnification) under fluorescent microscope. *HEK293 cells were fixed for immunofluorescence imaging 48hrs after single transfection (hVWF, p.Cys2184Tyr, p.Gly74Arg). Fixed cells were stained for vWF (red channel, middle panel) and for PDI (ER marker, red channel, upper panel). In the bottom panel (merged green and red channels), the ER containing vWF vs. observed (yellow) due to double staining with ER marker PDI. Images were acquired at 20X magnification*.

Finally, the cells were rinsed with PBS and mounted with Vecta shield (Peterborough, UK) containing DAPI (Molecular Probes, Eugene, Orlando). Images

were analysed using Olympus FluoView™ FV1000 Confocal Microscope with a 60X/1.40 NA oil objective [Figure 37].

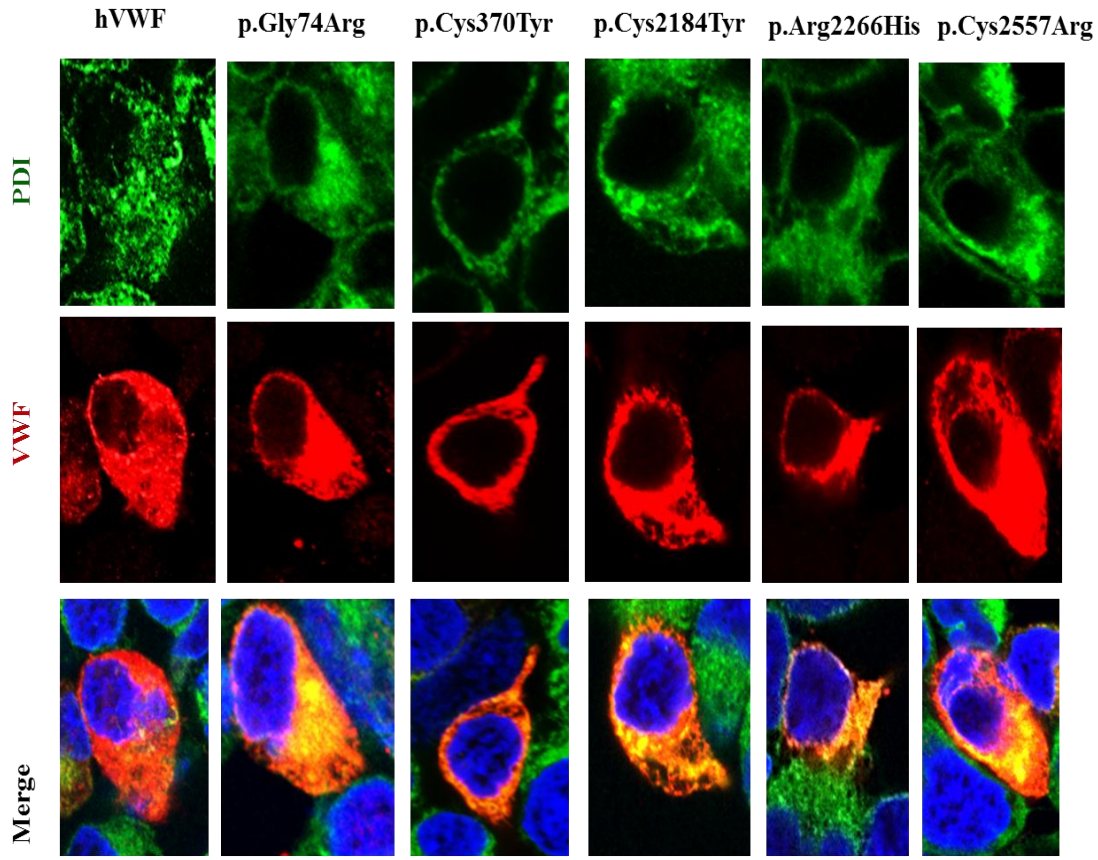


Figure 44: ***Intracellular distribution of WT-VWF and variants (at 60 X magnification) under confocal microscopy*** *HEK293 cells were fixed for immunofluorescence imaging 48hrs after single transfection (hVWF, p.Cys2184Tyr, p.Gly74Arg). Fixed cells were stained for vWF (red channel, middle panel) and PDI (ER marker, red channel, upper panel). In the bottom panel (merged), the ER containing vWF was observed (yellow) as a result of double staining with ER marker PDI. Images were acquired at 60X magnification under confocal microscopy.*

5. Discussion

Von willebrand factor is a large multimeric protein, which imparts a critical role in both primary and secondary hemostasis (Castaman et al., 2003). Absence of this factor results in von willebrand disease (VWD) (Lillicrap, 2013). VWD is classified as quantitative (Type 1 and 3) and qualitative disorders (Type 2A, B, M and N) (Goodeve, 2010). Type 3 VWD is the severe form of the disease. Patients with type 3 VWD are characterized by severely decreased VWF, and markedly reduced FVIII levels (Sadler et al., 2000). These patients are prone to severe bleeding episodes requiring frequent medical intervention (Sadler et al., 2000).

In our centre, based on the 10 year data obtained from the registry of new patients, maintained in the Department of Immunohematology and Transfusion Medicine, VWD ranks as the third most common inherited bleeding disorder next to hemophilia A and B (Sumitha et al., 2011). Type-3 VWD was found to outnumber the other subtypes in populations with high degree of consanguinity and under-diagnosis of the mild to moderate subtypes (Trasi et al., 2005). The prevalence of type 3 VWD varies among countries ranging from 0.1-5.3 per million (Eikenboom, 2001). In southern India the incidence of type 3-VWD is relatively higher, therefore requires more attention for diagnosis and treatment (Srivastava, 2005). Limited data exists on the molecular pathogenesis associated with type-3VWD in patients from India and their genotype-phenotype correlation (Ahmad et al., 2013a, Baronciani et al., 2000, Baronciani et al., 2003, Bowman et al., 2013, Kasatkar et al., 2014).

In this study we have evaluated the clinical spectrum of type-3 VWD in India, and established molecular diagnosis of this condition and assessed its genotype-phenotype correlation. The findings of this study will add significantly to the database of mutations in VWD. It has already helped us to establish carrier status of affected females and provide better genetic counselling, prenatal diagnosis as well as to understand its biology. Since the management of bleeding disorders is not optimal in our country (Srivastava, 2005), preventive strategies by mutation detection (Gupta et al., 2008), and validation of these genetic diagnosis strategies in a large cohort will help us to establish and optimise such services.

5.1 Clinical features and Bleeding profile of Type 3 VWD

5.1.1 Clinical features

The severity of bleeding symptoms in 102 study subjects was documented using ISTH-BAT questionnaire (Rodeghiero et al., 2010). Majority of patients in the present study presented with bleeding from minor wounds (86%), cutaneous (81%) and oral cavity bleeds (75%). Twenty-five out of 26 menstruating females (96%) in our study had menorrhagia. Literature studies on 150 type 3-VWD patients analysed for their bleeding symptoms showed that oral cavity bleeding (54%) was the most common bleeding symptom followed by muscle (28%) and joint (45%) bleeding accounting to 28% and 45% (Metjian et al., 2009). In a study from the western part of India where 85 patients with type 3 VWD studied, cutaneous bleeding was the common symptom followed by bleeding from a minor injury and oral cavity bleeding (Kasatkar et al., 2014).

The median age of presentation in our cohort was found to be two years (range: 0-60years) and the median age at diagnosis was found to be 14years (range: 1month-70years). Forty eight (47%) patients were diagnosed below 10 years of age and 54 (53%) patients were diagnosed above 10 years of age in the present study. A study by Metjian AD *et al*, have shown that the age of onset was 1.95 years (Metjian et al., 2009), while, Kasatkar *et al* reported the median age of presentation in their cohort was 10 years ranging from 40 days to 25 years (Kasatkar et al., 2014)

The median age at which the first transfusion was given in our study was found to be 11 years (range: 3 months-60 years). This is much later than the age of presentation. The age at first transfusion and number of transfusions is attributed to the awareness of the availability of therapeutic products and socioeconomic status. Educated patients with a good economic status seek medical help earlier than patients in the lower strata (Mohl et al., 2011, Kasatkar et al., 2014, Bowman et al., 2013, Sutherland et al., 2009b, Baronciani et al., 2003).

Sixty two patients were born of consanguineous marriage in the present study. A similar observation was observed in patients from Iran and western part of India (Shahbazi et al., 2009, Kasatkar et al., 2014). A strong association between consanguinity and severe VWD was depicted in 39%, 60%, and 91% of patients with this subtype in India, Iran, and Oman, respectively (Srivastava, 2005).

5.1.2 Bleeding score

Early detection of bleeding disorders depends on the sophistication of the health care system to detect such disorders. Delayed diagnosis of bleeding disorders

is a problem in many developing countries (Srivastava, 2005). Bleeding scores describes the severity of bleeding symptoms in patients with bleeding disorders (Federici et al., 2014). In a recent study, the BS were used to evaluate the tendency of the patient to bleed, in patients diagnosed with type 1, 2A, 2B, 2M (Federici et al., 2009, Castaman et al., 2011). Also the, BS also serves as a valuable tool to evaluate the genotype phenotype interactions (Rydz and James, 2012).

In the present study the bleeding score (BS) was found to range from 2-19, with a median BS of 9. As follow up was not long in most patients, only the BS at presentation was recorded. BS observed in the present study was similar to those reported in other populations (Bowman et al., 2013, Kasatkar et al., 2014). Out of 26 females in the reproductive age group 25 females were found to have menorrhagia with a median BS 11 (range: 6 -19) and required medical interventions. This is consistent with the previous data, which emphasizes menorrhagia as an important clinical feature of inherited bleeding disorders (Kadir et al., 1998, Kasatkar et al., 2014, Foster, 1995).

To study the factors influencing the BS, we made several categories to study their association. Initially we divided the patients based on the age of diagnosis (i) Age $\leq$ 10 (ii) Age >10. Then we divided them into those with BS $\leq$ 10 and BS > 10 based on a previous study (Federici et al., 2014). We compared the age of diagnosis with the BS. Forty eight patients were diagnosed under 10 years of age out of whom 42 (41%) had BS $\leq$ 10 and 6 (12.5%) had BS >10. Out of 54 (53%) patients diagnosed at age >10 years, 31 (57.4%) patients had BS $\leq$ 10 and 23 (43%) patients had BS >10 [Table 1]. The difference was statistically significant ($p = 0.001$).When the bleeding scores were compared with consanguinity 19.4%

born of consanguineous marriage had BS >10, while 43.6% born of non-consanguineous marriage had BS ≤10 [Table 1]. The difference was statistically significant (p = 0.026). When we compared the sex with BS >10 and BS ≤10 statistical difference was not observed between male and female (p = 0.731).

5.2 Genotypic analysis

Mutations were screened in 102 patients from 90 families by PCR-CSGE strategy already established by us (Jayandharan et al., 2003). In 80 patients disease causing mutations were identified using CSGE in both the alleles. In 3 patients mutations were identified only in a single allele using CSGE. In 19 patients where CSGE failed to identify the mutation, direct sequencing of the amplicons was carried out, were mutations could be detected in 10 patients in both the alleles. Mutations were not identified in 9 patients. Hence studies to identify deep intronic mutations and next generation sequencing methods can be adopted in the future to identify the causative mutation (Corrales et al., 2012) in these patients.

Gene dosage analysis was performed to identify any possible large deletions in patients without any identifiable point mutations. Single exonic deletion spanning exon 13 and multiexonic deletions spanning exon 1- 4 were identified in the present study. One major hindrance in understanding the molecular aetiology in patients with VWD is the amplification of pseudogene spanning exons 23-34, which mimics the authentic gene (Mancuso et al., 1991). The mutations identified in the present study were validated by using previously reported primers received on request (Hampshire et al., 2010). Mutations were found to span throughout the gene as observed in other studies (Bowman et al., 2013, Kasatkar et al., 2014, Shahbazi et al., 2009).

Various techniques have been used by different groups to identify mutations. A study from Italy, identified mutations using SSCP (single strand conformation polymorphisms) and RFLP approach in 37 patients, among whom mutations could be detected in 21 (57%) patients (Baronciani et al., 2003). In studies carried out Germany, in collaboration with other centres from India, using direct sequencing approach, mutations were identified in 20 (83%) of the total 24 patients screened (Gupta et al., 2008). Another study using sequencing-based approach was carried out in Hungary, where out of 23 families screened, mutation was not identified in one family (Mohl et al., 2011). A similar approach was used to screen mutations in 10 patients (Shahbazi et al., 2009) and (Ahmad et al., 2013) where they were not able to identify any mutation in 1 family and two families, respectively. In a recent report from Canada, using direct sequencing approach and multiple ligation probe amplification (MLPA) technique, mutations were identified in 29 out of the 34 (85%) families screened (Bowman et al., 2013). In another study from western part of India, mutations were identified in 77 (91%) of the 85 patients using various techniques namely direct sequencing, PCR-RFLP, and MLPA (Kasatkar et al., 2014). The frequency of mutations (91%) detected in our study using PCR-CSGE-sequencing is therefore similar to the results (80-90%) from other study groups (Bowman et al., 2013, Kasatkar et al., 2014, Mohl et al., 2008, Zhang et al., 1994, Sutherland et al., 2009b, Gupta et al., 2008).

5.2.1 Location of genetic variants in the VWF gene

Fifty five different mutations in 93 patients were identified in the present study. Mutation subtypes identified include frame shift (n =15, 27.27%), missense

(n = 13, 23.64%), non-sense (n = 12, 21.82%), splice site mutations (n = 10, 18%), large deletion (n = 2, 3.64%) and gene conversion (n=3, 5.45%). Thirty-five (63.6%) novel mutations were identified in the present study. Similar frequencies of the novel mutations were reported in patients from western India (57.6%) (Kasatkar et al., 2014), in Canadian cohort (64.5%) (Bowman et al., 2013) and patients from north-western England (66.6%) (Sutherland et al., 2009b). Other cohort studies including Hungarian population (Mohl et al., 2011) and the study by multi-ethnic group (Baronciani et al., 2000) reported a high frequency of novel mutations accounting for 71.4% and 83.3% respectively. A low frequency of novel mutations accounting for 42.8% was observed in the studies on Indian and Greek patients (Gupta et al., 2008) and Iranian population (Shahbazi et al., 2009).

In the present study, maximum number of mutations identified were localised in the propeptide region (27%), and the median BS in these patients was 9 (range: 2-18). A similar observation was observed in the Canadian cohort, where the majority (42%) of the mutations were clustered in the propeptide region (Bowman et al., 2013). Propeptide mutations identified in other studies include 30.5 % (Kasatkar et al., 2014), 38% (Mohl et al., 2011, Baronciani et al., 2000) and 33% (Ahmad et al., 2013, Sutherland et al., 2009b) and 16 % (Shahbazi et al., 2009).

5.2.2 Molecular Characterization in Type 3 VWD patients

5.2.2.1 Types of mutations

5.2.2.1.1 Nonsense Mutations

A total of 12 nonsense mutations were identified in 33 (32%) patients. Four of them were novel. Median BS of the patients harbouring nonsense mutation

in the present study is 8 (range: 2-18). Similar frequency of mutations of mutations were reported in Kasatkar et al., 2014 (33.9%), Hungarian population (38%) (Mohl et al., 2011), Iranian population (50%) (Shahbazi et al., 2009) and study from a multiethinic group (33.9%) (Baronciani et al., 2000). Three common nonsense mutations (p.Arg373*; p.Trp2107*, p.Gln1311*) were identified in the present study. Phenotype of the patients harbouring the mutation (p.Arg373*, p.Gln1311*) in this study was similar to the previous study [Table 26]. The novel mutation, p.Trp2107*, was identified in 7 patients. Bleeding symptoms in these patients varied from mild to severe [Table 26].

The mutation p.Arg1779* was previously reported as a common mutation in 5 patients from India (Kasatkar et al., 2014). Haplotype analysis suggested founder effect. These patients were from Uttar Pradesh (Gaderia, community from Northern India) (Kasatkar et al., 2014). We observed this mutation in only two patients [Table 26]. The difference may be due to ethnic variations within this vast country as patients in that study were from the northern part of the country while, patients in our study predominantly have a southern origin.

Interestingly two patients presented with a symptomatic bleed only at the age of 43 and 60 harboured the novel mutation p.Gln1463* [Table 26].

The nonsense mutations identified in the study might result in degradation of mRNA mediated by NMD (Nonsense-mediated Decay) pathway (Hentze and Kulozik, 1999) which explains the Type-3 VWD in this series of patients

Present study			Previous study		
Mutation	**No of Patients**	**Bleeding Score (Median)**	**Population Reported (Reference)**	**No of Patients**	**Bleeding Score (Median) / Phenotype**
p.Trp 222*	1	9	Italian [1, 2]	2	Severe
p.Arg373*	9	11 (4-17)	Indian [1,2,3] Canadian [4]	5 1	Severe 8
p.Tyr604*	1	9	Indian [2,5]	2	6, Severe
p.Gln895*	2	16 (16-17)	Novel		
p.Trp1120*	1	7	Novel		
p.Gln1311*	5	5 (2-11)	Indian [5,2,3] Iran [3] Caucasian[5] Canadian [4] Greece [3] Spanish [6]	7 1 1 1 1 4	Severe Severe Severe 8 Severe Severe
p.Gln1463*	2	11 (9-13)	Novel		
p.Arg1566*	1	9	Indian [8]	1	Severe
p.Arg1779*	2	4	Indian [2, 7]	7	13 (3-34)
p.Trp2107*	7	11 (4-17)	Novel		
p.Arg2535*	1	18	Swedish [9] Dutch [10,11] German [12] Italian Turkish [14] Indian [2]	1 4 1 1 1	Moderate None Severe
p.Gln2551*	1	5	Indian [2]	1	11

Table 26*: Nonsense mutations in patients with type 3 VWD.*

1. (Baronciani et al., 2000, Baronciani et al., 2003) 2. (Kasatkar et al., 2014) 3. (Gupta et al., 2008). 4. (Bowman et al., 2013) 5. (Sutherland et al., 2009b) 6. (Casana et al., 2000) 7. (Ahmad et al., 2013) 8. (Kakela et al., 2006) 9, 10. (Eikenboom et al., 1998, Zhang et al., 1994) 11. (Zhang et al., 1992b). 12. (Eikenboom et al., 1992) 13. (Schneppenheim et al., 1994) 14. (Hampshire et al., 2013).

5.2.2.1.2 Frameshift mutations

Fifteen frame shift mutations were identified in 24 patients (23.5%) in the present study. All of them were novel. Median BS in these patients was 10 (range: 3-18). Studies suggest predominance of frame shift mutations (Bowman et al., 2013). In the present study single nucleotide deletions were identified in 7 patients [Figure 45]. Deletion of two nucleotides was observed in 3 patients [Figure 45]. Deletion of 14 nucleotides was observed in a patient spanning D1 domain. The insertion-deletion mutation was identified in two patients [Figure 45].

Occurrence of the deletions and insertions was found to be influenced by the local DNA sequence environment (Bawono and Heringa, 2014, Ashkenazy et al., 2016). One patient harboured duplication of 4 nucleotides. These mutations may result in premature truncation of protein associated with the NMD pathway (Lykke-Andersen et al., 2000).

The common mutation p.Leu970del, identified in 12 patients in western India was not observed in our population (Kasatkar et al., 2014). The reason may be due the difference in the ethnicity since the patients in our study mostly were from southern India. The Baltic founder mutation, identified in the original family with VWD was not observed in our study subjects (Casana et al., 1998).

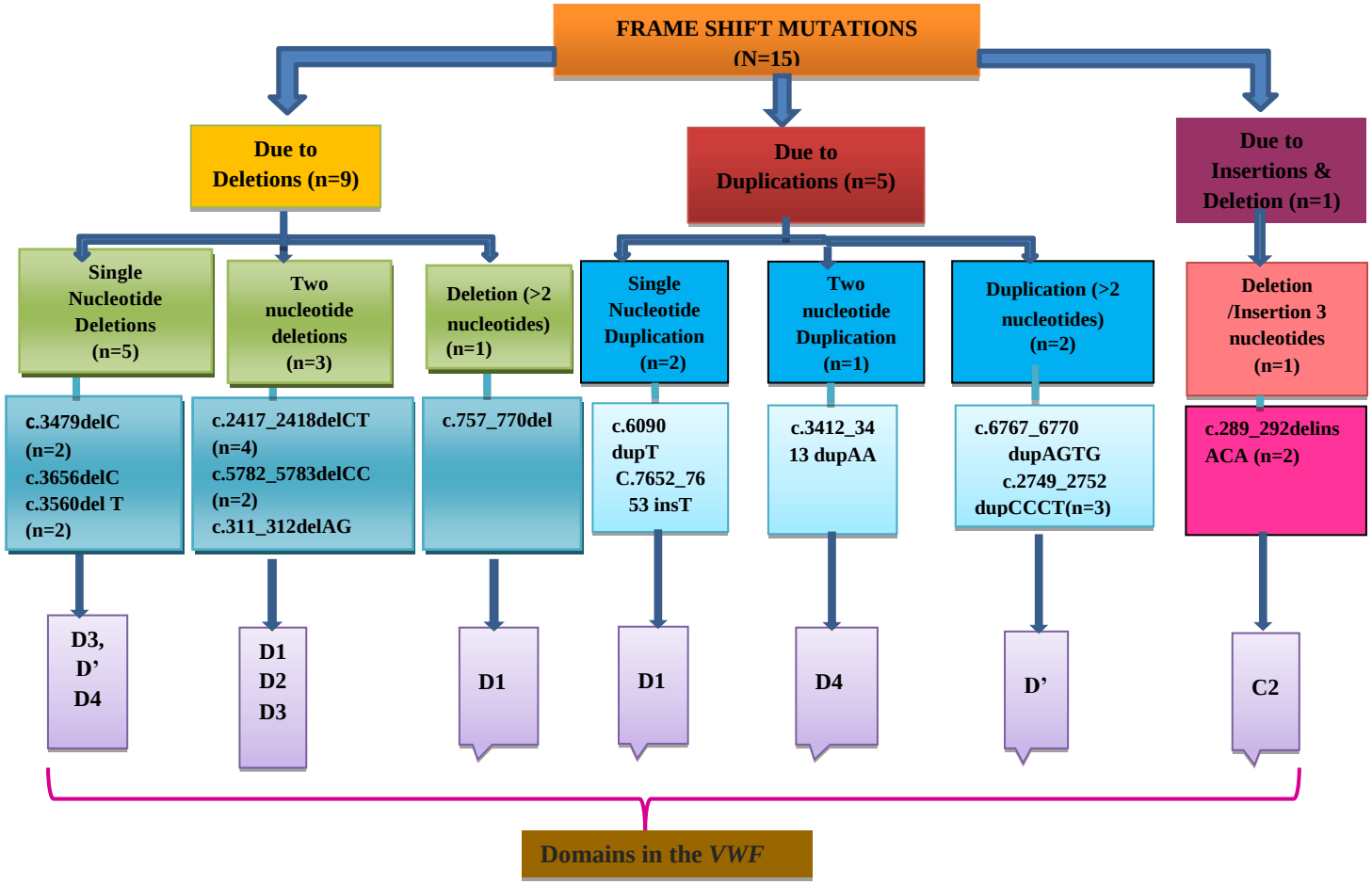


Figure 45: ***Schematic representation of frame shift mutations identified in the present study.***

5.2.2.1.3 Splice site Mutation

Ten splice mutations were identified in 24 (23.5%) patients in the present study of which 7 were novel. Median BS of the patients harbouring splice site mutation in the present study is 6 (range: 1-19). Previous studies suggest the occurrence of splice site mutation to be lower in comparison to other mutation types (Mohl et al., 2011, Kasatkar et al., 2014, Bowman et al., 2013, Sutherland et al., 2009b, Baronciani et al., 2003). In the present study three splice site mutations were found to affect guanine at position -1 and is likely to alter the normal splice site [Figure 46]. Common splice mutation identified in this study was (c.2443-1G>C), with a median BS was 6 (range: 4-15) [Figure 46]. This mutation is previously reported in patients from Iran, western India and in Turkish populations (Kasatkar et al., 2014, Shahbazi et al., 2009). One novel splice site mutation was identified in Intron 20 [Figure 46]. A previously reported splice site mutation was identified in intron 40 [Figure 46] (Baronciani et al., 2003). Other splice site mutations identified in the acceptor site in the present study include intron 14 and intron 37 [Figure 46]. These splice site mutations are predicted to result in exon skipping (Baronciani et al., 2003, Baronciani et al., 2000, Schneppenheim et al., 2012).

Six splice site mutations were found to affect the donor site (+1) in the VWF gene [Figure 46]. In the present study recurrent donor splice site mutation was identified in the D3 domain may impair the process associated with multimerization of VWF (Yee et al., 2014) [Figure 46]. Median BS in these patients was 8 (range: 6-15). In a previous study occurrence of the mutation (c.3379+1G>A) was found to be associated with p.Arg924Gln (Mohl et al., 2011), which was not observed in the present study.

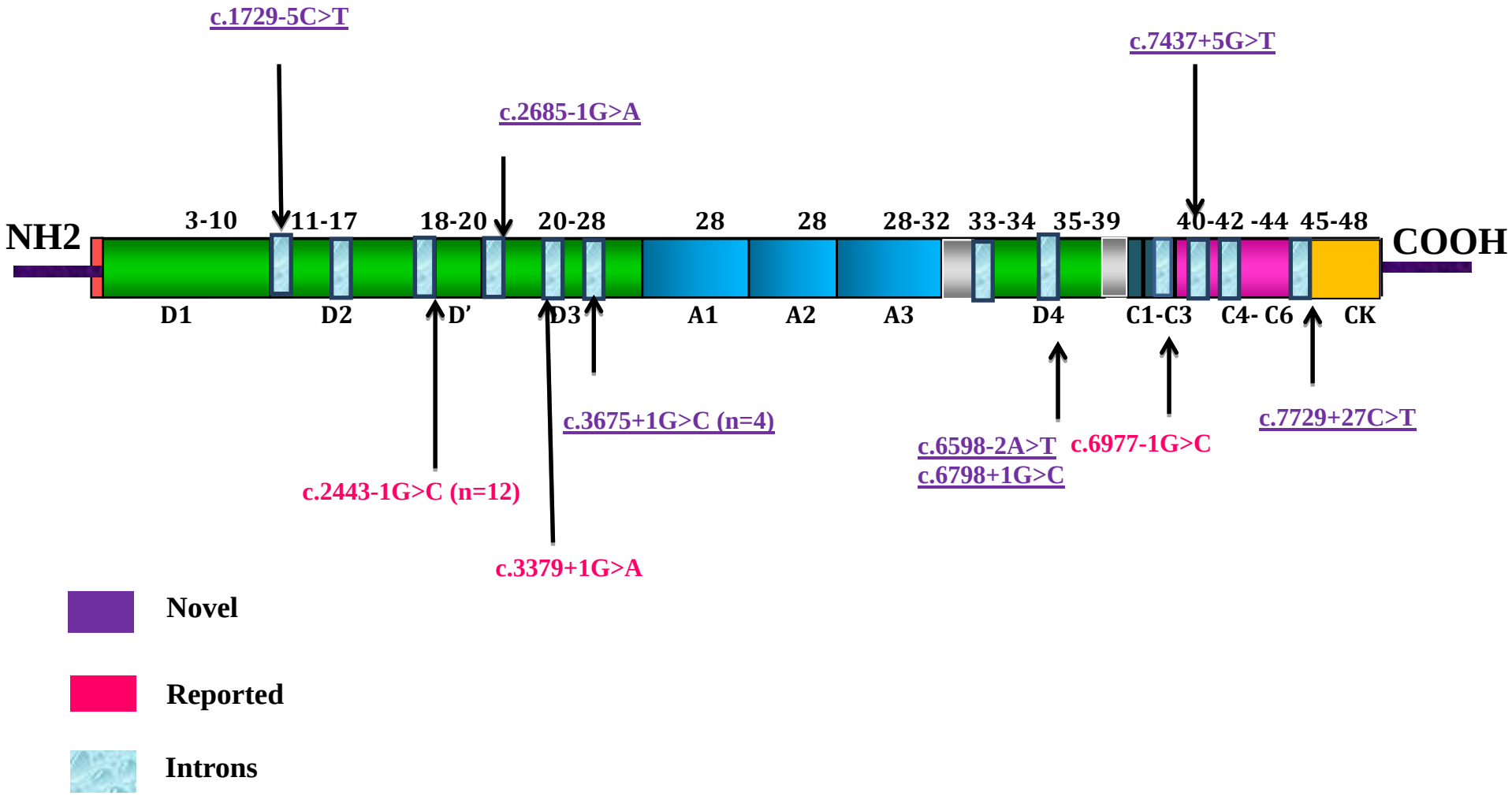


Figure 46*: Schematic representation of the splice site mutations identified in the present study*

We performed *in silico* analysis to validate the effect of the novel splice mutations (Choi and Chan, 2015). All these tools were supportive of the fact that the mutations identified in our study might disrupt the normal splicing process, however, functional effects of this mutation need to be confirmed at the RNA level (Zhang et al., 1994).

5.2.2.1.4 Missense mutations

Thirteen missense mutations were identified in 16 patients (15.7%). Seven of them were novel. Median BS of the patients harbouring the missense mutation in the present study was found to be 10 (range: 4-18). Studies suggest predominance of missense mutations (Ahmad et al., 2013). Structural analysis (in collaboration with Dr.Arijit Biswas & Dr.Johannes Oldenburg) and *in vitro* expression studies was performed for the 4 novel missense (p.Gly74Arg, p.Cys2184Tyr, p.Cys2557Arg, and p.Gln2266His) mutations identified in this study. *In vitro* studies were also carried out for a previously reported mutation (p.Cys370Tyr). The novel mutations identified in the present study were absent in the 50 normal alleles screened.

5.2.2.1.4.1 Mutation identified in propeptide region of VWF

Mutations identified in the propeptide region in the present study include p.Asp47Val, p.Gly74Arg p.Asp141Asn, p.Cys370Tyr and p.Met771Ile of which two were novel. The novel mutation p.Asp47Val identified in this study was located within the D1 domain of VWF (Schneppenheim et al., 2001). The residue p.Asp47 was found to be conserved across the species (Bawono and Heringa, 2014). The mutation, p.Asp47Val, when analyzed by SIFT, Polyphen (Ramensky et al., 2002, Sunyaev et al., 2001) had a damaging effect on their respective proteins.This mutation

introduces an amino acid with different properties, which can disturb this domain and abolish its function (Venselaar et al., 2010). Difference in charge between the wild-type and mutant amino acid was observed (Venselaar et al., 2010). The mutation p.Asp47Val introduces a hydrophobic group which may hamper the formation of multimers (Baronciani et al., 2000). Missense mutations, reported at the adjoining residue includes p.Asp47His and p.Gly39Arg, has been shown to inhibit the process associated with multimerization and secretion of VWF (Baronciani et al., 2000).

The second novel missense mutation identified in this study is p.Gly74Arg. Structural analysis (in collaboration with Dr.Arijit Biswas & Dr.Johannes Oldenburg) of this substitution suggests that replacement with a positively charged amino acid might lead to non-native interactions (i.e. putative hydrogen bond pairs) with the neighbouring negatively charged residues, i.e., Asp75, Asp183, and Asp186. These interactions can therefore introduce rigidity thereby destroying flexibility and the function associated with this region. Hence this mutation may impair the process associated with secretion of VWF. Other mutations identified in the adjoining region include Y87S, R273W (Bowman et al., 2013, Schneppenheim et al., 2012), were found to impair the secretion of VWF. Hence a similar mechanism is likely to contribute to the phenotype thereby suggesting the importance of D domains in multimerization. Previously reported mutations identified in the propeptide region in the present study include mutations p.Asp141Asn (Yin et al., 2015), p.Met771Ile (Goodeve et al., 2007) and p.Cys 370Tyr, were considered disease causative.

5.2.2.1.4.2 Mutations identified in the D3 domain of VWF

Two novel mutations identified in the D3 domain in the present study includes, p.Ala1150Pro and p.Met1055Lys. *In silico* analysis of p.Met1055Lys suggest difference in charge and less hydrophobic compared to wild type (Venselaar et al., 2010). Hence the interactions either on the surface or core of the protein, might be lost (Venselaar et al., 2010). Missense mutations identified in the adjacent residues include Q1053H, C1060R, and C1060Y (Hilbert et al., 2003, Wang et al., 2011). *In vitro* studies for Q1053H and C1060R were found to impair FVIII binding (Hilbert et al., 2003, Wang et al., 2011). Hence the mutation p.Met1055Lys identified in the present study may impair the process associated with FVIII binding (Hilbert et al., 2003).

Second novel mutation identified in the D3 domain was p.Ala1150Pro. The mutation p.Ala1150Pro when analysed by SIFT and Poly phen scores had a damaging effect on proteins and on MUpro analysis, was found to decrease the protein stability (Ramensky et al., 2002, Sunyaev et al., 2001). Mutations identified in the adjoining regions include C1130F, C1149R, C1149Y, W1144G, Y1146C, and C1153Y was found to impair the process associated with multimerization (Bodo et al., 2001, Schneppenheim et al., 2012). Hence a similar mechanism is likely to contribute to type 3-VWD seen in the patients observed in this study.

5.2.2.1.4.3 Mutations identified in A1 domain of VWF

A previously reported mutation p.Arg1315Cys was observed in a patient (Casana et al., 1998, Ribba et al., 2001, Zhang et al., 1994) was considered disease causative (Ribba et al., 2001). This mutation is reported in Swedish, Finish, and

German patients, diagnosed with type-2A, 2M phenotype, and in patients with type-3VWD (Casana et al., 1998, Ribba et al., 2001, Zhang et al., 1994). *In vitro* studies on the mutant p.Arg1315Cys was found to exhibit abnormal folding of the protein resulting in loss of both intermediate and HMW multimers and impaired binding to the platelets (Ribba et al., 2001).

5.2.2.1.4.4 Mutations identified in D4 domain of VWF

Two novel mutations (p.Cys2184Tyr, Gln2266His) were identified in two patients in this study. Structural analysis (in collaboration with Dr.Arijit Biswas & Dr.Johannes Oldenburg) for p.Cys2184 suggests that the residue is linked to Cys2150 by a disulphide bond, which provides stability to this surface, where an unbound Cys residue Cys2174 is closer to the D4-D4 dimeric interface. Unbound Cys residue may participate in disulphide exchanges which contribute to the stabilisation of the dimeric interface as predicted by structural analysis. Therefore the mutation p.Cys2184Tyr would disrupt the disulphide bond formation with the adjacent residues thereby disturb the surface orientation of this region, which might interfere with putative D4-D4 domain dimerization. Also, the accommodation of a large aromatic hydrophobic side chain in this tightly packed hydrophobic region might result in steric clashes with neighbouring Trp2107, Tyr2160 and His2182 residues, thereby contributing to domain instability. *In vitro* characterization of the mutations identified in the adjoining residues (p.Cys2190Tyr, Ser2179Phe) suggests absence of weibel palade body formation and decreased plasma survival of VWF (Castaman et al., 2012).

The novel mutation Gln2266His in a patient was localised close to the C1-D4 domain boundary of VWF. This region consists of a disulphide bond that is part of a beta sheet in this region Cys2257-Cys2283 at the N-terminal flexible region of the C1 domain. This cysteine is part of a disulphide bond (Cys2254-Cys2220) that connects this region to the D4 domain, and this can be observed at the C-terminal extended part of the D4 domain model. Structural implications (in collaboration with Dr.Arijit Biswas & Dr.Johannes Oldenburg) for p.Gln2266His suggest that the disulphide bonds formed by both these cysteines connect two domains (C1 and D4). Therefore this region contributes to inter-domain linkage therefore to flexibility and stability of the protein. The Gln2266 is surrounded by two aromatic residues, His2265 and Phe2267. Substitution of His in this region may contribute to aromatic stacking interactions between these three residues. Therefore this mutation might introduce rigidity in this area and prevent the inter-domain flexibility that is needed during the dimerization/zipping up process of these domains. Other mutations identified in the adjoining regions include Leu2207Pro Cys2257Ser, Cys2283Arg, was found to impair secretion of VWF (Yadegari et al., 2013, Eikenboom et al., 2009).

5.2.2.1.4.5 Mutations identified in C domain of VWF

Two mutations including p.Arg2535Gln and p.Cys2557Arg were identified in the C domain of VWF. A previously reported mutation identified in this study p.Arg2535Gln was considered causative (Kasatkar et al., 2014). The second mutation identified in C domain was a novel mutation p.Cys2557Arg. Structural analyses (in collaboration with Dr.Arijit Biswas & Dr.Johannes Oldenburg) suggest that

since this mutation p.Cys2557Arg occurs on a disulphide bond (Cys2557-Cys2574), it is more likely to alter the structure of VWF. Further addition of a positively charged side chain can lead to non-native interactions within the domain resulting in generation of incorrect interface. Therefore this mutation would most likely to impair the process associated with multimerization of VWF. Mutations identified in the adjoining regions including p.Cys2619Tyr and p.Cys2676Phe were found to disrupt intra-domain disulphide bonds formation required for the formation of dimeric bouquets resulting in an impaired storage and secretion of VWF (Yadegari et al., 2013). Hence mutations in this region may impair the process associated with dimerization of VWF (Goodeve et al., 2007).

5.2.2.1.4.6 Mutation identified in CK domain of VWF

A previously reported p.Cys2804Tyr was identified in four patients in the present study was considered disease causative (Yadegari et al., 2013). The Cys2184 residue is disulphide bonded to Cys2150, which provides stability to this surface were an unbound Cys residue Cys2174 is closer to the D4-D4 dimeric interface. Unbound Cys residue might participate in one of many disulphide exchanges which contributes to the formation and stabilisation of the dimeric interface. The mutation would disrupt the disulphide bond and also disturb the surface orientation of this region, therefore, might interfere with putative D4-D4 domain dimerization. Also, the accommodation of a large aromatic hydrophobic side chain in this tightly packed hydrophobic region might result in steric clashes with neighbouring Trp2107, Tyr2160 and His2182 residues thereby contributing to domain instability. Similar mutations identified in the region include Cys2190Tyr, Ser2179Phe are known to

impair secretion and absence of WPB formation or decreased plasma survival of VWF (Haberichter et al., 2006, Castaman et al., 2012).

5.2.2.1.5 Gene conversion

In the present study gene, gene conversion mutations were identified in 5 (4.9%) patients. The median BS of the patients associated with gene conversion in the present study was 5 (range: 2-17). Occurrence of gene conversion events between VWF and pseudogene (VWFp) is described previously in patients with type-3VWD (Baronciani et al., 2003, Kasatkar et al., 2014, Venselaar et al., 2010, Yadegari et al., 2013, Eikenboom et al., 2009). Recombination events occur due to the presence of chi or chi like sequences at the 3' of intron 27 (CCTGGTGG) or 5' part of exon 28 (GCTGGTGG). These sequences can mediate non-homologous recombination between the authentic gene and pseudogene localised in chromosome 22 (Castaman et al., 2012, Schneppenheim et al., 2012). This subtype was previously reported in >10% of the diseased chromosomes in a cohort of 50 type-3VWD patients (Venselaar et al., 2010). Occurrence of gene conversion events was identified in the homozygous condition in three patients and in heterozygous state in two patients in the present study. In one patient mutations including p.Gln1311*, p.Val1279Ile, p.Pro1266Leu was observed in heterozygous condition.

Various groups have reported the occurrence of the mutations (p.Gln1311*, p.Val1279Ile, p.Pro1266Leu) associated with gene conversion events (Gupta et al., 2008, Baronciani et al., 2003, Schneppenheim et al., 2012, Venselaar et al., 2010, Kasatkar et al., 2014). In another study were occurrence of the mutation p.Gln1311* was found to be associated with the development of inhibitors (Yadegari et al., 2013).

However, in our series of patients, we did not observe the presence of inhibitors. The mutation p.Pro1266Leu was previously described in Type 1 [New York/Malmo (Holmberg et al., 1993)] type 3 and 2B VWD patients (Venselaar et al., 2010).

Two patients harboured the mutation p.Val1279Ile and p.Gln1311* in homozygous condition in the present study. One patient was associated with recurrent transfusion history (>150 exposures). The causative mutation identified in the patient (p.Gln1311* and p.Arg1315His) was reported in a previous study (Goodeve et al., 2007). In the present study p.Gln1311* was found to occur predominantly in associated with gene conversion events. Hence it is predicted to undergo premature truncation of the protein related to the NMD (Nonsense-mediated decay) pathway, resulting in decay of the associated null transcripts (Lykke-Andersen et al., 2000).

5.2.2.1.6 Large deletions

Two (3.64%) large deletions were identified in the present study. Median BS of the patients harbouring large deletion was found to be 13 (range: 12-14). Single exonic deletion spanning exon 14 and multiexonic deletion spanning exon 1- 4 were identified in our series of patients resulting in removal of the essential sequence of VWF. Predominance of large deletion was identified in the Hungarian population (Mohl et al., 2008) when compared to other studies (Baronciani et al., 2003, Bowman et al., 2013, Gupta et al., 2008, Sutherland et al., 2009b, Zhang et al., 1994). Deletions appear as a result of recombination events between Alu Y and Alu SP repetitive sequences (Mohl et al., 2008). Alu sequences were found to be usually associated with transposition activity (Huang et al., 2012). The presence of a large

deletion identified in the present study was confirmed by the failure of amplification of the exons in the PCR followed by control gene PCR and gene dosage analysis to confirm the carrier status in the parents. In the earlier reports, patients with large deletions, including partial and complete gene deletion, were found to be associated with development of antibodies against VWF (Baronciani et al., 2003, Eikenboom et al., 1998, Schneppenheim et al., 2007, Mancuso et al., 1994, Ngo et al., 1988). In contrast, in the present study, both these patients did not develop antibodies despite multiple transfusions. A similar observation was made in the Hungarian population, where five patients, though homozygous for large deletion, did not develop anti-VWF antibodies against VWF (Mohl et al., 2008). Earlier studies suggest that Alu-mediated deletions are associated with the development of inhibitors, in patients with haemophilia A (Rossetti et al., 2004). This, in turn, puts forth a notion that there may be additional genetic and environmental modifiers coexisting in these patients, which remains unanswered (Mohl et al., 2008). Another study reported two large deletions: one spanning from intron 13 to exon 14, and the other from promoter to intron 6 (Ahmad et al., 2013). Deletion spanning exons 17-18, 22-43, 23-52, and exon 42 have already been described (Baronciani et al., 2000). The authors proposed double-strand break of DNA followed by homologous recombination as a mechanism for the deletions observed in the study (Mohl et al., 2008).

5.2.3 *Expression studies*

In the present study based on *in silico* analysis, novel mutations spanning propeptide (p.Gly74Arg, p.Cys370Tyr), D4 domain (p.Cys2184Tyr, p. Arg2266His) and C domains (p.Cys2557Arg) were chosen for further investigation. Plasmids

expressing desired nucleotide change and WT-VWF (pSVHVWF1) were transfected in HEK-293 cells (Wang et al., 2011). Forty eight hours after transfection of the WT-VWF in HEK-293 cells, intracellular storage organelles i.e. pseudo-weibel palade bodies (WPBs), resembling WPBs in the endothelial cell was observed. We compared the expression of the VWF in WT-VWF (pSVHVWF1) and in the variants. Results of the immunofluorescence studies increased expression of VWF in the mutants (p.Gly74Arg, p.Cys370Tyr, p.Cys2184Tyr, p.Cys2557Arg, and Arg2266His) compared to the WT-VWF.

Expression studies on the mutations Y87S, D141Y, G160W N166I and C275S identified in the propeptide region was found to impair the process associated with multimerization (Schneppenheim et al., 2001) as implicated by the secretion of dimeric form VWF (Montgomery et al., 1999, Kasatkar et al., 2014, Zhang et al., 1994, Schneppenheim et al., 1994, Eikenboom et al., 2009). Hence the mutations identified in the propeptide region (p.Gly74Arg, p.Cys370Tyr), can impair the secretion of VWF. *In vitro* studies on the mutations identified in the D4 domain (p.Cys2190Tyr, Ser2179Phe, Cys2257Ser, Cys2283Arg) was found to impair secretion of VWF or reduce the survival of VWF in the plasma (Yadegari et al., 2013, Eikenboom et al., 2009, Castaman et al., 2012). Hence the mutations identified in the present study (p.Cys2184Tyr, p.Gln2266His) may impair the secretion of VWF. Mutations identified in the adjoining residues spanning C domain include (p.Cys2619Tyr and p.Cys2676Phe) were found to impair the storage and secretion of VWF (Yadegari et al., 2013). Hence it is likely that the mutations (p.Cys2557Arg) can impair the secretion of VWF. However further studies including measurement of

basal and regulated secretion of VWF, shape of weibel palade bodies to predict the effect of mutations, and multimer analysis (Castaman et al., 2012) must be carried out to elucidate the true nature of the mutations identified in the present study.

5.2.4 Genotype-phenotype correlation

The phenotypic complexity of a monogenic disease may be modulated by multiple factors including genetic and non–genetic modifiers (Schafer et al., 2005). Genotype-phenotype correlation in VWD is complicated by the large heterogeneity associated with the same mutation (Baronciani et al., 2003, Gupta et al., 2008, Kasatkar et al., 2014, Bowman et al., 2013). The present study confirms the clinical and genetic heterogeneity in patients with type 3 VWD as described earlier in Indian patients (Baronciani et al., 2003, Gupta et al., 2008, Kasatkar et al., 2014). Information derived from the genotype-phenotypic correlations will aid management of the patients and genetic counselling decisions (Goodeve, 2010). We attempted to study the genotype-phenotype correlation as there is a limited data in the literature. In this study, we recalculated the BS excluding menorrhagia to negate the phenotype. The recalculated BS was then compared with the mutation subtypes and location of mutations to see if there is any association between the phenotype and the observed genotype.

Initially the bleeding scores were segregated into 4 quartiles (group 1 = ≤ 5; group 2 = 5.1-8; group 3 = 8.1-11 and group 4 = >11). We then compared these quartiles with the location of mutations in the VWF gene, where maximum no of patients were found to have mutations clustered on to the propeptide (27%) region of VWF. When mutation subtypes were compared within these quartiles, no

correlation was observed. Based on the report of BS of 10 being the possible divider of severity (Federici et al., 2014), we also evaluated mutation subtypes with BS >10 and BS ≤ 10. No statistical significant difference was observed between two groups (p value =0.960). We then compared the location of mutations in patients with BS >10 and BS ≤ 10. No significant difference was observed (p value =0.560). Thus we were not able to correlate genotype with phenotype even in this large cohort [Table 27].

Parameter	Number of Patients (n=102)	BS≤10		BS>10		p-value
		No of patients	Median (Range)	No of patients	Median (Range)	
Age	<10yrs=48	42	6(2-10)	6	13.5(11-18)	p = 0.001
	>10yrs=54	31	13(11-19)	23	7(2-10)	
Sex	Male =50	35	6(2-10)	15	14(11-19)	p=0.731
	Female=52	38	6(2-10)	14	13(11-18)	
Consanguinity	Present=62	50	6(2-10)	12	13(11-18)	p=0.026
	Absent=40	17	7(3-10)	22	13(11-19)	
FVIII:C	<2%=31	22	6.5(2-10)	9	13(11-15)	p=0.997
	2--5%=51	36	6(6-10)	15	13(10-19)	
	>5%=20	14	6(2-10)	6	13(11-18)	
Missense	32	24	7(2-9)	8	13(11-18)	p=0.560
Frame shift	24	18	6.5(3-10)	6	13.5(11-15)	
Splice site	24	16	6(2-10)	6	13.5(11-15)	
Missense	16	5	6(2-10)	11	13(12-16)	
Gene conversion	5	4	4(2-7)	1	13	

Table 27 : ***Bleeding scores in patients with type 3 VWD***

SD: standard deviation; BS: Bleeding score

In the present study in 9 patients for whom the pathogenic explanation could not be illustrated in both the alleles, had similar VWF:Ag, VWF:RCo and FVIII

levels when compared to the 90 patients for whom the mutations were identified in both the alleles. The median BS in these patients was found to be 6 (range: 2-19).

Data on VWF and non-VWF variation's that contribute to phenotypic variability between individuals with identical mutation and patient diagnosed with type 3 but no mutations identified in the VWF gene remains rudimentary (Goodeve, 2010). In the present study a common splice site mutation c.2443-1G>C was identified in 12 patients. Despite having the identical splice site mutation the site and intensity of mucocutaneous bleeding seemed to vary. The median BS in these patients was found to be 6 (range: 4-15). Commonest clinical manifestation with the splice site mutation c.2443-1G>C, was found to be bleeding from minor wounds and cutaneous. Also the predominant clinical manifestation observed in patients with p.Arg373*, p.Trp2107*, p.Gln1311*, c.3675+1G>C was found to be bleeding from minor wounds and cutaneous [Table 28].

S. No	Mutation	No of patients	Age of presentation years median (range)	Bleeding Score median (range)	Most common sites of bleeding
1	c.2443-1G>C	12	10.5 (1 month - 5yrs)	6 (4-15)	BMW, Cut
2	p.Arg373*,	9	6 (1 month-5yrs)	7 (2-9)	BMW, Cut
3	p.Trp2107*	7	3 (1month-13yrs)	11 (4-17)	Epi, Cut, BMW
4	p.Gln1311*,	5	1.5 (6 month-10yrs)	5 (2-11)	Cut, BMW
5	c.3675+1G>C	4	2.5 (6 month-10yrs)	10 (6-15)	OCB, Cut, BMW

Table 28: ***Clinical manifestations in patients with common mutation***

BMW = Bleeding from minor wounds Cut = Cutaneous; OCB = Oral cavity bleeding; Epi =Epistaxis.

A possible role for mutations in other genes (both cis and trans) is also to be evaluated. Whole exome sequencing could possibly provide some clues. The possibility of involvement of other regulatory/epigenetic genes in influencing the disease phenotype also cannot be ruled out. Further studies at the protein level could possibly explain the discrepancy in genotype-phenotype correlation. The limitation of this study is that even though the sample size is adequate, the number of patients with similar mutations is relatively small in each category. Also, we had BS calculated only at the time of diagnosis; further follow up data was not available for most patients.

6. Summary and Conclusions

1. The present study is one of the largest series, carried out to understand the molecular basis of Type3-VWD. Clinical details were documented using ISTH-BAT bleeding questionnaire. Commonest clinical manifestations observed in the study include bleeding from minor wounds and cutaneous bleeding. Consanguinity among the patients evaluated was accounting for 62%

2. Mutations were screened in 102 patients from 90 families. The strategy used in the present study was to first screen for mutations using CSGE followed by sequencing of relevant segments of the gene.

3. Two-factors complicate the molecular diagnosis of VWD. (i) Large size and (ii) presence of pseudogene. In the present study, two different sets of primers were used to minimize the chance of SNP on the primer binding site, resulting in the amplification of only a single allele.

4. The experimental approach designed to study the mutation spectrum was successful in identifying mutations in 93(91%) patients from 81(79%) families.

5. A total of 35 novel mutations were identified in the present study. Mutations were distributed throughout the VWF gene. Mutations identified in the present study were heterogeneous as reported in other populations. Maximum numbers of mutations were identified in the propeptide region (VWFpp), (27%) of VWF.

6. We identified two large deletions spanning exons 1-4 and 13-14. In contrast to several previous reports, none of the patients homozygous for the large deletion developed alloantibodies to VWF.

7. Pathological implications of the novel mutations identified in the present study were evaluated by *in silico* analysis.

8. Structural analyses were carried for four novel missense mutations identified in the present study. Results of the analysis suggest that the mutations (p.Gly74Arg, p.Cys370Tyr, p.Gln2266His, p.Cys2557Arg) may impair the process associated with secretion. The mutation (p.Cys2184Tyr) was found to affect the antigenic stability.

9. *In vitro* cell expression studies were carried out for 4 novel mutations and a previously reported mutation in the present study. These studies suggest that the mutations (p.Gly74Arg, p.Cys370Tyr, p.Cys2184Tyr, p.Gln2266His, p.Cys2557Arg) may impair the secretion of VWF.

10. Five common mutations (p.Arg373*, c.3675+1G>C, c.2443-1G>C, p.Gln1311*, p.Trp2107*) were identified. A common haplotype was observed only for p.Trp2107*. Hence common mutations could be initially screened to facilitate rapid and cost effective molecular diagnosis in patients with type 3 VWD.

11. Based on these results, facilities to control VWD through genetic counselling and prenatal diagnosis have been set up.

12. The present study highlights the molecular spectrum of type 3 VWD and the phenotypic heterogeneity in these patients in India.

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8. List of Publications

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Aaron Chapla, Giridhara Rao Jayandharan, Elayaperumal Sumitha, Govindanattar Sankari Devi, Paneerselvam Shenbagapriya, Sukesh Chandran Nair, Auro Viswabandya, Biju George, Vikram Mathews, Alok Srivastava, **Molecular basis of hereditary factor V deficiency in India: Identification of four novel mutations and their genotype phenotype correlation.** *Thromb Haemost*, 2011, 6:1120-3.

Sumitha E, Jayandharan GR, Arora N, Abraham A, David S, Devi GS, Shenbagapriya P, Nair SC, George B, Mathews V, Chandy M, Viswabandya A, Srivastava A. **Molecular basis of Quantative fibrinogen deficiency in patients from India**" *Haemophilia.* 2013 Jul; 19(4):611-8.

9. APPENDICES

9.1 Evaluation of bleeding in patients with type-3 VWD using this BAT score

1.	**Epistaxis**		
1.1	Have you ever had spontaneous epistaxis?	Yes	No or trivial (skip to 2)
1.2	Have the symptom ever required medical attention ?	Yes	No (resolve spontaneously; skip to 1.6)
1.3	If answer to 1.2 is yes, please specify	Consultation only Cauterization/ Packing Treatment with desmopressin / antifibrinolytics/ iron therapy Treatment with plasma, platelet or factor concentrates Blood (RBC) transfusion	
1.4	How many times in your life did you receive any of the above treatments (# 1.3)?	1 - 2 3 to 5 6 to 10 more than 10	
1.5	At what age did you first have symptoms?	Before 1 year Between 1-5 years of age Between 6-12 years of age Between 13-25 years of age After 25 years of age	
1.6	Approximate number of episodes NOT requiring medical attention	less than 1 per year 1 per year 1-5 every six month 1-3 every month 1 every week	
1.7	Duration of average single episode (min.) NOT requiring medical attention	1 minute or less 1 - 10 minutes more than 10 minutes	

2.	Cutaneous bleeding (Bruising, ecchymoses, purpura, subcutanueos hematomas)		
2.1	Have you ever had any of the above cutaneous bleeding?	Yes	No or trivial skip to 3
2.2	Have the symptom ever required medical attention?	Yes	No skip to 2.6
2.3	If answer to 2.2 is yes, please specify	Consultation only Treatment with desmopressin Treatment with plasma, platelets or factor concentrates Blood (RBC) transfusion	
2.4	How many times in your life did you receive any of the above treatments (# 2.3)?	1 - 2 3 to 5 6 to 10 more than 10	
2.5	At what age did you first have symptoms?	Before 1 year Between 1-5 years of age Between 6-12 years of age Between 13-25 years of age After 25 years of age	
2.6	Approximate number of episodes NOT requiring medical attention	less than 1 per year 1 per year 1-5 every six month 1-3 every month 1 every week	
2.7	Type of bleeding	Petechiae Bruises Hematomas	
2.8	Location	Exposed sites Unexposed sites Both	
2.9	Common size	≤ 1 cm >1 cm Extensive (palm sized or larger)	
2.10	How many bruises >1 cm in exposed areas in the most severe manifestation?	≤ 5 > 5	
2.11	Location of petechiae	Limited to lower limbs Diffuse	

3.	Bleeding from minor wounds (not requiring stitches in the average patient)		
3.1	Have you ever had prolonged bleeding from minor wounds?	Yes	No or trivial skip to 4
3.2	Have the symptom ever required medical attention ?	Yes	No skip to 3.6
3.3	If answer to 3.2 is yes, please specify	Consultation only Surgical hemostasis Treatment with desmopressin Treatment with plasma, platelet or factor concentrates Blood (RBC) transfusion	
3.4	How many times in your life did you received any of the above treatments (# 3.3)?	1 - 2 3 to 5 6 to 10 more than 10	
3.5	At what age did you first have symptoms?	Before 1 year Between 1-5 years of age Between 6-12 years of age Between 13-25 years of age After 25 years of age	
3.6	Approximate number of episodes NOT requiring medical attention	less than 1 per year 1 per year 1-5 every six month 1-3 every month 1 every week	
3.7	Duration of average single episode (min.)	1 to 10 minutes more than 10 minutes	

4. Hematuria

4.1	Have you ever had hematuria ?	Yes	No skip to 5
4.2	If answer to 4.1 is yes, please specify		
	Presence of associated urologic disease	Yes (skip to 5)	No
		Specify:	
		Stones Infection Kidney/ bladder disease	

Please answer the following questions only for SPONTANEOUS symptoms (answer No to 4.1)

4.3	Have the symptom ever required medical attention ?	Yes	No skip to 4.7
4.4	If answer to 4.3 is yes, please specify	Consultation only Surgery Treatment with desmopressin Treatment with plasma, platelet or factor concentrates Blood (RBC) transfusion	
4.5	How many times in your life did you received any of the above treatments (# 4.4)?	1 - 2 3 to 5 6 to 10 more than 10	
4.6	At what age did you first have symptoms?	Before 1 year Between 1-5 years of age Between 6-12 years of age Between 13-25 years of age After 25 years of age	
4.7	Approximate number of episodes NOT requiring medical attention	less than 1 per year 1 per year 1-5 every six month 1-3 every month 1 every week	

5.	**Gastrointestinal bleeding** (Hematemesis, Melena, Hematochezia)

5.1	Have you ever had gastrointestinal bleeding ?	Yes	No skip to 6
5.2	If answer to 5.1 is yes, please specify		
	Type of bleeding	Hematemesis Melena Hematochezia	
	Presence of associated GI disease	Yes Specify: Ulcer Portal hypertension Angiodysplasia	No

Please answer to the following questions only for SPONTANEOUS symptoms

5.3	Have the symptom ever required medical attention ?	Yes	No skip to 5.7
5.4	If answer to 5.3 is yes, please specify	Consultation only Surgical haemostasis Treatment with desmopressin Treatment with plasma, platelet or factor concentrates Blood (RBC) transfusion	
5.5	How many times in your life did you received any of the above treatments (# 5.4)?	1 - 2 3 to 5 6 to 10 more than 10	
5.6	At what age did you first have symptoms?	Before 1 year Between 1-5 years of age Between 6-12 years of age Between 13-25 years of age After 25 years of age	
5.7	Approximate number of episodes NOT requiring medical attention	less than 1 per year 1 per year 1-5 every six month 1-3 every month 1 every week	

6.	**Oral cavity bleeding** (Tooth eruption, spontaneous or after brushing/flossing, gum bleeding, bleeding after bites to lip & tongue)

6.1	Have you ever had oral cavity bleeding ?	Yes	No or trivial skip to 7
6.2	Have the symptom ever required medical attention ?	Yes	No skip to 6.6
6.3	If answer to 6.2 is yes, please specify	Consultation only Surgery (dental packing, suture, cauterization) Treatment with desmopressin / iron therapy Treatment with plasma, platelet or factor concentrates Blood (RBC) transfusion	
6.4	How many times in your life did you received any of the above treatments (# 6.3)?	1 - 2 3 to 5 6 to 10 more than 10	
6.5	At what age did you first have symptoms?	Before 1 year Between 1-5 years of age Between 6-12 years of age Between 13-25 years of age After 25 years of age	
6.6	Approximate number of episodes NOT requiring medical attention	less than 1 per year 1 per year 1-5 every six month 1-3 every month 1 every week	
6.7	Duration of average single episode (min.)	1 to 10 minutes more than 10 minutes	

7. Bleeding after Tooth/ Teeth extraction

7.1	Have you ever had bleeding after tooth (teeth) extraction ?	Yes	No skip to 8
7.2	If answer to 7.1 is yes, please specify		
	Number of extractions		

*Please fill in one of the following forms for **each** tooth extraction*

Age at extraction	**Type of extraction**	**Deciduous**	**Permanent Molar**
Actions taken to prevent bleeding	None		
	Antifibrinolytics		
	Desmopressin		
	Plasma or clotting factor concentrates		
	Platelet infusion		
Bleeding after extraction?	Yes	No	
Actions taken to control bleeding	Resuturing		
	Packing		
	Antifibrinolytics		
	Desmopressin		
	Plasma or clotting factor concentrates		
	Platelet infusion		
	Blood (RBC) transfusion		

8. Bleeding after Surgery or Major Trauma

8.1	Have you ever had bleeding after surgery or major trauma ?	Yes	No, skip to 9
8.2	If answer to 8.1 is yes, please specify		

Number of interventions

*Please fill in one of the following forms for **each** surgery or major trauma episode*

Age at intervention/trauma

Type of surgery

Tonsillectomy/Adenoids
Pharynx/Nose

Major-abdominal
Major-thoracic
Major-gynecology
Other

Actions taken to prevent bleeding

- None
- Antifibrinolytics
- Desmopressin
- Plasma or clotting factor concentrates
- Platelet infusion

Bleeding after intervention? Yes No

Actions taken to control bleeding

- None Resuturing
- Packing
- Antifibrinolytics
- Desmopressin
- Plasma or clotting factor concentrates
- Platelet infusion
- Blood (RBC) transfusion

9. Menorrhagia

9.1	Have you ever had very heavy menstrual bleeding (menorrhagia)?	Yes	No or trivial skip to 10
	If answer to 9.1 is yes, please specify	□ Changing pads/tampons more frequently than every 2 hours □ Bleeding more than 7 days □ Clot and flooding	
		Impairment of daily activities (work, housework, exercise, social activities):	□ Never or rarely □ Most menses
9.2	Have the symptom ever required medical attention ?	Yes	No skip to 9.6
9.3	If answer to 9.2 is yes, please specify	a Consultation only	
		b Pictorial Bleeding Assessment	Score _____
		c Antifibrinolytic therapy	
		d Iron therapy	
		e Hormonal therapy	
		f Combined antifibrinolytics & Hormonal therapy	
		g Hysterectomy / endometrial ablation / D & C	
		h Treatment with desmopressin, plasma or factor concentrates, platelet transfusion	
		i Blood (RBC) transfusion	
		l Hospital admission and emergency treatment	
9.4	How many times in your life did you received any of the above treatments (# 9.3 a-l)?	1 - 2 3 to 5 6 to 10 more than 10	
9.5	At what age did you first have symptoms?	At menarche Between 14-25 years of age After 25 years of age	
9.6	Have you had time off work/school for menorrhagia?	< twice a year > twice a year	
9.7	Duration of menorrhagia	Since menarche > 12 months < 12 months	
9.8	Have you had acute menorrhagia requiring emergency treatment/hospital admission	Yes How many times: ___	No

10.	Post-partum hemorrhage

10.1	Number of successful pregnancies (live births)		
10.2	Have you ever had post-partum haemorrhage?	Yes	No or trivial skip to 11
10.3	Did it occur	In the first 24 hours after delivery (Primary)	
		Between 24 hours and 6 weeks postpartum (Secondary)	
		Both Primary and Secondary	
10.4	How long did vaginal discharge (lochia) last?	< 6 weeks > 6 weeks	
10.5	Did it require changing pads/tampons more frequently than every 2 hours?	Yes	No
10.6	Did this bleeding cause delay of hospital discharge/ readmission to hospital?	Yes	No
10.7	Have the symptom ever required medical treatment?	Yes	No
10.8	If answer to 10.7 is yes, please specify	Consultation only /oxytocin i.v. infusion	
		Additional uterotonic medications	
		Iron therapy	
		Antifibrinolytic therapy	
		Treatment with plasma or factor concentrates, platelet transfusion	
		Blood (RBC) transfusion	
		Any procedure requiring examination under anaesthesia	
		Uterine balloon/package to tamponade the uterus	
		Any procedure requiring critical care or surgical intervention (includes: hysterectomy, internal iliac artery legation, uterine artery embolization, uterine brace sutures)	
10.9	Number of deliveries that required any of the above treatments (# 10.8)?		

11.	Muscle hematomas or hemarthrosis (spontaneous)

11.1	Have you ever had muscle hematomas or hemarthrosis ?	Yes	No or trivial skip to 12
11.2	Have the symptom ever required medical attention ?	Yes	No skip to 11.6
11.3	If answer to 11.2 is yes, please specify	Consultation only Surgical draining Treatment with desmopressin Treatment with plasma, platelet or factor concentrates Blood transfusion	
11.4	How many times in your life did you receive any of the above treatments (# 11.3)?	1 - 2 3 to 5 6 to 10 more than 10	
11.5	At what age did you first have symptoms?	Before 1 year Between 1-5 years of age Between 6-12 years of age Between 13-25 years of age After 25 years of age	
11.6	Approximate number of episodes NOT requiring medical attention	less than 1 per year 1 per year 1-5 every six month 1-3 every month 1 every week	

12	Other bleedings

12.1	Have you ever had one of the following?		
	Excessive umbilical stump bleeding	Yes	No
	Cephalohematoma	Yes	No
	Bleeding at circumcision	Yes	No
	Venipuncture bleeding	Yes	No
	Suction Bleeding	Yes	No
	Ovulation bleeding(in women)	Yes	No
12.2	Have one of these symptoms ever required medical attention?	Yes	No
12.3	If answer to 12.2 is yes, please specify	Consultation only	
		Surgery	
		Treatment with desmopressin	
		Treatment with plasma, platelet or factor concentrates	
		Blood (RBC) transfusion	
12.4	How many times in your life did you receive any of the above treatments (# 12.3) for this symptom?	1 - 2 3 to 5 6 to 10 more than 10	

Table 1. Bleeding score

SYMPTOMS (up to the time of diagnosis)	SCORE				
	0[§]	1[§]	2	3	4
Epistaxis	No/trivial	- > 5/year or - more than 10 minutes	Consultation only*	Packing or cauterization or antifibrinolytic	Blood transfusion or replacement therapy (use of hemostatic blood components and rFVIIa) or desmopressin
Cutaneous	No/trivial	For bruises 5 or more (> 1cm) in exposed areas	Consultation only*	Extensive	Spontaneous hematoma requiring blood transfusion
Bleeding from minor wounds	No/trivial	- > 5/year or - more than 10 minutes	Consultation only*	Surgical hemostasis	Blood transfusion, replacement therapy, or desmopressin
Oral cavity	No/trivial	Present	Consultation only*	Surgical hemostasis or antifibrinolytic	Blood transfusion, replacement therapy or desmopressin
GI bleeding	No/trivial	Present (not associated with ulcer, portal hypertension, hemorrhoids, angiodysplasia)	Consultation only*	Surgical hemostasis, antifibrinolytic	Blood transfusion, replacement therapy or desmopressin

Hematuria	No/trivial	Present (macroscopic)	Consultation only*	Surgical hemostasis, iron therapy	Blood transfusion, replacement therapy or desmopressin
Tooth extraction	No/trivial or none done	Reported in ≤25% of all procedures, no intervention**	Reported in >25% of all procedures, no intervention**	Resuturing or packing	Blood transfusion, replacement therapy or desmopressin
Surgery	No/trivial or none done	Reported in ≤25% of all procedures, no intervention**	Reported in >25% of all procedures, no intervention**	Surgical hemostasis or antifibrinolytic	Blood transfusion, replacement therapy or desmopressin
Menorrhagia	No/trivial	Consultation only* or - Changing pads more frequently than every 2 hours or - Clot and flooding or - PBAC score>100[#]	- Time off work/school > 2/year or - Requiring antifibrinolytics or hormonal or iron therapy	- Requiring combined treatment with antifibrinolytics and hormonal therapy or - Present since menarche and > 12 months	- Acute menorrhagia requiring hospital admission and emergency treatment or - Requiring blood transfusion, Replacement therapy, Desmopressin, or - Requiring dilatation & curretage or endometrial ablation or hysterectomy)
Post-partum hemorrhage	No/trivial or no deliveries	Consultation only* or - Use of syntocin or - Lochia > 6 weeks	- Iron therapy or - Antifibrinolytics	- Requiring blood transfusion, replacement therapy, desmopressin or - Requiring examination under anaesthesia and/or the use of uterin balloon/package to tamponade the uterus	- Any procedure requiring critical care or surgical intervention (e.g. hysterectomy, internal iliac artery legation, uterine artery embolization, uterine brace sutures)
Muscle hematomas	Never	Post trauma, no therapy	Spontaneous, no therapy	Spontaneous or traumatic, requiring desmopressin or replacement therapy	Spontaneous or traumatic, requiring surgical intervention or blood transfusion

Hemarthrosis	Never	Post trauma, no therapy	Spontaneous, no therapy	Spontaneous or traumatic, requiring desmopressin or replacement therapy	Spontaneous or traumatic, requiring surgical intervention or blood transfusion
CNS bleeding	Never	-	-	Subdural, any intervention	Intracerebral, any intervention
Other bleedings^	No/trivial	Present	Consultation only*	Surgical hemostasis, antifibrinolytics	Blood transfusion or replacement therapy or desmopressin

In addition to the guidance offered by the table, it is mandatory to refer to the text for more detailed instructions.

[§] Distinction between 0 and 1 is of critical importance. Score 1 means that the symptom is judged as present in the patient's history by the interviewer but does not qualify for a score 2 or more

* Consultation only: the patient sought medical evaluation and was either referred to a specialist or offered detailed laboratory investigation

** Example: 1 extraction/surgery resulting in bleeding (100%): the score to be assigned is 2; 2 extractions/surgeries, 1 resulting in bleeding (50%): the score to be assigned is 2; 3 extractions/surgeries, 1 resulting in bleeding (33%): the score to be assigned is 2; 4 extractions/surgeries, 1 resulting in bleeding (25%): the score to be assigned is 1

[#] If already available at the time of collection

^ Include: umbilical stump bleeding, cephalohematoma, cheek hematoma caused by sucking during breast/bottle feeding, conjunctival hemorrhage or excessive bleeding following circumcision or venipuncture. Their presence in infancy requires detailed investigation independently from the overall score