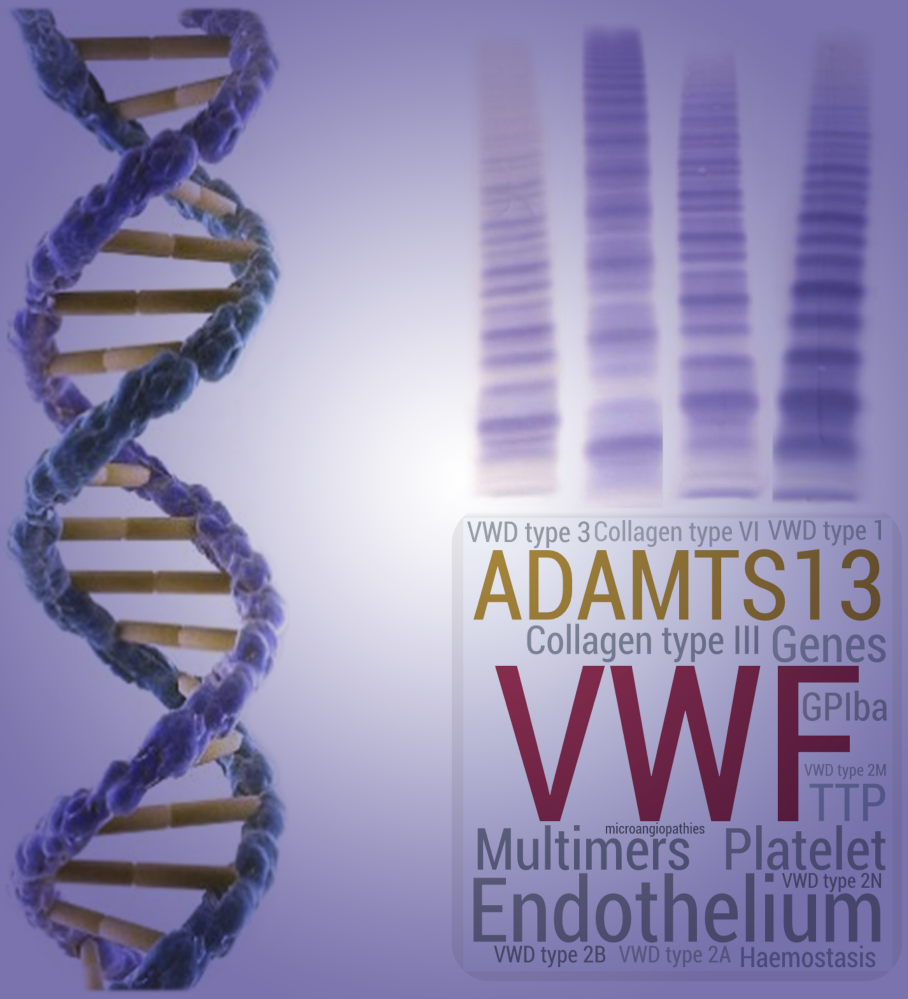

Teresa de Jesus Semedo Fidalgo

VON WILLEBRAND FACTOR AND ADAMTS13 DUALITY IN HEMORRHAGIC AND THROMBOTIC DISEASE

Tese de doutoramento em Biociências, ramo de especialização em Biologia Celular e Molecular, orientada pela Senhora Doutora Maria Letícia Ribeiro, pelo Senhor Professor Doutor Armando Cristóvão, e apresentada ao Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Dezembro de 2016

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Von Willebrand factor and ADAMTS13
Duality in hemorrhagic and thrombotic disease

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"Se quiseres chegar rápido vai sózinho,
se quiseres chegar longe vai acompanhado"

Provérbio africano

Publications

The following original articles have been published in peer-scientific journals, within the scope of the present PhD thesis:

Fidalgo T; Salvado R; Corrales I; Silva Pinto C; Borràs N; Oliveira A; Martinho P; Ferreira G; Almeida H; Oliveira C; Marques D; Gonçalves E; Diniz MJ; Antunes M; Tavares A; Caetano G; Kjöllerström P; Maia R; Sevivas T; Vidal F & Ribeiro ML. (2016) Genotype–phenotype correlation in a cohort of Portuguese patients comprising the entire spectrum of VWD types: impact of NGS. *Thromb Haemost,* **116**, 17-31.

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Fidalgo T; Ferreira G; Oliveira A.C.; Silva Pinto C; Martinho P, Salvado R; Duarte M; Mendes MJ; Ribeiro ML. Acquired von Willebrand Syndrome (AVWS) in hematologic malignancies – how the clinical-laboratory correlation improves a challenging diagnosis. *(Under review, Dec 2016)*

Fidalgo T; Martinho P; Silva Pinto C; Salvado R; Borràs N; Corrales I; Coucelo M; Oliveira A; Maia T; Mendes MJ; Barreto RO; Vidal F & Ribeiro ML. Combined study of ADAMTS13 activity and genetic analysis of complement genes in the diagnosis of thrombotic microangiopathies – the add-value of Next-Generation Sequencing. *(Under review, Dec 2016)*

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"Nenhum dever é mais importante do que a gratidão."
Cícero

O estudo do Factor de von Willebrand (FVW) representa um desafio permanente nos estudos de hemostase – pela sua complexidade, multiplicidade de interações e, também, pela dificuldade de escolher a abordagem laboratorial mais precisa para detetar défices e anomalias. Este desafio acompanhou a minha atividade profissional, fui implementando técnicas de estudos funcionais (agregometria, electroforese de Laurel, SDS e ELISA) e estudos moleculares (Sequenciação Sanger e recentemente NGS). Foram anos de trabalho que me possibilitaram a aquisição de novos conhecimentos, o desenvolvimento de um sentido crítico que me conduziram a uma capacidade de diagnóstico mais alargada e que me possibilitou desenvolver esta tese. Contudo, foi um percurso de colaboração porque o estudo destas patologias tão heterogéneas e complexas ilustra a necessidade de uma equipa multidisciplinar. A interpretação dos resultados foi um exercício constante de correlação clinico-laboratorial e de aprendizagem conjunta.

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TABLE OF CONTENTS

ABBREVIATIONS

ABBREVIATIONS

A

aa	Amino-acid
AAs	African Americans
ADAMTS13	A disintegrin-like and metalloprotease domain with thrombospondin type-1 motif, number 13
ADAMTS13	ADAMTS13 gene
aHUS	Atypical haemolytic uraemic syndrome
aPTT	Activated partial thromboplastine time
ASS	Acceptor splice site
AU	Arbitrary units
AVWS	Acquired von Willebrand syndrome

B

BAT	Bleeding assessment tools
Bp	Base pair
BSs	Bleeding scores

C

c.	Coding DNA reference sequence
C3	Complement component 3 gene
CD46	CD46 gene
CD46 (MCP)	Cluster of differentiation 46
CFB	Complement factor B gene
CFH	Complement factor H gene
CFHR	Complement factor H related gene
CFI	Complement factor I gene
CK	Cysteine knot
C-terminal	Carboxy-terminal

D

Da	Dalton
db	Database
dbSNP	Database single nuclear polymorphism
DDAVP	1-desamino-8-D- arginine-vasopressin
DGKE	Diacylglycerol kinase epsilon gene
dl	Deciliter
DNA	Deoxynucleic acid

dsDNA	Double strand DNA
DSS	Donor splice site

E

ECM	Extracellular matrix
EDTA	Ethylenediamintetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
EVS	Exome Variant Server
ExAC	Exome Aggregation Consortium

F

F8	Factor VIII gene
FH	Factor H database
FVIII	Factor VIII
FVIII:C	Factor VIII activity

G

g.	Genomic reference sequence
gDNA	Genomic DNA
GP1BA	Glycoprotein Ib platel*et al*pha subunit gene
GPIb	glycoprotein Ib
GPIIb–IIIa	Glycoprotein IIb–IIIa

H

HGMD	Human Gene Mutation Database
HMW	High-molecular weight
HMWM	High-molecular weight multimers
Hr	Hour
HSF	Human Splicing Finder

I

INH	Inhibitor
ISTH SSC	International Society on Thrombosis and Haemostasis Scientific and Standardisation Committee
IU	International unit

K

kb	Kilo base pair
kDa	Kilo Dalton

M

MaxEntScan	Maximum Entropy Scan (of Short Sequence Motifs with Applications to Splicing Signals)
MCP	Membrane Cofactor Protein
µg	Microgram
mg	Miligram
min	Minute
ml	Milliliter
MLPA	Multiplex ligation-dependent probe amplification
mRNA	Messenger RNA
MutationAssessor	Mutation Assessor (functional impact of amino-acid substitutions in proteins)
MutationTaster	Mutation Taster (evaluate DNA sequence variants for their disease-causing potential)
MutPred	Mutation Pediction (classify an amino acid substitution as disease- associated or neutral)

N

NetGene	Network Gene (service producing neural network predictions of splice sites in human)
ng	Nanogram
NGS	Next-generation sequencing
NNSPLICE	Neural network splice site
nt	Nucleotide
N-terminal	Amino-terminal

P

p.	Protein reference sequence
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction Protein
PEX	Plasma exchange
PFA 100	Platelet function analyzer 100
PI	Plasma infusion
PolyPhen	Polymorphism Phenotyping
PROVEAN	Protein Variation Effect Analyzer
PT-VWD	Platelet-type VWD

R

RER	Rough endoplasmic reticulum

RGD	Arg-Gly-Asp
RIPA	Ristocetin induced platelet aggregation
RNA	Ribonucleic acid
ROIs	Regions of interest

S

SCR	Short consensus repeat
SDS	Sodium dodecyl sulfate
SIFT	Sorting Intolerant From Tolerant
SLE	Systemic lupus erythematosus
SpliceView	Splice View (splice prediction by using consensus sequences)

T

TGN	Trans-Golgi network
THBD	Thrombomodulin gene
TMA	Thrombotic microangiopathy
TSP-1	Thrombospondin-1
TTP	Thrombotic thrombocytopenic purpura

U

UL-VWF	Ultra large von Willebrand factor

V

VWD	Von Willebrand disease
VWF	Von Willebrand factor
VWF	VWF gene
VWF:Ag	Von Willebrand factor antigen
VWF:CB	Von Willebrand factor: collagen binding
VWF:C^{III}B	Von Willebrand factor: collagen binding type III
VWF:C^{VI}B	Von Willebrand factor: collagen binding type VI
VWF:FVIIIB	Von Willebrand factor: FVIII binding
VWF:GPIbB	Von Willebrand factor: GPIb binding
VWF:RCo	Von Willebrand factor: ristocetin cofactor
VWFP1	VWF pseudogene
VWFpp	Von Willebrand factor propeptide

W

WPB	Weibel-Palade body
WT	Wild type

ABSTRACT & RESUMO

ABSTRACT

Haemostasis is a normal defence mechanism that requires the combined activity of vascular, platelet and plasma factors. Under physiologic conditions, a haemostatic balance is achieved through the effects of natural procoagulant and anticoagulant factors which, in equilibrium with each other, provide haemostasis at the sites of vascular injury. Abnormalities of these haemostasis factors can lead to excessive bleeding or thrombosis.

One of the key players in both processes is the von Willebrand factor (VWF), an adhesive glycoprotein of large dimensions with crucial functions in haemostasis derived from its ability to organise in multimers. The high-molecular-weight multimers (HMWM) of VWF are essential for primary haemostasis, mediating a molecular endothelium–platelet bridge for binding collagen and the platelet receptors glycoprotein (GP), GPIb and GPIIb/IIIa. In addition, VWF binds and stabilises factor VIII (FVIII) in the circulation, protecting it from rapid proteolytic degradation while delivering it to sites of vascular damage. Once secreted into the blood, VWF multimers are subject to competing processes of clearance and of proteolysis by ADAMTS13 (A Disintegrin And Metalloproteinase with a ThromboSpondin type 1 motif, member 13). The unusually large multimers of VWF are, under normal conditions, cleaved by ADAMTS13 to smaller, less adhesive multimers. As a result of this physiological process, VWF insufficiencies may cause haemorrhage by reducing platelet function, or by reducing the FVIII concentration. Abnormalities in VWF secretion, intravascular clearance of VWF, the assembly of VWF multimers, or exaggerated proteolytic degradation by ADAMTS13 can cause diverse forms of von Willebrand disease (VWD). Conversely, defects in proteolysis of VWF by ADAMTS13 can cause thrombotic thrombocytopenic purpura (TTP), a disease caused by the clumping of platelets by ultra-large VWF and defined clinically by microangiopathic hemolytic anaemia and thrombocytopenia.

There are two main forms of thrombotic microangiopathies (TMAs) that have overlapping clinical phenotypes: TTP and atypical haemolytic uraemic syndrome (aHUS). Conceptually, TTP has been distinguished from aHUS by more common neurological manifestations, whereas in aHUS, renal involvement is more pronounced. However, this rule is not always reliable; some aHUS patients have neurological complications and some TTP patients have renal failure. Over the last decade, noteworthy progress has been made to improve the clinical and laboratory approaches to predict the expected signs and symptoms, patient outcome and genotype-phenotype correlations. Despite this, the differential diagnosis of TTP/aHUS remains challenging.

While TTP is characterised by a severe deficiency of ADAMTS13, aHUS is characterised by hyperactivation of the alternative complement pathway resulting from either a loss-of-function mutation in a regulatory gene, *CFH, CFI, CD46 (MCP)* or *THBD*, or a gain-of-function mutation in an effector gene, *CFB or C3*.

The molecular analysis of *VWF, ADAMTS13* and complement genes are very useful in the diagnosis of VWD and differential diagnosis of TTP/aHUS. To better understand the clinical variability, which is found even within families, it is necessary to characterise the mutational profile. Nevertheless, up to now, the molecular analysis of *VWF* and genes implicated in TMAs was not affordable by diagnostic laboratories due to the high costs involved in the study of multiple genes by conventional Sanger DNA sequencing. The advent of next-generation sequencing (NGS) is changing this paradigm since NGS allows for simultaneous sequencing of large gene panels and generates competitive results at a lower cost and in a shorter period of time.

With this in mind, we conducted a study in a haemorragic disease (VWD) and a thrombotic disease (TTP/aHUS) with two main objectives: first, to expand our understanding of the molecular basis of different types of VWD, TTP and aHUS and establish phenotype-genotype correlations; second, to develop a diagnosis workflow based on VWF/ADAMTS13 activity and screening of mutations using a custom NGS gene panel. This approach allowed for the creation of a new algorithm that uses an

efficient and cheaper methodology to establish the diagnosis, prognosis and more accurate treatment for these diseases.

First, we conducted a study to determine the correlations between genotype and phenotype in 92 Portuguese individuals from 60 unrelated families with VWD; therefore, we directly sequenced VWF. We compared the classical Sanger sequencing approach and NGS to assess the value-added effect on the analysis of the mutation distribution in different types of VWD. Sixty-two different VWF mutations were identified, 27 of which had not been previously described. NGS detected 26 additional mutations, contributing to a broad overview of the mutant alleles present in each VWD type. Twenty-nine probands (48.3%) had two or more mutations; in addition, mutations with pleiotropic effects were detected, and NGS appropriately classified seven of them. Furthermore, the differential diagnosis between VWD 2B and platelet type VWD (n = 1), Bernard-Soulier syndrome and VWD 2B (n = 1), and mild haemophilia A and VWD 2N (n = 2) was possible.

Next, we evaluated two VWF dysfunctions that could be underdiagnosed: VWF collagen-(types III and VI) binding defects and acquired von Willebrand syndrome (AVWS) in hematologic malignancies.

The functional and molecular studies focused on VWF collagen-binding defects reported VWF sequence variations that may not interfere with VWF functional screening assays showing normal values, and can only be detected using type VI collagen. This analysis in our cohort of nine patients, albeit small, indicated that a laboratory approach based on the correlation of type III and type VI collagen-binding assays and molecular studies is indispensable for a more accurate diagnosis of type 2M VWD. Moreover, the detection of the specific type VI collagen-binding defect may contribute to the correct diagnosis of patients with mild bleeding disorders that are often classified as having an undefined cause.

AVWS is a relatively uncommon and under-diagnosed acquired bleeding disorder, and it occurs in individuals with no personal or familial history of bleeding diathesis. AVWS

is usually associated with an underlying disorder, namely lymphoproliferative disorders, cardiovascular diseases, myeloproliferative neoplasms, other cancers and autoimmune diseases.

The diagnosis is challenging because it greatly depends on clinical suspicion. On the other hand, the tests used to assess AVWS are the same as those for VWD, and the differential diagnosis between both can sometimes be difficult. We studied six patients with AVWS and different underlying diseases: Waldenstrom's Macroglobulinemia (WM), Marginal Zone Lymphoma (MZL), Chronic Myeloid Leukaemia (CML) and Essential Thrombocythemia (ET). The clinical-laboratory correlation in the six cases led to the diagnosis of AVWS. The detection of low VWF levels in two patients came before the diagnosis of the underlying lymphoproliferative disease. In another patient, the detection of the *VWF* variant could have led to a misdiagnosis, in this case, a congenital VWD. However, the discordant genotype-phenotype correlation and late haemorrhagic manifestation led to the suspicion of simultaneous AVWS. Including an evaluation of VWF analysis in late haemorrhagic episodes and/or in AVWS-associated disorders enhanced the efficacy of AVWS diagnosis, leading to a more tailored management approach in each patient.

Finally, we analysed genotype-phenotype correlations in 45 patients (11 TTP and 34 aHUS) based on the impact of the predicted pathogenicity of each variant found and the co-inherited known genetic risk factors for TMAs.

In total, 33 different rare variants, eight of which novel (in *ADAMTS13, CFH* and *CD46*), were identified across seven genes. The eleven TTP patients were homozygous (n = 6), compound heterozygous (n = 2) and heterozygous (n = 3) for 10 *ADAMTS13* variants (six pathogenic mutations). Among the 34 aHUS patients, 17 were heterozygous for 23 variants in the different complement genes with distinct consequences, ranging from single pathogenic mutations associated with complete disease penetrance to benign variants that cause aHUS only when combined with other variants and/or *CFH* and *CD46* risk haplotypes or *CFHR1-3* deletion. Our study provides evidence of the usefulness of the NGS panel as an excellent technology that enables more rapid

diagnosis of TMAs, and is a valuable asset in clinical practice to discriminate between TTP and aHUS.

The custom NGS panel has made it easier to study *VWF*, *ADAMTS13* and the complement genes. Moreover, this technology has changed the paradigm of routine molecular studies: in the face of multiple genetic changes found in every patient, the critical challenge became discriminating disease-associated variants from the broader background of variants present in all patients' genomes. This analysis led us to a well-established bioinformatics pipeline according to NGS guidelines, and evidences that a clinical-laboratory approach for each patient's genotypic data must be evaluated with consideration of their specific and differential clinical manifestations.

In conclusion, the study of these 144 patients contributes to a better understanding of the molecular genetics of *VWF*, *ADAMTS13* and complement gene-related phenotypes. Moreover, it provides evidence of the usefulness of the NGS panel as an excellent and advantageous technology that enables more rapid and cost-effective diagnosis. These findings show that this is a valuable asset in clinical practice given that a correct diagnosis is essential for determining the most effective treatment for each patient with these complex diseases.

Keywords: Gene mutations, von Willebrand disease, von Willebrand factor, Thrombotic thrombocytopenic purpura; Atypical haemolytic uraemic syndrome; ADAMTS13; Complement; molecular biology methods, Next-generation sequencing

RESUMO

A hemostase é um mecanismo de defesa que requer a atividade combinada do endotélio, plaquetas e fatores plasmáticos. Sob condições fisiológicas, o equilíbrio hemostático é conseguido através da atividade dos fatores procoagulantes e anticoagulantes naturais que, em equilíbrio entre si, proporcionam a formação de um tampão hemostático nos locais de lesão vascular. Alterações destes fatores hemostáticos podem dar origem a hemorragia ou trombose.

Um dos principais intervenientes em ambos os processos é o factor von Willebrand (FVW), uma glicoproteína adesiva de grandes dimensões com funções cruciais na hemostase devido à sua capacidade de se organizar em multímeros. Os multímeros de alto peso molecular (MAPM) do FVW são essenciais para a hemostase primária, ao mediar uma ponte molecular de endotélio-plaqueta para ligação ao colagénio e aos recetores plaquetares glicoproteína (GP) Ib e GPIIb/IIIa. Além disso, o FVW liga e estabiliza o factor VIII (FVIII) na circulação, protegendo-o da rápida degradação proteolítica, ao mesmo tempo que o distribui para os locais de lesão vascular. Uma vez na corrente sanguínea, os multímeros do FVW são submetidos a processos de *clearance* e de proteólise pela enzima ADAMTS13 (A Disintegrin And Metalloproteinase with a ThromboSpondin type 1 motif, member 13). Os multímeros do FVW de grandes dimensões são, em condições normais, clivados pela ADAMTS13 em multímeros mais pequenos e menos adesivos.

Como resultado deste processo fisiológico, deficiências no FVW podem causar hemorragia por redução da função plaquetar ou por redução da concentração do FVIII. Anomalias na secreção do FVW, *clearance* intravascular, formação dos multímeros, ou a degradação proteolítica excessiva pela ADAMTS13 podem dar origem a diversas formas da doença de von Willebrand (DVW). Por outro lado, as deficiências na proteólise do FVW pela ADAMTS13 podem originar púrpura trombocitopénica trombótica (PTT), uma doença causada por aglomerados de plaquetas e MAPM do FVW, e que se apresenta clinicamente com trombocitopenia e anemia hemolítica microangiopática.

Existem duas formas principais de microangiopatias trombóticas (MATs) que têm fenótipos clínicos sobrepostos: PTT e síndrome hemolítico urémico atípico (SHUa).

Conceitualmente, a PTT tem sido diferenciada do SHUa por ter manifestações neurológicas mais frequentes, enquanto no SHUa o envolvimento renal é mais pronunciado. No entanto, esta regra nem sempre é fiável: alguns doentes com SHUa, apresentam complicações neurológicas e alguns doentes com PTT apresentam insuficiência renal. Ao longo da última década foram feitos progressos notáveis para melhorar a abordagem clinico-laboratorial, para identificar sinais e sintomas, estabelecer o prognóstico dos doentes e analisar a correlação genótipo - fenótipo. Apesar disso, o diagnóstico diferencial PTT/SHUa ainda é um desafio.

Embora a PTT seja caracterizada por uma deficiência grave de ADAMTS13, o SHUa resulta da hiperactivação da via alternativa do complemento devida a uma mutação com perda-de-função num dos genes reguladores*, CFH*, *CFI*, *CD46 (MCP)* ou *THBD*, ou de uma mutação com ganho-de-função num gene efetor, *CFB* ou *C3*.

A análise molecular do *VWF*, *ADAMTS13* e genes do complemento é muito útil no diagnóstico da DVW e diagnóstico diferencial de PTT/SHUa. Para melhor compreender a variabilidade clínica, inclusivé dentro da mesma família, é necessário caracterizar o perfil mutacional. Contudo, até agora, a análise molecular do *VWF* e dos genes implicados nas MATs não estava acessível aos laboratórios de diagnóstico devido aos elevados custos envolvidos no estudo de múltiplos genes pelo método convencional de sequenciação do DNA (Método de Sanger). O aparecimento da sequenciação de nova geração (NGS-next generation sequencing) está a alterar este paradigma, uma vez que a NGS permite a sequenciação simultânea de grandes painéis de genes e gera resultados competitivos a um custo mais baixo e num curto período de tempo.

Baseados nestes dados, realizámos um estudo numa doença hemorrágica (DVW) e numa doença trombótica (PTT/SHUa) com dois objetivos principais: 1) alargar o nosso conhecimento sobre a base molecular dos diferentes tipos de DVW, PTT e SHUa e estabelecer correlações genótipo – fenótipo; 2) desenvolver uma estratégia de

diagnóstico com base na atividade de FVW/ADAMTS13 e desenhar um painel de genes para efetuar a pesquisa de mutações por NGS.

Esta abordagem permitiu-nos criar um novo algoritmo de estudo, que utiliza uma metodologia eficiente e mais barata, e que permite estabelecer o diagnóstico e o prognóstico e contribui para uma melhor definição da estratégia terapêutica destas patologias.

Em primeiro lugar, realizámos um estudo para determinar as correlações entre o genótipo e o fenótipo em 92 indivíduos Portugueses, de 60 famílias não relacionadas com DVW; assim, sequenciámos diretamente o *VWF*. Comparámos a abordagem clássica de sequenciação de Sanger e a metodologia NGS para avaliar a mais-valia na análise de mutações nos vários tipos de DVW. Foram identificadas 62 mutações diferentes no *VWF*, 27 das quais não tinham sido previamente descritas.

A NGS detetou 26 mutações adicionais, contribuindo para uma perspetiva alargada dos alelos mutados presentes em cada tipo de DVW. Vinte e nove propósitos (48,3%) apresentavam uma ou mais mutações; além disso, foram detetadas mutações com efeitos pleiotrópicos, tendo a NGS classificado adequadamente sete delas. Foi ainda possível o diagnóstico diferencial entre DVW 2B e DVW tipo plaquetar (n=1), síndrome de Bernard-Soulier e DVW 2B (n=1) e hemofilia A ligeira e DVW 2N (n=2).

De seguida, avaliamos duas disfunções do FVW que poderiam estar a ser subdiagnosticadas: defeitos de ligação FVW-colagénio (tipos III e VI) e síndrome de Von Willebrand adquirida (SVWA) em doenças hemato-oncológicas.

Os estudos funcionais e moleculares incidiram nas alterações de ligação ao colagénio associados a variações da sequência do *VWF* que podem não interferir nos testes funcionais de *screening*, que apresentam valores normais, e que só podem ser detetados utilizando colagénio tipo VI. Esta análise no nosso grupo de nove doentes, apesar de pequeno, indicou que uma abordagem laboratorial baseada na correlação entre os ensaios de ligação ao colagénio tipo III e tipo VI e os estudos moleculares é indispensável para um diagnóstico mais preciso da DVW tipo 2M. Além disso, a

deteção dos défices específicos da ligação ao colagénio tipo VI pode contribuir para o diagnóstico de doentes com perturbações hemorrágicas ligeiras que muitas vezes são classificados com tendência hemorrágica de causa desconhecida.

SVWA é uma patologia hemorrágica adquirida, subdiagnosticada e relativamente pouco frequente, que ocorre em indivíduos sem história pessoal ou familiar de diátese hemorrágica. SVWA está geralmente associado com uma patologia subjacente, nomeadamente doenças linfoproliferativas, doenças cardiovasculares, neoplasias mieloproliferativas, outros cancros e doenças autoimunes.

O diagnóstico é desafiador porque depende muito da suspeita clínica. Por outro lado, os testes usados para diagnosticar o SVWA são os mesmos utilizados para o diagnóstico da DVW, e a distinção entre as duas patologias pode ser difícil. Avaliámos seis doentes com SVWA e diversas patologias subjacentes: Macroglobulinemia de Waldenstrom (MW), Linfoma de Zona Marginal (LZM), Leucemia Mielóide Crónica (LMC) e Trombocitémia Essencial (TE). A correlação clínica – laboratorial nos seis casos permitiu o diagnóstico de SVWA.

A deteção de níveis baixos de FVW em dois doentes ocorreu antes do diagnóstico da doença linfoproliferativa subjacente. Noutro doente, a deteção da variante de *VWF* poderia ter levado a um diagnóstico incorrecto de DVW congénita. No entanto, a correlação genótipo - fenótipo discordante e a manifestação hemorrágica tardia, levou à suspeita da presença concomitante de SVWA. Incluir uma avaliação da análise do *VWF* em episódios hemorrágicos tardios e/ou em doenças associadas com SVWA aumentou a eficácia do diagnóstico de SVWA, e permitiu uma estratégia terapêutica mais individualizada.

Finalmente, analisámos as correlações genótipo-fenótipo em 45 pacientes (11 PTT e 34 SHUa) com base no impacto da patogenicidade prevista para cada variante encontrada e dos fatores hereditários de risco genético para MATs.

No total, foram identificadas 33 variantes raras diferentes em sete genes, oito das quais descritas pela primeira vez (em *ADAMTS13, CFH* e *CD46*). Os onze doentes com

PTT eram homozigóticos (n=6), heterozigóticos compostos (n=2) e heterozigóticos (n=3) para 10 variantes *ADAMTS13* (seis mutações patogénicas). Entre os 34 doentes com SHUa, 17 eram heterozigóticos para 23 variantes nos diferentes genes do complemento com consequências distintas, desde mutações patogénicas únicas associadas com penetração completa da doença, a variantes benignas que apenas causam SHUa quando associadas a outras variantes e/ou haplótipos de risco *CFH* e *CD46* ou delecção *CFHR1-3*.

O nosso estudo evidencia a utilidade do painel NGS como uma excelente tecnologia que permite um diagnóstico mais rápido de MATs, e uma mais-valia na prática clínica para discriminar entre PTT e SHUa.

O painel NGS personalizado tornou mais fácil o estudo do *VWF*, *ADAMTS13* e dos genes do complemento. Além disso, esta tecnologia mudou o paradigma dos estudos moleculares de rotina: face às múltiplas alterações genéticas encontradas em cada doente, o desafio principal foi discriminar as variantes associadas com a doença de entre as variantes presentes no genoma de todos os doentes.

Esta análise levou-nos a um algoritmo bioinformático bem estabelecido, de acordo com as *guidelines* NGS, e à demonstração de que os dados do genótipo de cada doente devem ser avaliados numa abordagem clínico – laboratorial, tendo em conta as suas manifestações clínicas específicas.

Em conclusão, o estudo destes 144 doentes contribui para um melhor entendimento da genética molecular de *VWF*, *ADAMTS13* e dos genes do complemento, assim como dos fenótipos associados. Além disso, evidencia a utilidade do painel NGS como uma tecnologia excelente e vantajosa que permite um diagnóstico mais rápido e mais económico.

Estes resultados mostram ainda que esta estratégia é uma mais-valia na prática clínica dado que um diagnóstico correto é determinante na escolha da melhor estratégia terapêutica para cada um destes doentes com patologias tão complexas.

Palavras-chave: Mutações, doença de von Willebrand, fator de von Willebrand, Púrpura trombótica trombocitopénica; Síndrome hemolítico urémico atípico; ADAMTS13; Complemento; métodos de biologia molecular, Sequenciação de nova geração

CHAPTER

1

GENERAL INTRODUCTION

CHAPTER 1. GENERAL INTRODUCTION

1.1. Discovery of von Willebrand factor

In 1926, Dr. Erik von Willebrand from Helsinki described a severe bleeding disorder in several members of a family living in Föglö, an island in the Åland Archipelago situated between Finland and Sweden. The proband was a 5-year-old girl who later bled to death during her fourth menstrual period. She had a normal coagulation time, but the bleeding time was prolonged, despite a normal platelet count. Von Willebrand thought that the underlying cause of this bleeding was severe platelet dysfunction combined with a vessel wall abnormality which, in contrast to haemophilia, affected both sexes, and called it hereditary pseudo-haemophilia (von Willebrand, 1926). In 1957, Nilsson *et al.* concluded that the impaired clot formation in these patients was due to the lack of a plasma factor, the von Willebrand factor (VWF), which was present in both normal and haemophilia A plasma (Nilsson *et al*, 1957). In 1971, Zimmermann developed an immunological test to differentiate VWF and FVIII that revealed VWF as a separate "FVIII carrier" (Zimmerman *et al*, 1971). This test made it possible to diagnose patients with von Willebrand disease (VWD) and to identify those with haemophilia A in whom isolated FVIII deficiency existed. In 1985, four laboratories simultaneously cloned the *VWF* cDNA (Sadler *et al*, 1985; Ginsburg *et al*, 1985; Lynch *et al*, 1985; Verweij *et al*, 1985) which initiated advances in the understanding of VWF assembly, function and the molecular basis of VWD.

1.2. The role of von Willebrand factor in haemostasis

Haemostasis is a normal defence mechanism that requires the combined activity of vascular, platelet, and plasma factors. Under physiologic conditions, a haemostatic balance is reached by the effects of natural procoagulant and anticoagulant factors which, in equilibrium with each other, provide haemostasis at the sites of vascular injury. Abnormalities of these haemostasis factors can lead to excessive bleeding or thrombosis (Franchini & Mannucci, 2008; Denis & Lenting, 2012).

One of the key players in both processes is the VWF, an adhesive glycoprotein of large dimensions with crucial functions in haemostasis derived from its ability to organise in multimers (Sadler, 1998; Sadler *et al*, 2006). The VWF multimers are composed of identical subunits of approximately 250 kDa (monomers) and range in size from dimers of approximately 500 kDa to species of more than 20,000 kDa. The high-molecular-weight multimers (HMWM) of the VWF are essential for primary haemostasis, mediating a molecular endothelium-platelet bridge for binding collagen and the platelet receptors, glycoprotein (GP), GPIb and GPIIb/IIIa. In addition, the VWF binds and stabilises factor VIII (FVIII) in the circulation, protecting it from rapid proteolytic degradation while delivering it to sites of vascular damage. Once secreted into the blood, VWF multimers are subject to competing processes of clearance and of proteolysis by ADAMTS13 (A Disintegrin And Metalloproteinase with a ThromboSpondin type 1 motif, member 13). The unusually large multimers of VWF are, under normal conditions, cleaved by ADAMTS13 to smaller, less adhesive multimers (Schneppenheim & Budde, 2011). As a result of this physiological process, VWF insufficiencies may cause haemorrhage by reducing platelet function or by reducing the FVIII concentration. In this way, the VWF is essential for both primary (platelet-mediated) and secondary (coagulation factor-mediated) haemostasis (Batlle J *et al*, 2011).

1.3. VWF

1.3.1. VWF gene

The VWF gene (*VWF*) mapping to 12 p13.3 is 178 kb in length of genomic DNA and comprises 52 exons that transcribe into an 8.8 kb mRNA. The sizes of the exons vary from 41 to 342 bp, and 1.4 kb for exon 28 that encodes several sites for essential ligand-binding and cleavage functions and is the most mutated region of *VWF*. A non-coding *VWF* pseudogene mapping to 22 q11.1 is 97.5% similar in sequence to the coding gene, corresponding to exons 23 to 34 of *VWF* (Mancuso *et al*, 1989). This homology leads to multiple recombination events causing the gene conversion between the true gene (*VWF*) and the *VWF* pseudogene that contributes to the

mutation spectrum in VWD. The presence of the homologous partial pseudogene, the higher prevalence of polymorphic variants, along with its large size, makes genetic analysis of *VWF* challenging (Gupta *et al*, 2005; Goodeve, 2010).

1.3.2. Protein structure and domain organisation

The VWF is synthesised exclusively by endothelial cells present in different tissues and by the platelet precursor, megakaryocytes. The VWF found in the subendothelial matrix in plasma are primarily produced in endothelial cells, whereas megakaryocytic production is responsible for the presence of VWF in the α-granules of platelets. Both endothelial cells and platelets store ultra-large VWF multimers (> 10 000 kDa) in Weibel-Palade bodies (WPBs) and α-granules, respectively, for regulated release (Montgomery & Haberichter, 2011; Luo *et al*, 2012).

The primary translation product of the *VWF* mRNA is a single-chain pre-pro-polypeptide (pre-pro-VWF) monomer of 2813 amino acids (aa) composed of a signal peptide (1-22 aa), a propeptide (23-763 aa) and the mature VWF (764-2813 aa) domain organisation that contains a variety of specific ligand-binding sites (Figure 1. A–D). This pro-protein is highly repetitive, and contains several structural domains, which were initially designated: D1–D2–D'–D3–A1–A2–A3–D4–B1–B2–B3–C1–C2–CK.

Recently, the organisation of domains has been redefined in accordance with new structural data on VWF and consists of D1–D2–D'-D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK) (Figure 1. B–D) (Zhou *et al*, 2012).

The D1 and D2 domains comprise the propeptide (VWFpp) which are cleaved during proteolytic processing by furin to generate the mature VWF. The remaining domains (D'–CK) in the mature VWF carry out specific functions. These domains may be characterised as structural or functional, depending on whether their primary role is related to the VWF structure or its interaction with other factors (Figure 1. C, D).

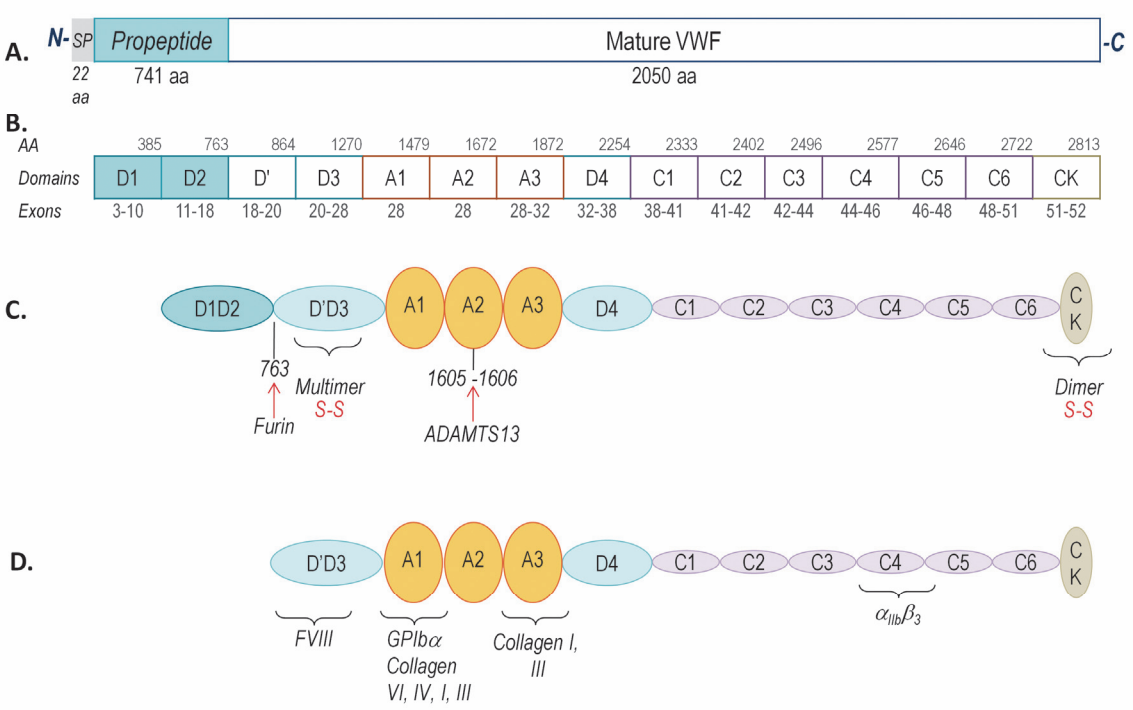

Figure 1. Schematic representation of the von Willebrand factor (VWF) monomer structure. (A) VWF contains a signal peptide, propeptide, and mature VWF portion. (B) The scheme represents the redefined domain structure of VWF showing the 52 exons of *VWF* and the corresponding encoding domains (adapted from Zhou *et al*., 2012). (C) VWF domain with cleavage sites for furin and ADAMTS13; dimerisation and multimerisation regions with S-S sites of intermolecular disulphide bond formation. (D) VWF domains with specific binding sites.

Structural domains are involved in the post-translational processing of VWF and include the cysteine knot (CK) domain that is required for dimerisation of VWF monomers and the D1, D2 and D3 domains for proper multimerisation of VWF dimers. These 'D' domains act as a protein disulphide isomerase to form disulphide bonds between the N-termini of VWF dimers. Functional domains include: i) FVIII binding site by D'-D3 domains; ii) binding site within A1 domain for GPIbα on platelets; iii) collagen type IV and type VI binding sites via the A1 domain, and collagen type I and type III binding sites via the A1 and A3 domains; iv) VWF-cleaving protease site (Tyr1605-Met1606) for ADAMTS13 within the A2 domain; and v) an Arg-Gly-Asp (RGD) sequence for binding to α2β3 on platelets within the C4 domain (Schneppenheim & Budde, 2011; Schneppenheim, 2011; Ruggeri & Ware, 1993).

1.3.3. VWF biosynthesis

VWF is subjected to extensive intracellular processing. Upon translocation to the endoplasmic reticulum (ER), the signal peptide is removed, the protein is folded, and the disulphide bonds are formed involving the majority of the VWF's 234 cysteine residues (Haberichter, 2015; Lenting & Christophe, 2015). This process of intraprotein cysteine bonding occurs to facilitate folding of the individual domains. Subsequent "tail-to-tail" interprotein disulphide bridge formation involving the C-terminal CK domains allows for the formation of prodimers. Furthermore, the first building blocks for N-linked glycosylation sites are coupled to the growing polypeptide chain (Figure 2.1).

Upon arrival in the Golgi apparatus, the presence of a slightly acidic pH and relatively high Ca^{2+} concentration promote the organisation of the prodimers into a dimeric bouquet structure i.e. dimers of domains C1–CK assemble into a long stem composed of six globular domains, and the A2, A3 and D4 domains adopt a flower-like arrangement (Valentijn & Eikenboom, 2013; Springer, 2013). Moreover, this environment favours multimerisation via disulphide bridging that couples adjacent N-terminal D3 domains, a process that is catalysed by the VWFpp that acts as chaperone in a low-pH-dependent oxidoreductase reaction (Sadler, 2009). While the multimerisation process takes place, the expanding multimer organises into a right-handed helical structure, allowing for a 100-fold compaction of the protein.

In this helical structure, the VWFpp (D1–D2 domains) and the D'–D3 domains form the wall of the hollow tube. The remainder of the VWF protein (A1-CK domains) protrudes outward from the helical architecture, occupying the space between the tubules of Weibel–Palade bodies (WPBs). VWF tubules assemble into so-called ministacks that represent the first WPB-like structure (Springer, 2013; Lenting & Christophe, 2015).

During the passage of VWF through the Golgi, maturation of the 12 *N*-linked glycans proceeds while 10 *O*-linked carbohydrate structures are also added to the protein. In some N-linked oligosaccharides, sulfation also occurs (Figure 2.2). The N-linked oligosaccharides of VWF contain ABO blood group oligosaccharides. The antigens of

the ABO blood group system exist as complex carbohydrate structures. It has been demonstrated that ABH structures are carried on the N-linked oligosaccharide chains of VWF according to the blood type of the individual. This extensively glycosylation of VWF is both essential for secretion and influences its proteolysis (Mcgrath *et al*, 2010; Jenkins & O'Donnell, 2006).

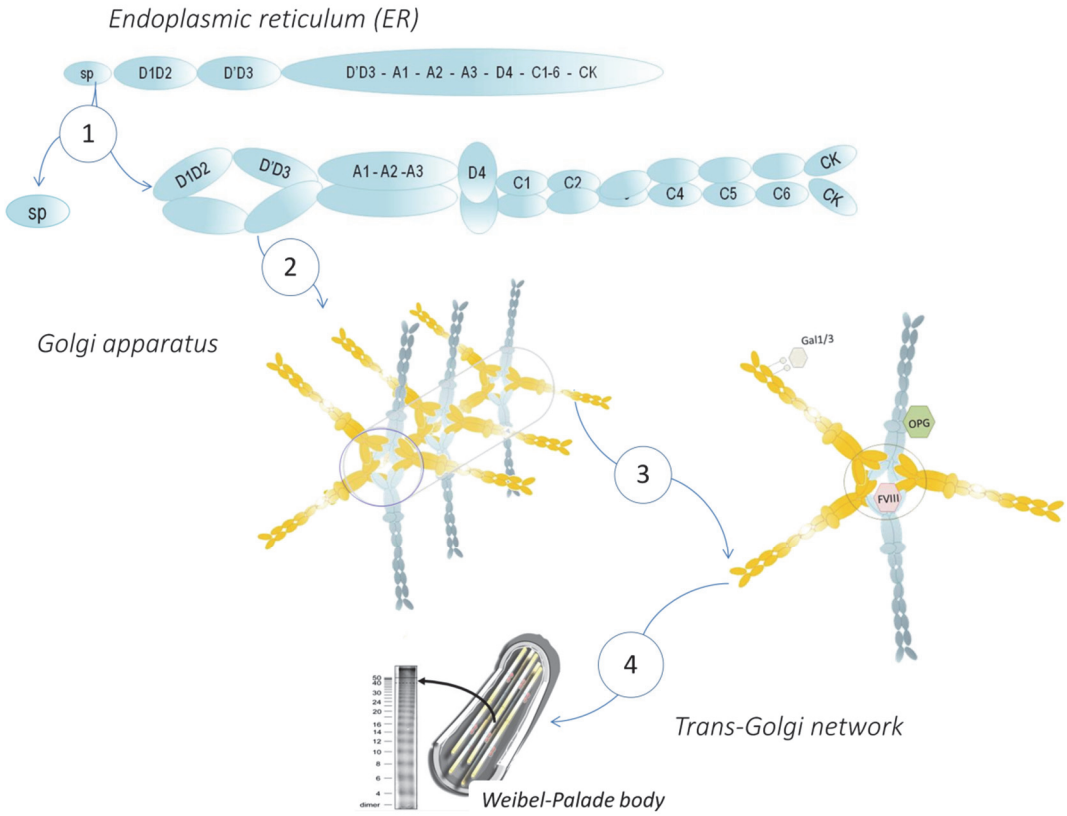

Figure 2. Biosynthesis of VWF and intracellular processing. *(1) Endoplasmic reticulum (ER)* – "tail-to-tail" interprotein disulphide bridge formation involving the C-terminal CK domains allows for the formation of prodimers; *(2,3) Golgi apparatus* – organisation of the prodimers into a dimeric bouquet structure; multimerisation via disulphide bridging, "head to head", that couples adjacent N-terminal D3 domains, a process that is catalysed by the VWFpp. Maturation of the N-linked glycans; addition of O-linked carbohydrate; incorporation of proteins that co-reside with VWF: FVIII; osteoprotegerin, galectins-1 and -3; *(4) Trans-Golgi network* – co-packaging of VWF-containing ministacks promotes maturation and formation of larger WPBs. Furin mediates the proteolytic separation of the propeptide (VWFpp) from the mature VWF subunits. Multimer analysis of endothelial VWF has revealed the presence of ultra large (UL) VWF multimers that exceed the size of multimers found in plasma. Adapted from (Lenting & Christophe, 2015).

In the WPBs formation some proteins coresides with VWF in this organelle: the FVIII interact with the D'–D3 region, suggesting that FVIII may locate to the inner core of the helix; osteoprotegerin (which binds to the A1 domain) and galectins-1 and -3 (which

bind to VWF glycans) are more likely to be present in the intertubular space (Figure 2.3) (Lenting & Christophe, 2015; Metcalf *et al*, 2008).

In the Trans-Golgi network, copackaging of VWF-containing ministacks promotes maturation and formation of larger WPBs. In addition, furin mediates the proteolytic separation of the VWFpp from the mature VWF subunits. However, after cleavage by furin, the mature VWF and VWFpp remain non-covalently associated with the D'-D3 domains; this connection is needed for the final folding of the VWF multimers into the structures of storage organelles (Figure 2.4) (Lenting & Christophe, 2015).

The majority of the newly synthesised endothelial-derived VWF is secreted via the constitutive pathway, which contributes to approximately 95% of plasma VWF; the remaining VWF is stored within WPBs and secreted via the regulated pathway on stimulation by secretagogues (Ruggeri & Ware, 1993). Conversely, VWF produced by megakaryocytes is stored in α-granules that are later partitioned into platelets, and VWF is predominantly released upon platelet activation. Therefore, α-granules-derived VWF has not been shown to contribute significantly to plasma VWF (Lenting & Christophe, 2015).

The VWF stored in the WPBs or in the α-granules of platelets is enriched in ultra-large (UL) VWF multimers (> 20,000 kDa in size), whereas the constitutively secreted VWF consists of different sizes, and includes low (L), intermediate (I), and high (H) molecular-weight forms that are characteristic of the circulating pool of VWF, ranging from a single dimer to up to 20 dimers (~10 000 kDa) (Sadler, 1998).

On secretion, a proportion of UL-VWF multimers that are released from WPBs remains attached to the surface of activated endothelial cells. This tethering elongates the UL-VWF multimers to extremely long strings that unravel, bind platelets, and wave in the direction of the flow. This UL-VWF is hyperreactive compared with normal circulating plasma VWF because they can induce spontaneous microaggregate formation in the blood. The probable mechanism is a combination of its extraordinary length, the higher number of platelet and collagen binding sites, and its propensity to adopt an

open, platelet-binding conformation. UL-VWF multimers are potentially harmful in the normal circulation and must be processed into smaller, less reactive molecules that will not precipitate unwanted platelet aggregation. Given this hyperreactivity, ADAMTS13 rapidly cleaves newly secreted UL-VWF multimers on the endothelial cells (Stockschlaeder *et al*, 2014; Valentijn & Eikenboom, 2013; Sadler, 2009).

The UL-VWF multimers can be secreted into the plasma via a tightly regulated pathway following stimulation by specific secretagogues. Various stimuli for release of these UL-VWF multimers have been identified and include exposure to physiological and pharmacological agents such as adrenaline, adenosine diphosphate, collagen, fibrin, histamine, thrombin, complement proteins, and the vasopressin analogue desmopressin (DDAVP) (Stockschlaeder *et al*, 2014; Luo *et al*, 2012).

1.3.4. VWF function

VWF exists both in the circulation and in the extracellular matrix (ECM) and acts as a vessel wall damage sensor at sites of vascular injury (Crawley *et al*, 2011). The VWF protein has three main recognised haemostatic functions, which are to mediate interactions of platelets to the subendothelial matrix and platelets to platelets (platelet adhesion and platelet aggregation), and to serve as a carrier molecule for procoagulant factor VIII (FVIII), thereby protecting FVIII from rapid clearance, and thus increasing its plasma half-life (Luo *et al*, 2012). Recent studies have stated that VWF may have other, non-haemostatic functions in angiogenesis, cell proliferation, inflammation and tumour cell survival (Luo *et al*, 2012; Lenting *et al*, 2012).

1.3.5. Regulation of VWF by ADAMTS13

Control of VWF size requires a specific regulatory mechanism by ADAMTS13 that cleaves VWF as soon as it is released from endothelial cells, resulting in the shedding of UL-VWF from the endothelial cell surface and fragmentation of VWF strings (Bryckaert *et al*, 2015). Indeed, the UL-VWF is not normally detected in circulation, but instead a series of lower molecular weight multimeric forms, ranging in size typically

from 500 to 10,000 kDa. By cleaving VWF, ADAMTS13 down regulates platelet aggregation and prevents excessive thrombus growth (Crawley *et al*, 2011).

Furthermore, the size of multimeric VWF may be regulated by the trimeric glycoprotein thrombospondin-1 (TSP-1) that acts to further reduce the size of VWF multimers after ADAMTS13 processing. Each TSP-1 subunit is composed of N- and C-terminal globular domains connected by a thin strand. Like VWF, TSP-1 is released from endothelial cells and megakaryocytes and tethers to sites of vascular damage. TSP-1 can then alter the multimer size of proximal VWF molecules by splitting the disulphide-bonds that link VWF monomers at its N- and/or C-terminal ends; in addition, TSP-1 shares homology with ADAMTS13 and can compete for the same VWF binding site in A2 and A3 domains, thus protecting it from proteolytic cleavage. TSP-1 has also been shown to regulate the multimer size of circulating VWF, or VWF released from activated platelets (Pimanda *et al*, 2004; Dognon & Clavague, 2011; Bryckaert *et al*, 2015).

Once secreted or proteolytically released in free circulation (L, I and HMW), VWF multimers adopt a globular conformation (Crawley *et al*, 2011). In this respect, the behaviour of the VWF A1-A2-A3 domains is central to VWF function. In its globular form, the collagen-binding site within the VWF A3 domain is constitutively exposed on the surface, as it must provide the initial contact point for the newly exposed collagen. Conversely, the glycoprotein Ibα (GPIbα) binding site in the VWF A1 domain remains largely hidden until required to prevent spontaneous or unnecessary platelet binding. The intervening VWF A2 domain that harbours the ADAMTS13 cleavage site (Tyr1605-Met1606) is folded such that the cleavage site and VWF A2 domain exosites are hidden or buried. While in this globular form, VWF exhibits functional quiescence. Thus, the consequence of a globular form is that certain interaction sites are buried and inaccessible to their ligands, which enables VWF to patrol the intact vasculature without binding unnecessarily to platelets or to plasma proteins (Crawley *et al*, 2011).

1.3.6. Influence of higher shear stress

Higher shear stress is an important determinant of VWF function that not only modulate the large form VWF, but can modulates the exposure of both the VWF A1 domain platelet binding sites and the VWF A2 domain ADAMTS13 binding/cleavage site(s) (Crawley *et al*, 2011; Denis & Lenting, 2012; Luo *et al*, 2012). Furthermore, attachment of VWF multimers to collagen and/or platelets further facilitates unfolding because of increased tensile force acting on the VWF molecule. Larger VWF multimers unravel more readily under high shear. VWF unfolding is thought to involve both the uncoupling of the VWF A1-A2-A3 tridomain cluster, and conformational changes within individual domains, most notably the VWF A2 domain (Figure 3).

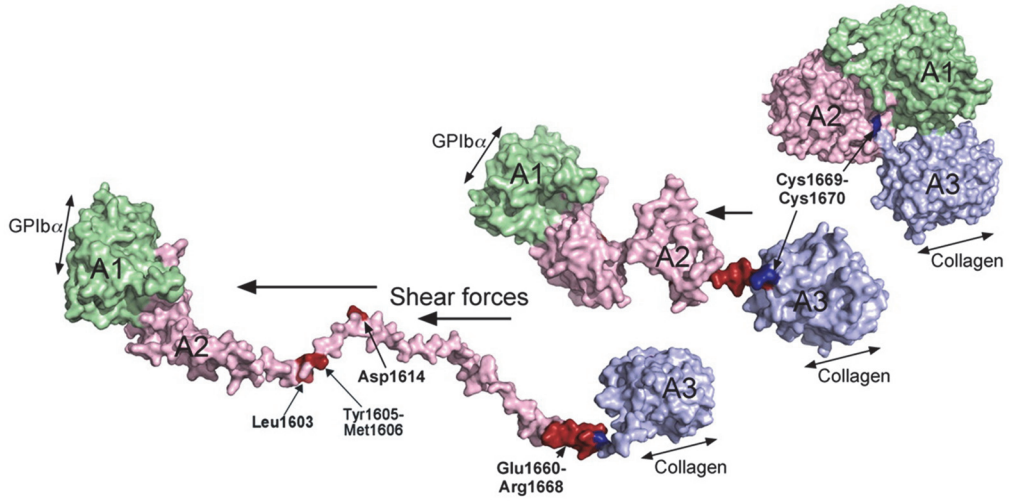

Figure 3. Molecular models of the unfolding of the VWF A1-A2-A3 domains. In globular VWF, the A3 domain collagen binding site is exposed. Elevated shear forces on VWF cause uncoupling of the A domains, extraction of the Cys1669-Cys1670 vicinal disulphide plug, and unravelling of the A2 domain. This exposes the GPIbα binding site in the A1 domain, cryptic ADAMTS13 binding sites, and the cleavage site Tyr1605-Met1606 in the A2 domain (red). Image adapted from (Crawley *et al*, 2011).

1.3.7. VWF and its role in primary haemostasis

The quiescence of circulating VWF is perturbed when a vessel membrane is injured, which results in the exposure of the collagen-rich matrix that normally underlies the protective endothelial cell monolayer. Globular VWF (via the VWF A3 collagen-binding site) recognises this newly exposed collagen leading to its specific recruitment to the

damaged vessel wall. The major binding site for type I and type III collagen lies within the VWF A3 domain (Crawley *et al*, 2011; Berndt *et al*, 2014).

The VWF functions as a vessel wall damage sensor and initiator of primary haemostasis is highly dependent on its multimeric size. The larger VWF multimers in plasma are the most hemostatically reactive not only because they contain more ligand-binding sites, but also because they are more conformationally responsive to vascular shear forces (Stockschlaeder *et al*, 2014).

VWF then undergoes a unique structural transition whereby it is unfolded by local shear forces exerted on the tethered molecule by the flowing blood. In this way, VWF adopts an elongated, "active" conformation that exposes previously hidden platelet binding sites that mediate the capture of circulating platelets to the site of vascular injury. This exposes VWF A1 domains that, in turn, reveal the binding sites for the platelet GPIbα receptor (Crawley *et al*, 2011).

The VWF-GPIbα interaction (platelet-vessel wall adhesion) is characterised by a fast association/dissociation rate, which enables the tethering of platelets to the endothelium where they "roll" over the VWF surface and provide the opportunity to establish firm platelet adhesion through the collagen receptors. VWF binds fibrillar types I and III collagen via the A3 and A1 domains, whereas it binds subendothelial microfibrillar type VI collagen exclusively via the A1 domain (Ceunynck *et al*, 2012). These different types of collagen are situated predominantly in different regions of the extracellular matrix, enabling VWF to participate in the initiation of coagulation when the vessel wall is damaged to different degrees. These first steps in the genesis of the primary platelet plug illustrate the importance of VWF conformation for its haemostatic function. The high-affinity, rapid and reversible interaction between the VWF A1 domain and GPIbα tethers platelets to the endothelium, where they roll until they are immobilised by integrin-mediated binding, which has slower binding kinetics. The RGD (Arg-Gly-Asp) sequence within the C4 VWF domain is responsible for the VWF binding to GpIIb/IIIa. The addition of a second layer of platelets (aggregation) involves binding VWF to the GPIb and GPIIb/IIIa platelet receptors and fibrin to GPIIb/IIIa,

resulting in the formation of a platelet plug that seals the damaged vessel wall (Savage *et al*, 1996).

1.3.8. VWF and its role in secondary haemostasis

In addition to its role in primary haemostasis, VWF also has a role in secondary haemostasis, acting as a carrier protein for FVIII in the plasma. VWF forms a complex with FVIII in plasma through a non-covalent interaction. FVIII is a cofactor of the intrinsic clotting cascade, and its deficiency manifests as haemophilia A. VWF protects FVIII from degradation and may also serve to localise FVIII to the site of the clot. This phenomenon may be mediated by one or a combination of the following mechanisms: (i) structural stabilisation of FVIII; (ii) inhibition of phospholipid-binding proteins that target FVIII for proteolytic degradation; (iii) inhibition of FVIII binding to activated FIX to stimulate the coagulation pathway; and (iv) prevention of FVIII cellular uptake via scavenger cell receptors. FVIII binds to VWF within the N terminal of the D' and D3 domains of VWF corresponding to residues 763-1035 (Luo *et al*, 2012; Federici, 2003; Terraube *et al*, 2010).

The haemostatic potential of VWF multimers in primary and secondary haemostasis differs depending on its molecular size (Table 1). Whereas VWF function in primary haemostasis (binding to GpIb, GpIIb/IIIa and collagen) strongly correlates with VWF multimerisation, with the largest multimers being the most active, the FVIII binding of VWF is size-independent (Fischer *et al*, 1998) (Table 1, Figure 4). The concentration of VWF in plasma ranges from 5 to 15 μg/mL while the concentration of FVIII is 200 ng/mL. Conversely, the VWF collagen-binding capacity and the functional ability of VWF to bind platelets decrease as the multimer size of VWF decreases. These effects may be because of fewer binding sites of the smaller multimers and lower binding affinity between low VWF multimers and platelets, reducing both platelet adhesion and aggregation (Stockschlaeder *et al*, 2014).

Table 1 - Multimers of von Willebrand factor and their physiological characteristics

Multimer	Nº of mult (dimers)	Size (kDa)	Primary distribution	Hemostatic function
Low	1–5	500–2500	Circulating plasma	FVIII carrier only
Intermediate	6–10	3000–5000	Circulating plasma	Low platelet binding affinity; FVIII carrier
High (large)	11–20	5500–10000	Circulating plasma	High platelet adhesion and aggregation; FVIII carrier
Ultra-large	>20	>10000	Uncleaved form of VWF*	Cleavage to smaller multimers that are characteristic of the circulating pool of VWF

Nº, number; mult, multimer; VWF, von Willebrand factor. * stored in Weibel–Palade bodies and α-granules; rapidly cleaved once released from storage. Adapted from (Stockschlaeder *et al*, 2014).

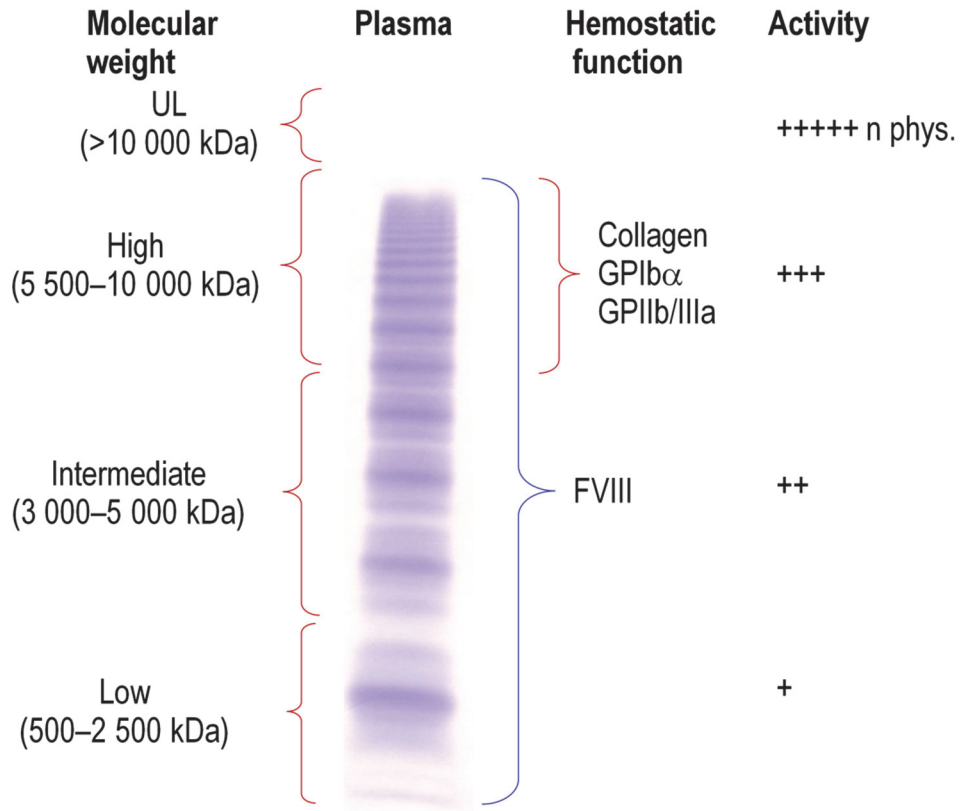

Figure 4. The haemostatic potential of VWF multimers and its dependency on molecular size. Dimerisation and multimerisation of plasma circulating VWF and the corresponding bands, as determined by sodium dodecyl sulphate agarose gel electrophoresis. Multimers vary in size from the smallest detectable dimer (~500 kDa) to the largest multimers that exceed 10,000 kDa. An increase in multimer size parallels an increase of VWF binding affinity for collagen and platelet glycoproteins Ib (GPIbα), IIb/IIIa (GpIIb/IIIa). VWF activity is dependent on the extent and pattern of multimerisation, as indicated by the schematic: indicating the strength (from low to high: '+' to '++++'). FVIII binding of VWF is size-independent. N phys, non-physiological. Adapted from (Schneppenheim & Budde, 2005; Fischer *et al*, 1998)

1.3.9. VWF in Diseases

VWF illustrates the duality between haemorrhagic and thrombotic mechanisms because is a key player in both processes.

Deficient or defective VWF results in VWD the most common inherited bleeding disorder with a reported incidence of 0.01–1% in the general population. Abnormalities in VWF secretion, intravascular clearance of VWF, in the assembly of VWF multimers, or exaggerated proteolytic degradation by ADAMTS13 can cause diverse forms of VWD.

Conversely, defects in proteolysis of VWF by ADAMTS13 can cause thrombotic thrombocytopenic purpura (TTP), a thrombotic microangiopathy (TMA) caused by clumping of platelets by ultra-large VWF and defined clinically by microangiopathic haemolytic anaemia and thrombocytopenia (Sadler *et al*, 2006; Mannucci, 2005).) Another form of TMA is the atypical hemolytic uremic syndrome (aHUS) caused by uncontrolled activation of the complement system. However, these two diseases have similar clinical manifestations although aHUS is principally a kidney disorder and TTP is more a systemic disorder. Recently, studies have shown interactions between VWF and complement system, which could explain the clinical overlap between these two thrombotic microangiopathies (Feng *et al*, 2013a; Feng *et al* 2015).

1.4. VWF and VWD

1.4.1. Diagnosis and classification

VWD is characterised by excessive mucocutaneous bleeding, which manifests clinically as epistaxis, menorrhagia, easy bruising and nose or oral cavity bleeding. Patients with severe forms of VWD may also suffer from joint, muscle and central nervous system bleeding (Sadler *et al*, 2006; Batlle J *et al*, 2011).

The disease shows no geographical or ethnic predilection, but although both sexes inherit mutant VWF alleles with equal frequency, females outnumber males by approximately 2:1 in most VWD populations, presumably because of the burden of

excessive mucocutaneous bleeding in reproductive-age women (Lillicrap, 2013; Rodeghiero *et al*, 2009a).

The diagnosis of VWD is based on the nature of the VWF deficiency. According to the ISTH classification (Sadler *et al*, 2006), VWD is classified into three different types: partial or complete VWF quantitative deficiencies (types 1 and 3, respectively) and qualitative deficiency (type 2) (Table 2). Type 1 VWD, a mild to moderate reduction in functionally normal VWF, constitutes 65% to 80% of cases; type 2 disease, involving the expression of functionally abnormal VWF, occurs in 20% to 35% of patients; and type 3 disease, the virtually complete absence of VWF, affects approximately 1 in 1 million people (Lillicrap, 2013; Sadler *et al*, 2006; Goodeve, 2010).

Table 2 - Classification of von Willebrand disease

VWD type	Description
	Quantitative deficiency of VWF
Type 1	Partial quantitative deficiency of VWF (structure and distribution of plasma VWF multimers indistinguishable from normal)
Type 3	Virtually complete deficiency of VWF
Type 2	**Qualitative deficiency of VWF**
2A	Decreased VWF-dependent platelet adhesion with selective deficiency of HMWM (either from defective multimer assembly or increased sensitivity to ADAMTS13 cleavage)
2B	Increased affinity for platelet GPIb (due to enhanced interaction of mutant VWF with platelet GPIb)
2M	Decreased VWF-dependent platelet adhesion without selective deficiency of HMWM, despite normal VWF multimer assembly (results from mutations that disrupt VWF binding to platelets or subendothelium)
2N	Markedly decreased binding affinity for FVIII (due to mutations that impair FVIII binding capacity)

FVIII, factor VIII; HMWM, high-molecular-weight multimer; VWF, von Willebrand factor. According to (Sadler *et al*, 2006; Goodeve, 2010; Stockschlaeder *et al*, 2014).

Type 2 VWD is characterised by functional defects that lead to impaired activity and is accordingly divided into four secondary categories, 2A, 2B, 2M and 2N. Functional defects lead to enhanced 2B or reduced 2A, 2M platelet interaction or impaired binding to FVIII, 2N (Meyer *et al*, 2011; Schneppenheim & Budde, 2011).

All these defects can be identified by laboratory tests that assess the functional characteristics of VWF (Table 3). VWF analysis was performed according to an algorithm based on established recommendations (Castaman *et al*, 2014): *screening tests* (VWF:Ag, VWF:RCo and FVIII:C) and *specific tests* to determine disease subtype (RIPA, VWF:FVIIIB, VWF:CB, VWFpp and VWF multimer profile).

Table 3 - Phenotypic analysis of VWD

Test	Type	Measurement
VWF:Ag	Screening	Antigen; quantity of protein
VWF:RCo	Screening	Ristocetin cofactor activity; ability to bind platelet GpIb in the presence of ristocetin
FVIII:C	Screening	FVIII coagulant activity
VWF:Ac	Screening	Monoclonal antibody binding to a functional epitope of the A1 loop: immunoassay of GpIb binding
RIPA	Subtyping	Ability to aggregate platelets at varying doses of ristocetin
VWF:FVIIIB	Subtyping	FVIII binding capacity
VWF:CB	Subtyping	Collagen (type III, type VI) binding capacity
VWFpp	Subtyping	Quantity of propeptide. Elevated VWFpp/VWF:Ag ratio indicates enhanced clearance rate from plasma
Multimer pattern	Subtyping	Aberrant profiles can indicate reduction in dimerisation /multimerisation, HMW multimer loss, enhanced or reduced ADAMTS13 cleavage, enhanced clearance and mutations that replace/introduce cysteine residues, affecting disulphide bonding

Abbreviations approved at ISTH-SSC on VWF (Sadler *et al*, 2006). Adapted from (Goodeve, 2010; Castaman *et al*, 2013)

The VWF deficiency is diagnosed by evaluating the following criteria (Figure 5) (Castaman, 2013; Goodeve, 2010):

- VWD diagnosis should be considered within the context of an appropriate personal and/or familial bleeding history. The use of a standardised questionnaire for history collection is advisable to appreciate the severity of the bleeding tendency.

- If personal and/or familial bleeding history is significant, VWF:RCo, VWF:Ag and VWF:CB assays should be measured on the same sample.

- If any of these tests are below 30%, the diagnosis of VWD should be strongly considered. Other family members with a possible bleeding history should be evaluated. Finding another member with bleeding and reduced VWF strongly supports the likelihood of diagnosis.

- A VWF:RCo/VWF:Ag ratio close to 1 suggests type 1 VWD; the absence of VWF (or the presence of only trace amounts) and a FVIII:C below 5% distinguishes type 3 VWD from severe type 1 VWD; a discrepant VWF:RCo/VWF:Ag ratio of < 0.7 suggests type 2 VWD; and a discrepant FVIII:C/VWF:Ag ratio of < 0.5 suggests type 2N VWD (to be confirmed by a binding study of FVIII to the patient's VWF).

– Aggregation of patient platelet-rich plasma (RIPA) in the presence of increasing concentrations of ristocetin (0.25, 0.5, 1.0 mg/mL, final concentration) should be assessed. Aggregation at low concentrations (< 0.5 mg) suggests type 2B (or platelet type) VWD.

– Multimer pattern using an intermediate resolution gel should be evaluated. The presence of a full complement of multimers suggests type 1, 2N or 2M. A lack of HMWM suggests type 2A and/or 2B. In type 3, all multimers are absent. Analysis of the triplet structure will help identify variants with increased or decreased cleavage. Repetition of the multimer gel in low resolution agarose may be helpful in confirming the presence/lack of HMW multimers.

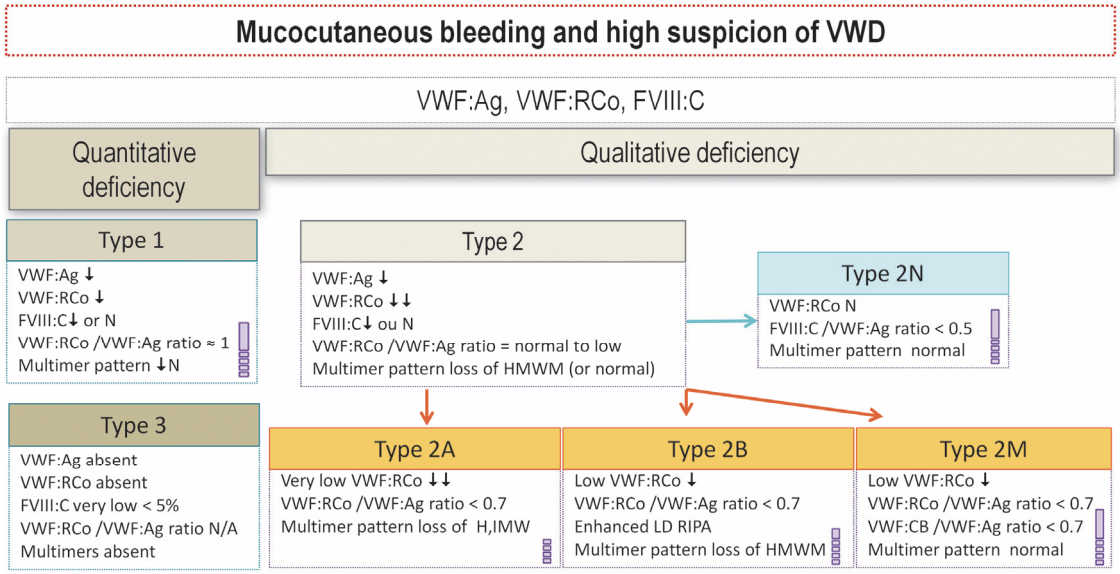

Figure 5 - Algorithm for the evaluation of the patient with mucocutaneous bleeding and high suspicion of VWD. ↓ represents decreased values; N, normal values; H,IMW, high and intermediate molecular weight; LD, low dose of ristocetin (≈ 0.5/mL).

It is crucial to correctly diagnose the subtype of VWD because the therapeutic approaches are different for each subtype. However, this diagnosis remains difficult because the clinical and laboratory phenotypes are very heterogeneous, and a clear discrimination between some of these subtypes can be challenging (Castaman *et al*, 2014; Budde U, Favaloro E, 2011).

1.4.2. Molecular genetics of VWD

Mutation analysis of VWF has helped to understand the structure-function relationship of this complex protein and can be helpful in establishing the correct diagnosis of VWD when conventional laboratory parameters are inconclusive (Schneppenheim & Budde, 2011).

The transmission of VWD is often autosomal dominant (types 1, 2A, 2B and 2M), but can also be rarely inherited in a recessive manner: type 3, severe type 1, type 2N and a particular subtype of type 2A/IIC. In families with more than one VWF deficiency may result in complex inheritance patterns and heterogeneous clinical presentations of the family members (Figure 6) (Schneppenheim & Budde, 2011; Goodeve, 2010, 2014).

1.4.2.1. Type 1 VWD

The most common VWD form (type 1) is associated with an extremely heterogeneous phenotype, with VWF levels ranging from 5% to 30% depending on the molecular pathogenesis. Moreover, it is characterised by low penetrance (not all who inherit the mutation show signs of clinical disease) and variable expressivity (those who inherit the same mutation show variable clinical signs). This phenomenon usually leads to a great deal of difficulty in consistently diagnosing type 1 correctly (Branchford & Di Paola, 2012; Goodeve *et al*, 2007; Tosetto *et al*, 2006).

The molecular epidemiological studies of type 1 VWD indicated that candidate *VWF* mutations are present in approximately 65% of index cases and that approximately 70% of these sequence variants represent missense substitutions (Lillicrap, 2013). Indeed, the more severe the VWF deficiency, the greater the likelihood of identifying a causative *VWF* mutation (Rodeghiero *et al*, 2009b). The potential pathogenetic mechanisms characterised in type 1 VWD to date include impaired biosynthesis, clearance (decreased survival) and intracellular retention of VWF (Goodeve, 2010). Recessive severe type 1 VWD is associated with very low VWF and FVIII:C levels due to missense mutations identified in the VWF propeptide (D1 and D2 domains) and the dimerisation site (cysteine knot, CK, domain) (Schneppenheim & Budde, 2011).

Therefore, type 1 VWD can be difficult to diagnose due to factors influencing VWF levels in plasma including environmental and genetics factors. One of the major determinants of plasma VWF:Ag levels is the ABO blood group of an individual. The O blood group has been known to be more prevalent in type 1 VWD than in the normal population and is associated with VWF levels approximately 25% lower than the population average. The effect of the O blood group appears to be through increased VWF clearance from the plasma; individuals in the O blood group have VWF plasma levels 30% lower than those in the AB blood group. ABO glycosyltransferase alleles encode different transferase enzyme specificities. The enzyme is non-functional in the O blood group due to a null allele. The A and B blood groups' glycosylation protects VWF from clearance, whereas its absence in the O blood group results in more rapid clearance (Goodeve, 2010; Goodeve *et al*, 2007; Millar & Brown, 2006).

With the recent evidence that 35% of mild type 1 VWD have no identifiable *VWF* mutations and the search for additional genetic modifiers of this phenotype has increased in the last few years. It should be noted that beyond the VWF deficiency, the coexistence of other mild bleeding disorders might also modify the clinical phenotype in these patients (Lillicrap, 2013).

1.4.2.2. Type 3 VWD

Type 3 VWD is characterised by the complete lack of VWF protein with undetectable VWF:Ag and VWF:RCo levels and very low FVIII:C levels (< 5%) that represent the steady state of FVIII without being chaperoned by VWF. Multimers are absent and the bleeding symptoms of patients with type 3 VWD are usually very severe (Branchford & Di Paola, 2012).

This virtually complete VWF deficiency results from homozygosity or compound heterozygosity for two *VWF* null alleles (gene deletions, stop codons, frame shift mutations, splice site mutations and absence of mRNA) in the majority; or, more rarely, are compound heterozygous for a null allele and a missense mutation or

homozygous for a missense mutation (Schneppenheim & Budde, 2011; Goodeve, 2010).

The phenotype analysis is generally sufficient for diagnosis of type 3 VWD, although discriminating from severe type 1 VWD can be dependent on assay sensitivity. Molecular analysis may be useful where carrier status determination or pre-natal diagnosis is required (Bowman *et al*, 2013; Surdhar, 2001).

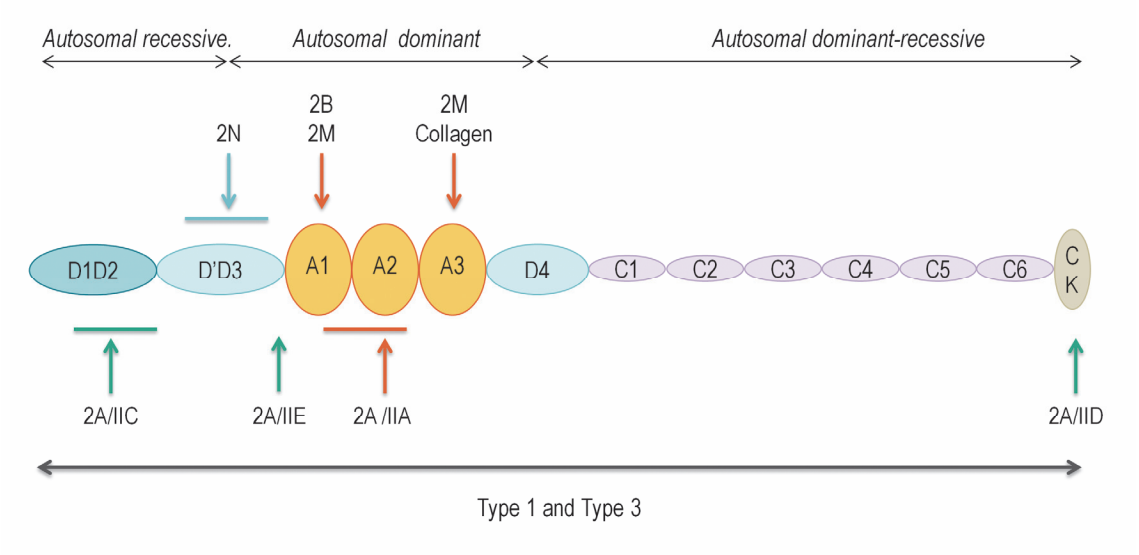

Figure 6. The scheme illustrating the location of mutations resulting in the various VWD subtypes. Types 1 and 3 VWD are caused by different types of mutation throughout the sequence. In contrast, the type 2VWD mutations are localised to distinct functional domains of VWF, with VWF affecting multimer structure (2A), binding to FVIII (2N), platelets (2B and 2M), and collagen (2M).

1.4.2.3. Type 2 VWD

Type 2 VWD is a qualitative defect caused by mutations in *VWF* that result in abnormal interactions with several of its ligands (Figure 6).

Type 2A VWD is the most common form of type 2 (20–25%), which is associated with reduced platelet binding due to a significant reduction or absence of VWF multimer profile showing a loss of HMWM and sometimes intermediate-molecular-weight multimers. The hallmark of type 2A disease is a low VWF:RCo to VWF:Ag ratio (< 0.7) and impaired RIPA. Type 2A (IIA) VWD results from *VWF* mutations located in the A1 and A2 domains, which cause intracellular retention of HMWM (Group I), or those with normal synthesis and release but with increased proteolytic susceptibility to

ADAMTS13 (Group II). Type 2A VWD can also be caused by structural defects due to mutations interfering with multimerisation assembly which is either located in the multimerisation region (IIC and IIE) or in the dimerisation region (IID) (Michiels & van Vliet, 2009; Schneppenheim, 2011; Schneppenheim & Budde, 2011).

ADAMTS13 cleavage results in the characteristic triplet satellite bands seen on multimer electrophoresis. HMW multimer loss and differences in patterns of satellite bands can help to identify the VWD subtype and mechanism responsible for disease. Dimerisation defects yield VWF that is terminated by a monomer and cannot form inter-chain disulphide bonds or does so inefficiently. This results in a characteristic 2A (IID) multimer pattern showing HMW multimer loss and aberrant satellite bands (Goodeve, 2010; Budde *et al*, 2008).

The multimer profile in 2A (IIE) demonstrates both severely reduced HMW multimers and an aberrant triplet structure indicating reduced proteolytic cleavage. Reduced affinity for GpIbα and resulting impaired platelet tethering renders mutant *VWF* less frequently cleaved by ADAMTS13 (Gadisseur *et al*, 2009a; Goodeve, 2010).

D2 domain mutations (exon 11–17) can prevent full multimerisation and are recessively inherited. Large multimers are severely reduced, proteolytic bands are absent, and there is an increase in dimers resulting in subtype 2A (IIC). Patients are either homozygous for a missense mutation or compound heterozygous, with a null second allele (Michiels *et al*, 2009; Schneppenheim, 2011).

Type 2B VWD is due to gain-of-function mutations in *VWF* that cause conformational changes, and enable the A1 domain to bind GPIbα spontaneously, leading to rapid clearance of the HMWM and mild thrombocytopenia. This can be detected through enhanced ristocetin induced platelet aggregation (RIPA) with low dose ristocetin (~0.5/mL). Patients with classic type 2B may have HMWM loss and thrombocytopenia, and in some cases, only during stressful situations, such as severe infection, surgery or pregnancy (Goodeve, 2010). This phenomenon is seen in patients with type 2B VWD or in patients with the phenocopy platelet-type pseudo VWD (PT-VWD), a rare disorder caused by mutations in platelet GPIbα. It is important to differentiate these two

phenotypically similar entities, because the therapeutic approach is very different: type 2B VWD is treated with VWF concentrates (because the molecular defect is in VWF), and pseudo-VWD is treated with platelet transfusions (because it is caused by mutations in platelet GPIbα). Mixing studies using patient and control plasma and platelets can discriminate the two disorders. Mutation analysis of *GP1BA* exons 1–2 may identify missense mutations, affecting directly or indirectly the confirmation of a GPIbα loop that interacts with the VWF A1 domain, leading to spontaneous binding to VWF. PT-VWD may be responsible for diagnosing 5% more patients with type 2B VWD and it is important to identify, as patients may require platelet transfusions in some circumstances (Goodeve, 2010; Federici *et al*, 2011; Othman & Favaloro, 2008; Othman & Emsley, 2014).

Type 2M ("M" for "multimer") VWD can be caused by the loss-of-function mutations in *VWF* and fits the well-characterised profile: a normal multimer distribution but a reduction in VWF-platelet interactions (in A1 domain) or the existence of collagen-binding defects (A1 and A3 domain). The A1 domain mutations cluster on the face, which interact with GPIbα, reducing or preventing the interaction and resulting in discrepantly low VWF:RCo/VWF:Ag ratios. The mutations lie on the opposite side of the A1 domain to those responsible for 2B VWD (Goodeve, 2010; Hermans & Batlle, 2009; Castaman *et al*, 2012a; Batlle *et al*, 2008; Pérez-Rodríguez *et al*, 2009; Gadisseur *et al*, 2009c).

Other missense *VWF* mutations which affect the collagen-binding A3 domain have also been described. Although they interfere with binding to subendothelium, they fit the 2M VWD definition but with discrepantly low VWF:CB/ VWF:Ag ratios. Indeed, VWF binds fibrillar types I and III collagen via the A3 and A1 domains but binds subendothelial microfibrillar type VI collagen exclusively via the A1 domain (Flood *et al*, 2012a). The standard functional assays for VWD detection do not always evaluate the collagen-binding function of VWF (VWF:CB), and assays for diverse collagen types are even less common, which may have contributed to the scarcity of reported VWF:CB deficiencies (Flood *et al*, 2012b). Functional and molecular studies focused on

this VWF dysfunction have increasingly reported VWF sequence variations that affect the ability of VWF to bind type I, type III and type VI collagen (Larsen *et al*, 2013; Flood *et al*, 2012b; Riddell *et al*, 2009; Legendre *et al*, 2013). However, some of these mutations may not interfere with the screening assays, which show normal values and can only be detected using type VI collagen. Hence, this VWF dysfunction could be underdiagnosed, even though it is typically reported in patients with a mild bleeding history (Flood *et al*, 2012b).

Type 2N ("N" for "Normandy") VWD can be caused by *VWF* mutations causing reduced binding to FVIII, allowing for increased clearance. Therefore, this variant presents as an autosomal form of mild/moderately VWD severe FVIII deficiency (Jacquemin, 2009). In contrast to the other type 2 variants of VWD, type 2N disease is transmitted in a recessive fashion with a variety of homozygous and compound heterozygous genotypes (Castaman *et al*, 2009). The VWF:RCo and VWF:Ag levels can both be within the normal range, while FVIII:C is typically 5–25%. The multimer profile is normal in the majority of cases. All of the *VWF* mutations responsible for this phenotype are located either within the N-terminal D'D3 assembly FVIII-binding region of VWF (> 95% of cases) or at the furin cleavage site for removal of the VWF propeptide (Lillicrap, 2013).

This VWD subtype in which VWF binds FVIII poorly or not at all mimics mild haemophilia A and is named VWD "2N" after "Normandy", the birth province of the first index case described (Jorieux *et al*, 1992). Symptoms largely result from a reduced FVIII level. An inheritance pattern may highlight whether 2N VWD or haemophilia A is more likely, but in some cases, bleeding history in family members is insufficiently widespread. Differential diagnosis can be achieved using the VWF:FVIIIB assay (Goodeve, 2010).

1.4.3. VWF and Acquired von Willebrand syndrome

Acquired von Willebrand syndrome (AVWS) is a rare acquired bleeding disorder, with clinical and laboratory characteristics similar to congenital VWD (Veyradier *et al*, 2000;

Federici *et al*, 2001; Lison *et al*, 2012). Most AVWS types mimic type 1 VWD or type 2A. The AVWS is a relatively uncommon and an under-diagnosed acquired bleeding disorder, with a prevalence estimated from approximately 0.04 to 0.13%, which occurs in individuals with no personal or familial history of bleeding diathesis. It is usually associated with an underlying disorder, namely lymphoproliferative disorders (48%), cardiovascular diseases (21%), myeloproliferative neoplasms (15%), other cancers (5%) and autoimmune diseases (2%). More rarely, it can arise in the context of hypothyroidism, uraemia, and viral infections, or it can be related to drugs, such as ciprofloxacin (Sucker *et al*, 2009; Perez-Rodriguez *et al*, 2011; Federici, 2008).

A variety of pathogenic mechanisms have been proposed to cause structural or functional deficiencies of VWF. These include autoantibodies, either interfering with platelets or collagen binding or increasing VWF clearance from the plasma. Loss of HMWM was demonstrated in patients with hematologic disorders due to the adsorption to clonal plasma cells or platelets. Therefore, the disease is the result of the occurrence of various phenomena, namely: (i) the presence of specific or nonspecific autoantibodies that inactivate VWF; (ii) adsorption of VWF to neoplastic cell clones; (iii) loss of HMWM after exposure to high shear stress; and (iv) increased proteolytic degradation of VWF by circulating proteases. Usually, treating the underlying disease normalises VWF values (Veyradier *et al*, 2000; Federici *et al*, 2001; Tiede *et al*, 2011).

1.5. VWF and Thrombotic Microangiopathies

Thrombotic microangiopathies (TMAs) are characterised by microvascular occlusion, thrombocytopenia and non-immune haemolytic anaemia. The two main forms of TMAs are TTP and aHUS. These two types of TMAs have overlapping clinical phenotypes. Conceptually, TTP has been distinguished from aHUS by more common neurological manifestations, whereas in aHUS, the renal involvement is more pronounced. However, this rule is not always reliable; some aHUS patients have neurological complications and some TTP patients have renal failure (George & Nester, 2014; Mannucci & Cugno, 2015; Kremer Hovinga & Lämmle, 2012; Crawley & Scully, 2013). Over the last decade, noteworthy progress has been made to improve the

clinical and laboratory approaches to predict the expected signs and symptoms, patient outcome, and genotype-phenotype correlations. Despite this, the differential diagnosis of TTP/aHUS remains challenging (Mannucci & Cugno, 2015).

TTP is mainly caused by a severe plasma deficiency, i.e. < 10%, of the cleaving protease of the VWF – ADAMTS13. A severe ADAMTS13 deficiency is due, more often, to anti-ADAMTS13 autoantibodies (acquired) or, rarely, to homozygous or compound heterozygous *ADAMTS13* gene mutations (hereditary, also called Upshaw-Schulman syndrome) (Levy *et al*, 2001; Sadler, 2008). However, heterozygosity for *ADAMTS13* mutations was observed among patients diagnosed with acute acquired TTP and severe ADAMTS13 deficiency (Meyer *et al*, 2007; Camilleri *et al*, 2008). In all forms, the predominant pathogenic factors of TTP are the increased number of ultra-large multimers of VWF resulting from the lack of proteolytic regulation by ADAMTS13, which lead to platelet clumping in the microcirculation of various organs (Sadler, 2008).

While TTP is characterised by the severe deficiency of ADAMTS13, aHUS is characterised by hyperactivation of the alternative complement pathway resulting from either a loss-of-function mutation in a regulatory gene, *CFH, CFI, CD46 (MCP*) or *THBD*, or a gain-of-function mutation in an effector gene, *CFB* or *C3* (Marina Noris & Remuzzi, 2009; George & Nester, 2014). The mutations were found mainly in the heterozygous state, and approximately 5% of patients have combined mutations, usually in *CFH* with either *CD46* or *CFI*. Homozygosity for risk haplotypes of *CFH* (*CFH-H3*) and *MCP* (*MCPggaac*) have been shown to significantly increase disease penetrance and severity (Caprioli *et al*, 2003; Esparza-Gordillo *et al*, 2005; Bresin *et al*, 2013). Additional genetic risk factors include the *CFHR1–3* deletion in the homozygous state caused by non-allelic homologous recombination of *CFHR3* and *CFHR1* (Moore *et al*, 2010). Finally, recessive mutations in *DGKE*, which encode diacylglycerol kinase-ε and are expressed in endothelial cells, platelets and podocytes, were identified in children with the onset of aHUS in the first year of life (Loirat *et al*, 2015).

Poor penetrance is a common clinical feature, and adult onset occurs in patients with severe deficiencies. In addition, both deficiencies predispose patients to a TMA after a triggering event, such as pregnancy, bacterial and viral infections, neoplasia, autoimmune disorders and exposure to certain drugs (Lämmle *et al*, 2008; Scully *et al*, 2014; Auer Von *et al*, 2015).

Severe ADAMTS13 deficiency (< 10%) is an important indicator in the differential diagnosis of TTP/aHUS (Mannucci & Peyvandi, 2007). However, several studies in aHUS patients have shown reduced ADAMTS13 activity that may predispose patients to the TMA phenotype (Remuzzi *et al*, 2002; Feng *et al*, 2013b). Indeed, *ADAMTS13* polymorphisms associated with partial deficiency of ADAMTS13 in the presence of a primary trigger or when co-inherited with a mutation in a complement gene have been described in these patients (Plaimauer *et al*, 2006; Feng *et al*, 2013b; Noris *et al*, 2005). These findings suggest that TTP and aHUS could exhibit overlap not only in their clinical characteristics but also in their pathophysiological mechanisms.

Recent studies have shown that CFH binds to VWF and enhances cleavage of VWF by ADAMTS13 (Feng *et al* 2013). This might be due to a conformational change in VWF induced by binding of CFH to VWF-A2 domain that makes the cleavage site more accessible to ADAMTS13. This binding was demonstrated to be in region of the 10-20 short consensus repeats (SCR) of CFH domains. Curiously, the mutations in CFH, which are the most common genetic alteration detected in patients with aHUS, mainly are located in SCRs 18-20, encompassing the region of CFH directing cell surface binding.

In another study, the same authors (Feng *et al* 2015) have shown that CFH co-localized with VWF to the Weibel-Palade bodies in endothelial cells, that CFH binds with high affinity to VWF, and that the cofactor function of CFH in the degradation of C3b by CFI was enhanced by increasing concentrations of VWF. These data suggests a role for VWF in the proteolytic inactivation of complement C3b by factor CFI. Whereas smaller VWF multimers, especially dimers, tetramers, and hexamers, enhance C3b inactivation by CFI, large and UL-VWF multimers are devoid of this cofactor activity and, therefore, they enhance complement activation by the alternative pathway C3 convertase,

C3bBb. These results suggested that in normal plasma, VWF multimers enhance degradation of C3b. Conversely, ULVWF multimers, as are present in patients with thrombotic microangiopathy, lack an inhibitory effect on complement and permit complement activation. Although these results are only based on biochemical studies in vitro, they have shown a functional interaction between CFH, VWF and ADAMTS13 which could contribute to the pathogenesis of thrombotic microangiopathy in both TTP and aHUS and provide a explanation for the frequently observed clinical overlap between TTP and aHUS (Lämmle B, 2015).

1.6. Molecular genetic analysis using next-generation sequencing (NGS)

Studies of large-size genes, such as *VWF* and the multiple genes implicated in TMAs, were not affordable by diagnostic laboratories due to the high cost of conventional Sanger DNA sequencing. The advent of NGS is changing this paradigm and greatly improves the molecular analysis of these diseases.

DNA sequencing technology has made impressive advances in recent years (Liu *et al*, 2012). NGS allows for the fast generation of thousands to millions of base pairs of DNA sequences of an individual patient and brings genetic diagnostics into a new era. The benefit of implementing NGS in diagnostics is the introduction of testing many genes at once in a relatively short time and at a relatively low cost, and thereby yielding more molecular diagnoses (Metzker, 2010; Gonzalez-Garay, 2014).

Several commercial NGS instruments, referred to as NGS platforms, have become available. All platforms require some level of DNA pre-processing into a library, a collection of DNA fragments that together represent the genome of an organism suitable for sequencing. Generally, all DNA library protocols involve (fragments of) DNA or RNA molecules fused with adapters that contain the necessary elements for immobilisation on a solid surface and sequencing (Cunha *et al*, 2015).

NGS-targeted gene panels are being introduced into clinical practice and provide substantial benefits for definitive diagnoses in haematological diseases as several recent reports have demonstrated (Corrales *et al*, 2012; Batlle *et al*, 2015; Simeoni *et*

al, 2016; Del Orbe Barreto *et al*, 2016). An essential contribution to this is the recently published guidelines for the evaluation and validation of NGS applications for the diagnosis of genetic disorders (Matthijs *et al*, 2015).

Moreover, this technology has changed the concept of routine molecular studies: in the face of the multiple genetic changes found in every patient, the critical challenge became discriminating disease-associated variants from the broader background of variants present in all patients' genomes. For this, variants must be classified as pathogenic, likely pathogenic, uncertain significance, likely benign or benign, based on the available evidence and according to the practice guidelines for the evaluation of pathogenicity recently published by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (Richards *et al*, 2015).

The NGS workflow includes a well-established bioinformatics pipeline and a clinical-laboratory approach to analyse identified variants in the context of each patient's specific phenotype. For this purpose, its interplay with a detailed clinical data registry and familial studies is crucial.

CHAPTER

2

AIMS & OUTLINE OF THIS THESIS

CHAPTER 2. AIMS & OUTLINE OF THIS THESIS

The VWF illustrates the duality between haemorrhagic and thrombotic mechanisms because is a key player in both processes.

Deficient or defective VWF results in VWD the most common inherited bleeding disorder with a reported incidence of 0.01–1% in the general population. However, the diagnosis of VWD is difficult because the clinical and laboratory phenotypes are very heterogeneous. Molecular analysis is very useful but the large size of VWF has been a disadvantage for studying and a clear diagnosis remains challenging.

Defects in proteolysis of VWF by ADAMTS13 can cause thrombotic thrombocytopenic purpura (TTP), a thrombotic microangiopathy (TMA). The two main forms of TMA are TTP and atypical haemolytic uraemic syndrome (aHUS). The aHUS is characterised by hyperactivation of the alternative complement pathway resulting from either a loss-of-function mutation in a regulatory gene, *CFH*, *CFI*, *CD46* (*MCP*) or *THBD*, or a gain-of-function mutation in an effector gene, *CFB* or *C3*. The overlap of TTP and aHUS clinical characteristics makes the differential diagnosis challenging.

Advances in next-generation sequencing (NGS) have improved the molecular study of large-size genes, such as *VWF* or the large number of genes implicated in TMAs.

With this in mind, we conducted a study in a haemorrhagic disease (VWD) and a thrombotic disease (TTP/aHUS) with two main objectives: first, to expand our understanding of the molecular basis of different types of VWD, TTP and aHUS to establish genotype-phenotype correlations; second, to develop a diagnosis workflow based on VWF/ADAMTS13 activity and screening of mutations using a custom NGS gene panel. This approach allowed for the creation of a new algorithm that uses an efficient and cheaper methodology to establish the diagnosis, prognosis and more accurate treatment for these diseases.

In Chapter 3, we explored genotype and phenotype characteristics of a cohort of 92 patients with VWD with the aim of analysing the distribution of different *VWF* mutations in different types of VWD and correlate them with clinical disease severity.

Moreover, the prediction of the pathogenicity of variants was assessed by in silico tools and the recommendations of the American College of Medical Genetics and Genomics that was crucial for understanding the great inter-individual variability.

In Chapter 4, we evaluated VWF collagen-binding defects (types III and VI) in a cohort of nine type 2M VWD patients. The main objective was to analyse the value added by the incorporation of VWF:C^{III}B and VWF:C^{VI}B assays into the initial approach for studying VWD, and thus improve the detection of underdiagnosed VWF deficiencies.

In Chapter 5, we analysed six patients with acquired von Willebrand syndrome (AVWS) and hematologic malignancies: Waldenstrom's Macroglobulinemia (WM), Marginal Zone Lymphoma (MZL), Chronic Myeloid Leukaemia (CML) and Essential Thrombocythemia (ET). The main objective was to validate a clinical-laboratory approach for detecting this uncommon and/or underdiagnosed acquired bleeding disorder.

In Chapter 6, to better understand the genotype-phenotype correlations, we detailed phenotypic characterisation in our cohort of 45 patients with TMAs (11 TTP and 34 aHUS) who had mutations in *ADAMTS13* and/or in complement genes. For this purpose, we designed and validated an NGS-based gene panel (*ADAMTS13, CFH, CFHR1, CFHR3, CFHR4, CFHR5, CFI, CFB, C3, THBD* and *DGKE*) to facilitate genetic testing in TTP and aHUS. We also evaluated the impact of the predicted pathogenicity of each variant found and the co-inherited known genetic risk factors for TMAs.

CHAPTER

3

GENOTYPE–PHENOTYPE CORRELATION IN A COHORT OF PORTUGUESE PATIENTS COMPRISING THE ENTIRE SPECTRUM OF VWD TYPES: IMPACT OF NGS

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CHAPTER 3. GENOTYPE–PHENOTYPE CORRELATION IN A COHORT OF PORTUGUESE PATIENTS COMPRISING THE ENTIRE SPECTRUM OF VWD TYPES: IMPACT OF NGS

3.1. Summary

The diagnosis of von Willebrand disease (VWD), the most common inherited bleeding disorder, is characterised by a variable bleeding tendency and heterogeneous laboratory phenotype. The sequencing of the entire VWF coding region has not yet become a routine practice in diagnostic laboratories owing to its high costs. Nevertheless, next-generation sequencing (NGS) has emerged as an alternative to overcome this limitation. We aimed to determine the correlation of genotype and phenotype in 92 Portuguese individuals from 60 unrelated families with VWD; therefore, we directly sequenced VWF. We compared the classical Sanger sequencing approach and NGS to assess the value-added effect on the analysis of the mutation distribution in different types of VWD.

Sixty-two different VWF mutations were identified, 27 of which had not been previously described. NGS detected 26 additional mutations, contributing to a broad overview of the mutant alleles present in each VWD type. Twenty-nine probands (48.3%) had two or more mutations; in addition, mutations with pleiotropic effects were detected, and NGS allowed an appropriate classification for seven of them. Furthermore, the differential diagnosis between VWD 2B and platelet type VWD (n = 1), Bernard–Soulier syndrome and VWD 2B (n = 1), and mild haemophilia A and VWD 2N (n = 2) was possible.

NGS provided an efficient laboratory workflow for analysing VWF. These findings in our cohort of Portuguese patients support the proposal that improving VWD diagnosis strategies will enhance clinical and laboratory approaches, allowing to establish the most appropriate treatment for each patient.

Keywords:

Gene mutations, von Willebrand disease, von Willebrand factor, molecular biology methods, NGS

3.2. Introduction

Von Willebrand disease (VWD) is the most common inherited bleeding disorder, with a reported incidence of 0.01–1% (Sadler *et al*, 2006; Batlle J *et al*, 2011). This disease is caused by a deficiency of von Willebrand factor (VWF), an adhesive glycoprotein of large dimensions with crucial functions in haemostasis derived from its ability to organise in multimers. The high-molecular-weight (HMW) multimers of VWF are essential for primary haemostasis, i.e., mediating a molecular endothelium–platelet bridge for binding collagen and the platelet receptors GPIb and GPIIb/IIIa. In addition, VWF binds and stabilises factor VIII (FVIII) in the circulation, protecting it from rapid proteolytic degradation while delivering it to sites of vascular damage. As a result of this physiological process, VWF insufficiencies may cause haemorrhage by reducing platelet function or by reducing the FVIII concentration (Batlle J *et al*, 2011; Sadler, 2009; Sadler *et al*, 2006).

The diagnosis of VWD is based on the nature of the VWF deficiency. VWD is classified into three different types: partial or complete VWF quantitative deficiencies (types 1 and 3, respectively) and qualitative deficiency (type 2). Type 2 VWD is characterised by functional defects that lead to impaired activity and is accordingly divided into four secondary categories (2A, 2B, 2M and 2N). Functional defects lead to enhanced (2B) or reduced (2A, 2M) platelet interaction or impaired binding to FVIII (2N); structural defects due to impaired dimerisation/multimerisation can lead to type 2A/IID, 2A/IIC and 2A/IIE VWD (Sadler *et al*, 2006; Schneppenheim, 2011). Type 2A VWD can also be caused by mutations that impair the production of the larger multimers (Group I) and those with normal synthesis and release but with increased proteolytic susceptibility to ADAMTS13 (Group II) (Michiels & van Vliet, 2009). All these defects can be identified by laboratory tests that assess the functional characteristics of VWF. It is crucial to correctly diagnose the subtype of VWD because the therapeutic approaches are different for each subtype. However, this diagnosis remains difficult because the clinical and laboratory phenotypes are very heterogeneous, and a clear discrimination between some of these subtypes can be challenging (Castaman *et al*, 2014; Budde U, Favaloro E, 2011).

Molecular analysis of *VWF* is very useful in the diagnosis and classification of VWD; type 2 mutations are localised to specific functional domains in exons 18–25 (type 2N), exon 28 (types 2A, 2B and 2M) and exons 11–16, 24–26 and 51-52 (type 2A), whereas the type 1 and type 3 VWD mutations require analysis of all the essential *VWF* regions. Moreover, compound heterozygosity for *VWF* mutations in different functional domains of the VWF molecule causes pleiotropic effects and produces different phenotypes (Corrales *et al*, 2009; Goodeve, 2010).

To elucidate the clinical variability, which is found even in the same family, it is necessary to identify the mutational profile; however, the large size of *VWF* has been a disadvantage for studying using conventional Sanger DNA sequencing in diagnostic laboratories owing to its high costs. Nevertheless, the prospects for molecular diagnosis will be improved with the introduction of next-generation sequencing (NGS) platforms; massively parallel sequencing has reduced the cost and increased the throughput of DNA sequencing (Corrales *et al*, 2012; Goodeve, 2014).

With this in mind, we conducted a study with two main objectives: first, to better understand the genotype–phenotype correlation, we detailed the phenotypes of 92 Portuguese patients from 60 unrelated families with different types of VWD and *VWF* mutations; second, to decide the most appropriate strategy for VWD diagnosis in our Department of Haematology, including feasibility and costs, we compared the results obtained using conventional Sanger sequencing and NGS. This approach allowed the creation of a new algorithm that uses an efficient and cheaper methodology to establish the diagnosis, prognosis and a more accurate treatment for VWD.

3.3. Materials and Methods

3.3.1. Patients and controls

This study included a group of 92 patients of Portuguese origin belonging to 60 apparently unrelated families; the patients were diagnosed with different types of VWD between 2007 and 2014. Sixty probands and 32 affected relatives (parents and siblings) were distributed as follows: 56 patients (31 families) regularly attended the

Department of Haematology at Centro Hospitalar Universitário de Coimbra for clinical and laboratorial follow-up. The remaining 36 patients (29 families) were referred from external centres in different regions of Portugal: Lisbon (three central hospitals, one paediatric) and Faro (one central hospital). These hospitals all diagnose and treat patients with VWD and had sent samples from patients with different types of VWD to our centre for confirmation of their results, for multimer pattern analysis and for molecular study. The patients included in this study were from the central and southern regions of Portugal.

The clinical characteristics of patients were recorded through a validated questionnaire focusing on personal and family history of significant mucocutaneous haemorrhage using bleeding scores (BSs) calculated with the *bleeding assessment tools* (BAT) recommended by the International Society on Thrombosis and Haemostasis and Standardization Committee on von Willebrand Factor guidelines (Rodeghiero *et al*, 2009b). In accordance with the Declaration of Helsinki, informed consent was obtained from all probands and their family members.

The 92 individuals in this study included both adults and children with a median age of 28 years (1–76) and a sex distribution of 58F : 34M. The adult probands (n = 42) had a median age of 41 years with a range of 19–76 years, and the child probands (n = 18) had a median age of 8 years with a range of 1–15 years.

Forty-two healthy volunteers acted as a control group for measurements of the PFA-100 closure time (PFA:ADP; PFA:Epi), FVIII coagulant activity (FVIII:C), VWF antigen level (VWF:Ag), ristocetin cofactor activity (VWF:RCo) and/or VWF collagen binding (VWF:CB) (Table 1). The control group included unrelated individuals (32F : 10M) without an individual or family history of mucocutaneous haemorrhage, with a mean age of 33.5 ± 11.12 yrs.

3.3.2. Samples and sample processing

Functional and immunological studies were performed on blood collected into vacuum tubes containing 3.2% sodium citrate and centrifuged within 15 min at room

temperature for 20 min at 2500 g. The obtained platelet-poor plasma was then separated into aliquots and kept frozen at −80°C until use. The separated plasma was later subjected to coagulation and multimer assays. Genomic DNA was extracted from EDTA whole blood by automatic isolation on an iPrep^{TM} instrument using a gDNA Blood Kit (Invitrogen, Carlsbad, USA). The DNA concentration was adjusted to a range of 25–50 ng/µl.

3.3.3. Algorithm for phenotypic analysis - coagulation and multimer assays

VWF analysis was performed according to an algorithm based on established recommendations (Castaman *et al*, 2014): (i) *screening tests* – platelet function analysis via the PFA-100 closure time (Siemens Healthcare, Marburg, Germany) in addition to measurements of the FVIII:C level, VWF:Ag level, VWF-platelet GPIb binding activity (Innovance) and VWF:RCo that were performed on a BCS XP coagulation analyser according to the manufacturer's instructions (Siemens Healthcare, Marburg, Germany); (ii) *specific tests* – to determine disease subtype, ristocetin-induced platelet aggregation (RIPA) in platelet-rich plasma with final ristocetin concentrations of 0.5 mg/ml and 1.2 mg/ml was performed using an in-house aggregometry assay. VWF:CB was measured (except in type 3 and 2N VWD) using type III collagen (Technozym, Austria), and the capacity of VWF to bind exogenous FVIII (VWF:FVIIIB) (Stago, France) was evaluated using ELISA. The multimer pattern was evaluated by SDS-agarose gel electrophoresis (low-resolution gel electrophoresis using 0.9% low-gelling temperature agarose or medium resolution using 1.6% agarose), followed by Western blotting and detection with rabbit anti-human VWF antibody (Dako, Glostrup, Denmark) using the alkaline phosphatase staining method (Pérez-Rodríguez *et al*, 2009).

The potential probands with VWF deficiency were diagnosed by evaluating the following criteria: presence of reduced (< 30%) VWF:RCo levels; a discrepant VWF:RCo/VWF:Ag ratio of < 0.7 that suggested type 2 VWD; a discrepant FVIII:C/VWF:Ag ratio of < 0.5 that suggested type 2N VWD; the absence of VWF (or

presence of only trace amounts) and a FVIII:C below 5% that distinguished type 3 VWD from severe type 1 VWD (Sadler *et al*, 2006).

The rate of VWF mutations found in type 1 VWD decreases when the VWF level is > 30% (Sadler *et al*, 2006). To avoid high costs, in our approach, patient samples with borderline VWF levels were not pursued for molecular analysis and Sanger sequencing.

3.3.4. Alloantibodies to VWF

To test alloantibodies to VWF in type 3 patients, an in-house ELISA-based assay was used as previously described (Siaka *et al*, 2003).

3.3.5. Strategy for VWF mutation analysis

The molecular analysis in this study used two different direct sequencing methodologies:

(i) Sanger direct sequencing: For the seven years of this study, the following sequences were determined using an approach based on VWD subtypes: type 1 severe and type 3 VWD – all coding *VWF*; types 2B and 2M VWD - only exon 28; type 2A - first exon 28 and then exons 11–16, 24–26 and 51–52 if there was no mutation in exon 28; and type 2N - exons 18–25. To confirm differential diagnosis between VWD 2B and platelet type VWD (PT-VWD), exon 2 of *GP1BA.*

(ii) NGS: The coding *VWF* [regions of interest (ROIs)] were analysed in all samples (DNA samples were sent to Unitat de Diagnòstic i Teràpia Molecular, Banc de Sang i Teixits (BST, Barcelona)).

3.3.5.1. Sequencing of VWF using NGS and identification of genetic variants

A recently described automated method for the Access Array™ platform (Fluidigm, South San Francisco, California, USA) derived from a previous NGS protocol (Corrales *et al*, 2012) was used for *VWF* amplification. Briefly, the resulting products from all patients included in this study were pooled and simultaneously sequenced in a MiSeq

Desktop Sequencer (Illumina, San Diego, California, USA) run. Barcoded sequences were de-multiplexed and individually analysed. The ROIs included in our NGS design were approximately 1300 bp of the *VWF* promoter region, exons 1–52 and the intronic flanking regions (at least 20 bp). The depth of coverage and primer design is described in detail in the Suppl. Material. The NGS pipeline output, in paired sequence files (FASTQ format), was used as an input for the analysis, which began with CLC Genomic Workbench software (Qiagen) and then proceeded to VariantStudio (Illumina). The optimal analysis parameters (coverage, minor allele counts, percent of variant alleles, etc.) were adjusted to obtain the optimal performance for variant detection (see Suppl. Material). This workflow allows the alignment of the resulting sequences against the human genome sequence (hg19) and concurrent *in silico* analysis, permitting the identification of potential pathogenic variants, discriminating pseudogene sequences and filtering the known polymorphisms described to date in the dbSNP (National Center for Biotechnology Information dbSNP (build 137)) and 1000 Genomes databases (The 1000 Genomes Project Consortium, 2015).

For confirmation of the variants identified by NGS, the corresponding region was amplified using PCR and sequenced by Sanger direct sequencing, as described in the Suppl. Material.

3.3.5.2. Multiplex ligation-dependent probe amplification (MLPA)

DNA samples from patients lacking identified pathogenic variants using PCR/direct sequencing, were screened using SALSA MLPA P011 and P012 VWF kits (version B2; MRC-Holland) to detect deletions/duplications in *VWF*. Fragment size analysis was performed using an ABI 3130 Genetic Analyzer (Applied Biosystems). Data normalisation was performed using four healthy controls and Coffalyser. Net software (MRC-Holland) was used for analysis using the fragment analysis files (.fsa) obtained from the Applied Biosystems Genetic Analyzer as input.

3.3.5.3. Mapping of the VWF exon 31 deletion breakpoints

The exact locations of the deletion breakpoints in one type 1 VWD proband in whom the mutation was initially identified by MLPA were determined by primer walking followed by Sanger sequencing. The genomic region from the end of exon 30 to intron 31 was amplified using 5 primer sets. The following primer pair sequences were used in the specific assay for the detection of the deletion: forward primer 5'-GCTGCAGTATGGAAGCATCA-3' and reverse primer 5'-GAGTTGAGGTGAGGCTGGAG-3'.

3.3.5.4. In silico analysis

The impact of missense changes was further analysed according to the recommended criteria that included evolutionary conservation of an amino acid or nucleotide, location and context within the protein sequence and the biochemical consequence of the amino acid substitutions (Richards *et al*, 2015; Gonzalez-Garay, 2014). This analysis was assessed using five different *in silico* algorithms: PROVEAN, SIFT, PolyPhen-2, MutationAssessor and MutationTaster (Suppl. References) (last accessed 21 September 2015). Missense variants were considered deleterious if at least three of the five prediction programs suggested a pathogenic effect.

Potential disease-causing variants due to aberrant splicing were evaluated using a set of five different *in silico* algorithms: Human Splicing Finder (HSF), MaxEntScan, NNSPLICE, NetGene2 and Splice View (Suppl. References) (last accessed 21 September 2015). This approach improves the accuracy of the splicing analysis of intronic, deep intronic and exonic variants that may directly disrupt constitutive splice sites or indirectly create cryptic splice sites (Jian *et al*, 2014; Wallis *et al*). MutPred Splice was used to confirm probable exonic splicing variants (Mort *et al*, 2014). A local splice site effect was considered potentially deleterious when at least three (intronic variants) or four (exonic variants) of these predictions for the variant vicinity were significant.

3.3.5.5. Genetic databases

As recommended by the guidelines (MacArthur *et al*, 2014; Richards *et al*, 2015; Wallis *et al*), we defined those variants that are not reported in the international databases (population and disease specific) as well as in published literature as 'novel'. Therefore, in addition to using the polymorphism databases (dbSNP and 1000 Genomes databases) mentioned above, we checked variants for inclusion in the Exome Aggregation Consortium (ExAC) (Exome Aggregation Consortium (ExAC), Cambridge, MA), Exome Variant Server (EVS) (Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA), Human Gene Mutation Database (HGMD Human gene mutation database (HGMD® Professional) from BIOBASE Corporation) and VWF international mutation databases (International Society on Thrombosis and Haemostasis-Scientific and Standardisation Committee of von Willebrand Factor (ISTH-SSC); VWF Online Data-base (VWFdb); European Association for Haemophilia and Allied Disorders (EAHAD); Coagulation Factor Variant Databases, Hemobase).

3.3.5.6. Assessment of the pathogenicity of variants

The following criteria were used to evaluate the pathogenicity of the variants: 1) whether the variant was a stop/frameshift variant, which was considered to most likely be disease causing, 2) co-segregation in the family, 3) whether the variation had been previously identified in international databases, 4) *in silico* evaluation and 5) presence of the second mutant allele in the case of autosomal recessive inheritance.

Variants were classified as pathogenic, likely pathogenic, uncertain significance, likely benign or benign based on the available evidence, according to the practice guidelines for the evaluation of pathogenicity recently published by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (Richards *et al*, 2015).

The amino acid numbering and nomenclature used is according to the international recommendations for the description of sequence variants of the Human Genome

Variation Society (http://www.HGVS.org) and applied to genetic variants in haemostasis (Goodeve *et al*, 2011).

3.3.6. Statistical analysis

Differences between groups were analysed by chi-squared testing for categorical variables and one-way ANOVA followed by Bartlett's test for all linear variables. Tests were assumed significant whenever the 2-tailed P-value was < 0.05. These statistical analyses were performed using GraphPad Prism 6.0 for Windows (GraphPad Software, La Jolla, CA, USA; www.graphpad.com).

3.4. Results

3.4.1. Laboratory haemostasis findings

The present study included a cohort of 60 probands from different regions of Portugal with severe/intermediate types of VWD. According to the study algorithm, in the first approach, a standardised BS and VWF:Ag, VWF:RCo and FVIII:C levels were taken into account to diagnose these patients. BS showed an overall inverse correlation with the plasma levels of VWF:Ag (P = 0.0022, Spearman's rho = 0.3822), VWF:RCo (P = 0.0013, Spearman's rho = 0.3991) and FVIII:C (P = 0.0020, Spearman's rho = 0.3829).

Forty-eight probands presented with low levels of VWF:RCo (9.15 ± 9.19%), and 12 presented with a reduced FVIII:C/VWF:Ag ratio (0.36 ± 0.15); these features prompted a detailed phenotypic study and mutational analysis.

The phenotypic characteristics of the probands were mainly distributed as follows: severe type 1 VWD (n = 7), type 3 VWD (n = 15) and type 2 VWD (n = 38). Specific tests indicated the subdivision of type 2 VWD as follows: type 2A (n = 9), type 2B (n = 6), type 2M (n = 11) and type 2N (n = 12) (Table 4). Familial studies (n = 32) included 25 affected relatives, three type 3 carriers and four type 2N carriers (data not shown).

Table 4 – Demographic and phenotypic data of 60 probands at diagnosis.

	Type 1 (n = 7)	Type 3 (n = 15)	Type 2N (n = 12)	Type 2A, 2B, 2M (n = 25)	P-value
Male/Female	4/3	1/14	5/7	8/17	0.0384
Median age, years (range)	17 (1-34)	15 (1-55)	41 (13-69)	36 (2-76)	NS
Median bleeding score (range)	14 (5-18)	18 (10-23)	12.5 (2-21)	11 (1-24)	NS
PFA (COL/ADP), s PFA (COL/Epi), s	>300 >300	>300 >300	121 (99-188) 96 (82-125)	>300 >300	NS
FVIII:C* (%) (range)	26 (11-33)	2.1 (<0.25-6)	27.5 (7-38)	37.5 (15-100)	< 0.0001
VWF:Ag* (%) (range)	9.9 (3-16)	<4	68.0 (45-120)	30.0 (14-85)	< 0.0001
VWF:RCo* (%) (range)	7.2 (2-15)	<4	64.0 (47-100)	11.0 (<4 -32)	< 0.0001
VWF:RCo/VWF:Ag* (range)	0.8 (0.67-1.00)	-	1.0 (0.83-1.11)	0.4 (0.14-0.73)	< 0.0001
VWF:CB* (%) (range)	4.5 (2-16)	-	-	13.0 (<4 -53)	< 0.0001

Normal range: PFA (COL/ADP), 93-161 s; PFA (COL/Epi), 67-99 s; FVIII:C, 49-149%; VWF:Ag, 50-160%; VWF:RCo, 48-173% and VWF:CB, 60-130%. PFA-platelet function analysis; s - seconds; *Median and range; Chi-squared testing for categorical variables; One-way ANOVA followed by Bartlett's test for all linear variables; NS, no significance.

The NGS method confirmed all variants detected by the Sanger method (100% sensitivity) and detected 26 additional variants: six missense, three synonymous and 17 intronic [15 of 17 (88%) were deep intronic]. In total, 62 different variants, whose frequency in the different populations studied in 1000 Genomes was below 1%, are summarised in Suppl. Table 1: 30 missense variants (48.5%), three nonsense (4.8%), two small deletions (3.2%), one large deletion (1.6%), one duplication (1.6%), five synonymous (8%) and 20 intronic variants (32.3%). The frequencies of those variants were also checked in the ExAC and EVS_EA population databases, which revealed differing values for some variants (Suppl. Table 1).

In total, 27 variants had never been reported in the population databases and international *VWF* databases (Suppl. Table 1).

3.4.2. Prediction of pathogenic variants

Twenty-six out of 31 missense variants were predicted to be deleterious (Suppl. Table 2). Fifteen of 56 variants (missense, synonymous and intronic) were detected using some *in silico* tools with probable impact on splicing; however, only six reached the required score (≥ 3 for intronic and ≥ 4 for exonic mutations) and were considered potential splice site variants (Suppl. Table 3). According to the practice guidelines (Richards *et al*, 2015), these *in silico* analyses combined with other evidence data (population, functional and segregation) permitted the following classification of the

variants: pathogenic (n = 29; 46.9%), likely pathogenic (n = 4; 6.4%), uncertain significance (n = 4; 6.4%), likely benign (n = 18; 29%) and benign (n = 7; 11.3%) (Suppl. Table 1). Accordingly, for the classification of these variants, we used the term 'mutation' only to refer to pathogenic variants.

Variants were identified in all probands of this cohort (100%), and 29 (48.3%) had two or more variants along *VWF*.

3.4.3. Potential functional impact of novel mutations

Eleven of the 27 novel variants (41%) were pathogenic: five missense changes (c.440A>G, p.Gln147Arg; c.2637C>A, p.Asp879Glu; c.4117G>T, p.Asp1373Tyr; c.5140G>C, p.Ala1714Pro; and c.7400A>C, p.Gln2467Pro); two nonsense mutations (c.4666C>T, p.Gln1556*; c.7086C>A, p.Cys2362*); two small deletions (c.100delT, p.Arg34Aspfs*49; c.5414_5415del, p.Val1805Glyfs*8); one large deletion [c.5312-104-_5455+642del (890 bp)] and one potential splice site mutation at the consensus 5'-GT donor splice site (c.1533+1G>A) (Suppl. Table 1). The remaining 16 variants accounted for two missense changes that were signalled as benign variants (c.3590C>A, p.Pro1197Gln; c.3686T>C; p.Val1229Ala) and 14 deep intronic variants that were considered with uncertain significance (2) and likely benign (12) (Suppl. Table 1).

3.4.4. Phenotype–genotype correlations

The probands' phenotypic and molecular data were separated according to the VWD type, and only the variants with predicted pathogenicity (mutations) are summarised in Tables 5–9. For four probands, the phenotype-based classification was changed after mutation analysis.

3.4.4.1. Type 1 VWD

Seven probands had a severe type 1 VWD profile with very low VWF:Ag and VWF:RCo levels but with moderately reduced FVIII:C levels (Table 5). In fact, five of these seven patients had an increased FVIII:C/VWF:Ag ratio with a median value of 3.7 (range 2.6–4.5). Thirteen variants were distributed along several regions of *VWF* with alterations

in domains D1, D2, A3, D4, C2, C3 and C4 (Figure 7). As shown in Table 5, 10 mutations (five not previously described) were associated with this phenotype.

Table 5: Phenotypic and molecular data of seven probands with severe type 1 VWD.

Laboratory values								**Molecular data**			
Probands ID (n)	Gender/age (M/F year)	Bleeding Score	FVIII:C (%)	VWF: Ag (%)	Ratio*	VWF: RCo (%)	VWF: CB (%)	Exon/ Intron	Nucleotide Change	Amino Acid Change	Dom.	Gen.
P17 (1)	M/24	18	11	3	3.7	2	2	43	**c.7400A>C**	**p.Gln2467Pro**	C3	Hmz
P19 (1)	M/27	12	26	16	1.6	15	16	31	**c.5312-104_ 5455+642del**		A3	Htz
P65 (1)	F/34	6	26	10	2.6	7	6	38	c.6699_6702 dupAGGC	p.Cys2235 Argfs*8	D4	Htz
P18 (1)	M/5	4	33	11	3.0	9	NA	5	**c.440A>G**	**p.Gln147Arg**	D1	CHtz
								42	**c.7086C>A** ‡	**p.Cys2362***	C2	
P15 (1)	F/5	16	31	7	4.4	5	3	15	c.1892C>T	p.Ala631Val	D2	Htz
								46/45	c.7730-4C>G‡		C4	Hmz
P12 (1)	M/10	16	27	6	4.5	6	3	38	c.6699_6702 dupAGGC	p.Cys2235 Argfs*8	D4	CHtz
								43	c.7437G>A‡	p.(=)	C3	
P48 (1)	F/5	5	17	13	1.3	10	12	29	**c.5140G>C**	**p.Ala1714Pro**	A3	CHtz
								29/29	c.5170+10C>T‡		A3	

*Ratio, FVIII:C / VWF:Ag; NA- not available; the mutations marked in **bold** were not previously reported; ‡ indicates mutation in *trans*; Dom., Domain; Gen., Genotype; Hmz, homozygous; Htz, Heterozygous, CHtz, compound heterozygous

All probands except three had compound heterozygous mutations: one had a novel homozygous missense mutation, p.Gln2467Pro, located in the C3 domain, another had a novel heterozygous large deletion of exon 31 located in the A3 domain, and the third had a duplication mutation c.6699_6702dupAGGC, p.Cys2235Argfs*8. MLPA analysis detected the large deletion, and PCR followed by direct DNA sequencing mapped the intronic breakpoints c.5312-104_5455+642del, indicating that the deletion was 890 bp in size (Figure 8).

The remaining four probands showed compound heterozygosity in trans for missense and null mutations (nonsense and splice site). Two probands had a recessively inherited missense mutation p.[Gln147Arg];[Cys2362*] and [p.Ala631Val];[c.7730-4C>G] located in the VWF propeptide (VWFpp) region (D1–D2 domain). Both had a typical multimeric pattern with a heavy predominance of VWF dimers (IIC). The proband P12 had a duplication mutation c.6699_6702dupAGGC, p.Cys2235Argfs*8 and the synonymous mutation c.7437G>A in the splice region, resulting in severely

decreased VWF:Ag levels. The same duplication mutation was detected in the homozygous state in type 3 VWD patients (Table 6).

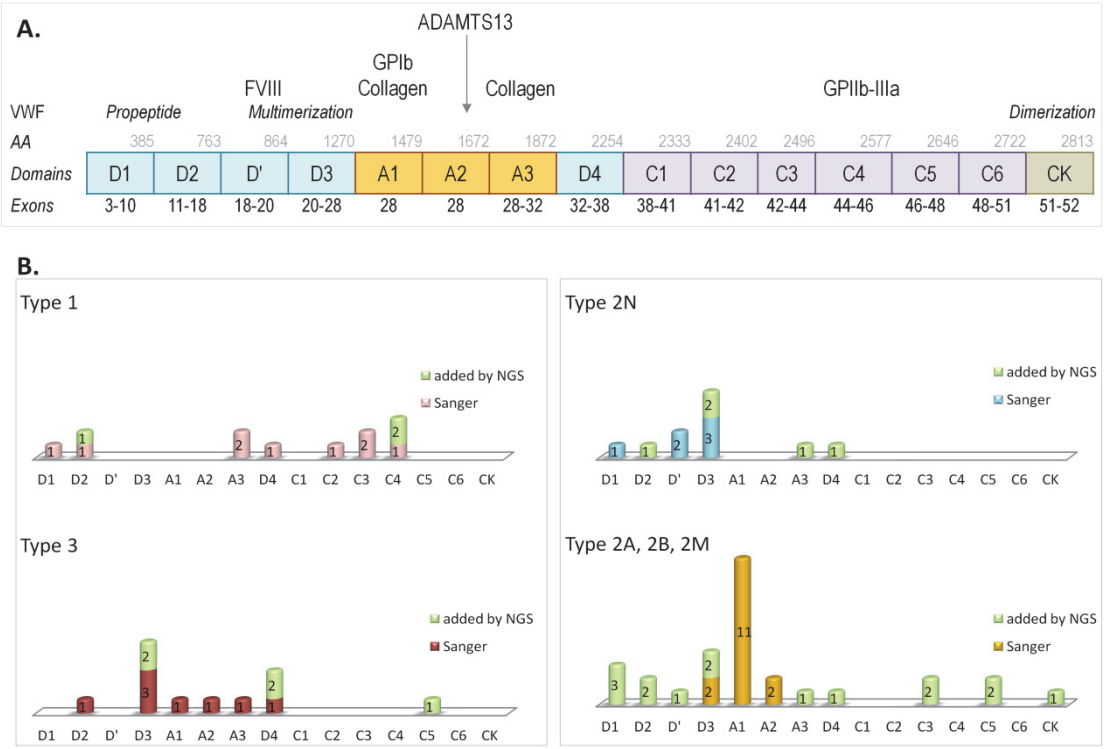

Figure 7 – Distribution of 62 different VWF mutations identified in 60 probands with VWD. (A) The scheme represents *VWF* and the corresponding domains of the VWF protein (Zhou *et al*, 2012; Lenting & Christophe, 2015). (B) The graphics show the mutation distribution associated with the VWF domains for severe type 1, type 3, type 2N and type 2A, 2B, 2M VWD. Each graphic discriminates between the mutations previously identified by Sanger sequencing and the mutations added by NGS. No evident cluster of alterations was observed for type 1 and type 3 VWD, and the spreading along *VWF* was accentuated. For type 2N VWD, a mutation cluster in the region coding for D'-D3 was preserved. For type 2A, 2B and 2M VWD, mutation spreading was evident, but a mutation cluster emerged in the region coding for the A1 domain.

Finally, the proband P48 had a compound heterozygosity in *trans* for the novel missense mutation p.Ala1714Pro in The A3 domain and the potential splice site variant c.5170+10C>T. This missense mutation in the proband's mother was inherited as a dominant trait with a type 2M VWD phenotype, normal multimeric pattern and low VWF:CB/VWF:Ag ratio (0.54), thus showing a pleiotropic effect.

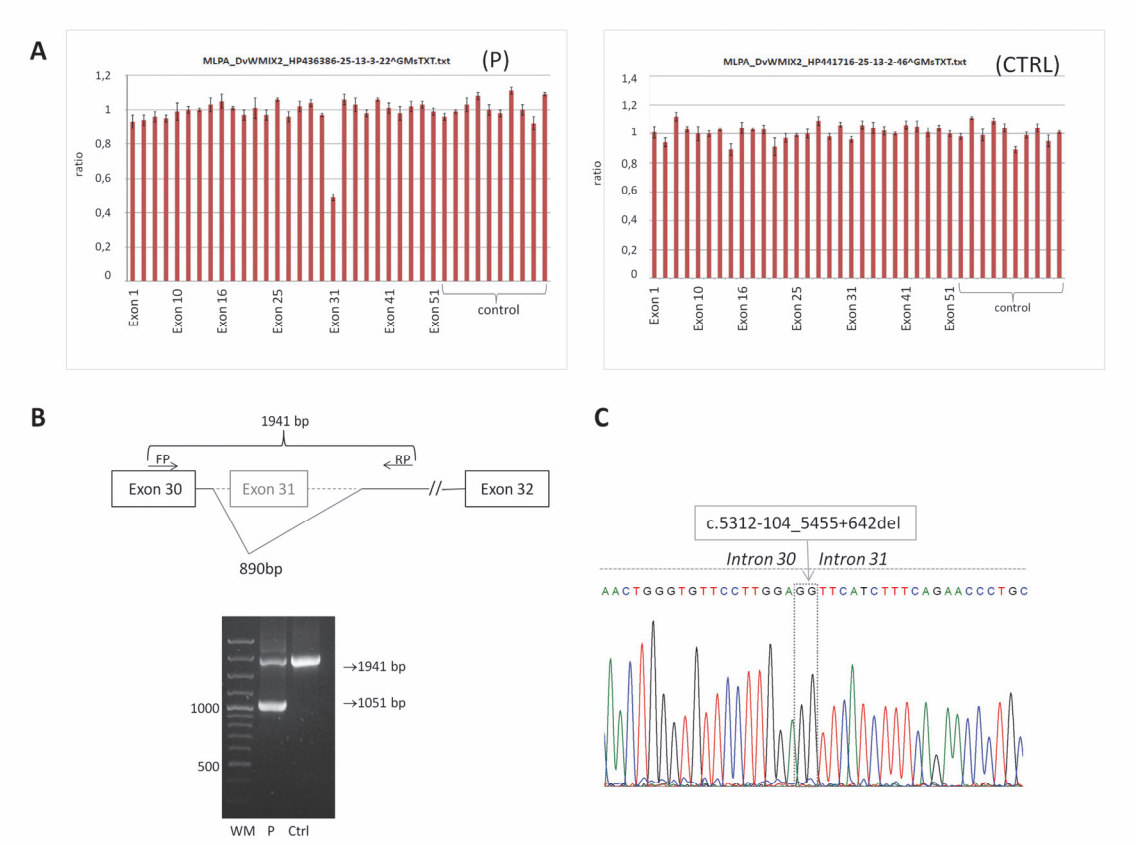

Figure 8 – Detection of the VWF exon 31 deletion breakpoints. (A) Detection of exon 31 deletion following dosage analysis using MLPA in the proband (P) and a healthy control (CTRL). (B) The PCR designed to detect a deletion (890 bp) shows the heterozygous state in the proband (P). FP - forward primer; RP, reverse primer and (C) deletion breakpoints identification (c.5312-104_5455+642del) by Sanger sequencing.

3.4.4.2. Type 3 VWD

The severity of 15 probands with type 3 VWD was evidenced by the low median age (15 years) and higher BSs, which were consistent with the low levels of FVIII:C (median 2%, range < 0.25–6), VWF:Ag (< 4%) and VWF:RCo (< 4%) and the absent VWF multimeric pattern (Tables 4, 6). Mutations were identified in 14 probands, 13 of whom were homozygous for only one mutation (93%). In total, 13 different variants and six mutations were scattered throughout *VWF* (Figure 7).

Three novel mutations were identified in our cohort of type 3 VWD patients: the nonsense mutation c.4666C>T (p.Gln1556*), the small deletion c.5414_5415delTG (p.Val1805Glyfs*8) and the splice site mutation c.1533+1G>A.

Table 6 – Phenotypic and molecular data of 14 probands with type 3 VWD.

Laboratory values						Molecular data				
Probands ID (n)	**Gender/age (M/F year)**	**Bleeding Score**	**FVIII:C* (%)**	**VWF: Ag*(%)**	**VWF: RCo*(%)**	**Exon/ Intron**	**Nucleotide Change**	**Amino Acid Change**	**Dom.**	**Gen.**
P2, P3, P4, P5, P63, (5)	F/21; M/24; F/15; F/1; F/21	21, 17,11, NA, 10	<2.5 (<2.5-4)	<4	<4	28	c.3931C>T	p.Gln1311*	A1	Hmz
P6, P7, P8, P9 (4)	F/13;F/12; F/41; F/46	21, 19, 22,13	<2.5 (<2.5-2.6)	<4	<4	28	**c.4666C>T**	**p.Gln1556***	A2	Hmz
P10 (1)	F/55	17	6	3	<4	31	**c.5414_5415 delTG**	**p.Val1805Glyfs*8**	A3	Hmz
P11, P13, P14 (3)	F/2; F/14; F/8	14, 22, 23	2.2 (2-6)	<4	<4	38	c.6699_6702 dupAGGC	p.Cys2235Argfs*8	D4	Hmz
P64 (1)	F/1	NA	<4	<4	<4	26	c.3437A>G	p.Tyr1146Cys	D3	CHtz
						13/13	**c.1533+1G>A**‡		D2	

*Median and range; NA- not available; the mutations marked in **bold** were not previously reported. ‡ indicates mutation in *trans*; Dom., Domain; Gen., Genotype; Hmz, homozygous; CHtz, compound heterozygous

The majority of the probands (13/15; 87%) were homozygous for a null mutation that comprised the nonsense mutations c.3931C>T (p.Gln1311*) and c.4666C>T (p.Gln1556*), the small deletion c.5414_5415delTG (p.Val1805Glyfs*8) and the duplication c.6699_6702dupAGGC (p.Cys2235Argfs*8). One proband was compound heterozygous for [p.Tyr1146Cys];[c.1533+1G>A]; a study of the parents allowed the tracing of the mutation inheritance in each allele: the father was heterozygous for p.Tyr1146Cys, showing a mild type 1 VWD, whereas the mother was heterozygous for c.1533+1G>A but was asymptomatic.

The remaining type 3 VWD proband and her brother were homozygous for a dinucleotide change c.3485_3486delinsTG that results in the variant p.Pro1162Leu, which affects the D3 domain; however, the real contribution of this variant to the severe quantitative phenotype must still be clarified. Thus, causative mutations for type 3 VWD were found in 14 out of 15 probands (93%).

Among those patients who were homozygous for the c.3931C>T (p.Gln1311*) mutation, one proband (P2) showed an ineffective response after replacement therapy, and the presence of an inhibitory alloantibody against VWF was confirmed by an anti-VWF ELISA assay. On the basis of these data, the patient received FVIII bypass therapy according to their clinical situation.

3.4.4.3. Type 2N VWD

All the 12 type 2N VWD probands had reduced FVIII:C levels with median levels of 27.5% (range 7–38), normal or near normal median VWF:Ag levels of approximately 68% (range 45–120), normal VWF:RCo levels of 64% (range 47–100) and a normal VWF multimeric pattern (Table 7). The reduced FVIII:C/VWF:Ag ratio (0.4; 0.07–0.6) was suggestive of type 2N VWD, which was confirmed by reduced VWF:FVIII binding and/or molecular studies.

In total, 11 different variants and seven mutations were found in the VWF regions affecting the domains D1, D', D3, A3 and D4. Despite this distribution, five missense mutations were clustered in exons 19 and 26 of *VWF,* which encode the FVIII-binding D'–D3 domains (Figure 7). The frequently described missense mutations c.2561G>A (p.Arg854Gln) and c.2446C>T (p.Arg816Trp) repetitively occurred in our cohort, being the most recurrent mutations (75% of cases) (Table 7).

Table 7 – Phenotypic and molecular data of 12 probands with type 2N VWD

Laboratory values							**Molecular data**				
Probands ID(n)	**Gender/age (M/F year)**	**Bleeding Score**	**FVIII: C*(%)**	**VWF: Ag*(%)**	**VWF: RCo*(%)**	**VWF: FVIIIB (%)**	**Exon**	**Nucleotide Change**	**Amino Acid Change**	**Dom.**	**Gen.**
P54 (1)	F/50	7	7	100	100	NA	19	c.2446C>T	p.Arg816Trp	D'	Hmz
P52 (1)	F/51	21	11	52	47	0.49	19	c.2446C>T	p.Arg816Trp	D'	Htz
							3	**c.100delT‡**	**p.Arg34Asp fs*49**	D1	Htz
P51; P55, P56,P58, P60, P61 P53 (7)	M/41;F/31;F/61; F/23; F/69; M/40, F/43	3; 10;14; 8;21;16;15	27.5 (19-33)	74.5 (45-88)	70.5 (50-93)	0-2	20	c. 2561G>A	p.Arg854Gln	D'	Hmz
P57 (1)	F/35	2	18	46	50	NA	20	c. 2561G>A	p.Arg854Gln	D'	Hmz
							39	c.6890C>T‡	Pro2297Leu	C1	Htz
P59 (1)	M/40	11	38	66	68	6	20	c.2561G>A	p.Arg854Gln	D'	CHtz
							20	**c.2637C>A‡**	**p.Asp879Glu**	D3	
P62 (1)	M/13	6	32	120	100	NA	19	c.2451T>A	p.His817Gln	D'	Htz
							26	c.3485_3486 delinsTG ‡	p.Pro1162Leu	D3	Hmz

*Median and range; NA- not available; the mutations marked in **bold** were not previously reported. ‡ indicates mutation in *trans*; Dom., Domain; Gen., Genotype; Hmz, homozygous; Htz, Heterozygous; CHtz, compound heterozygous

The missense mutation p.Arg816Trp was found in the two probands who had the lowest FVIII:C levels (Table 7), one in a homozygous state and the other compound heterozygous with a null allele p.[Arg816Trp];[Arg34Aspfs*49] (2N/3). Eight out of nine

individuals were homozygous for p.Arg854Gln, and one was compound heterozygous for a novel mutation, c.2637C>A (p.Asp879Glu).The FVIII:C/VWF:Ag ratio was not significantly different in homozygotes or heterozygotes for the p.Arg854Gln mutation. In a 13-year-old African male (P62) who was previously diagnosed with mild haemophilia A (based only on functional studies), heterozygosity for the missense variant c.2451T>A (p.His817Arg) and homozygosity for the variant c.3485_3486delinsTG (p.Pro1162Leu) were detected in the D' and D3 domains, respectively. The two variants, p.Pro1162Leu and p.His817Arg, located in the VWF region, which affects the FVIII binding capacity, could both contribute to the type 2N phenotype.

In this group of 2N VWD patients, two probands (P61 and P62) had previously been misclassified as having mild haemophilia A.

3.4.4.4. Type 2B VWD

The six probands classified as 2B VWD had a very low median VWF:RCo level of 11% (range 10–24) and a VWF:RCo/VWF:Ag ratio of 0.4 (0.35–0.50); the VWF multimeric patterns showed partial or complete loss of the HMW multimers (Table 8).

Table 8 – Phenotypic and molecular data of five probands with type 2B VWD and one with PT-VWD

Laboratory values											**Molecular data**		
Probands ID (n)	**(M/F year)**	**BS**	**FVIII: C*(%)**	**VWF: Ag*(%)**	**VWF: RCo*(%)**	**VWF: CB (%)**	**Ratio****	**Plat. X10^3/µl**	**RIPA**	**VWF Mult.**	**Exon**	**Nucleotide Change**	**Amino Acid Change**	**Dom.**
P26, P27, P28 (3)	F/41; F/11;F/30	20, 8,5	47 (23-78)	25 (20-30)	11 (10-12)	12 (9-16)	0.44 (0.40-0.50)	79, 230, 242	NA,E NA	CL	28	c.3916C>T	p.Arg1306Trp	A1
P25 (1)	F/41	5	26	29	11	13	0.38	15	E	CL	28	c.3916C>T	p.Arg1306Trp	A1
											20	c.2561G>A℘	p.Arg854Gln	D'
P50 (1)	F/11	10	37	60	24	22	0.35	130	NA	CL	28	c.3946G>A	p.Val1316Met	A1
P66 (1)	M/40	1	101	122	47	50	0.4	50	E	PL	2, *GP1BA*	c.751G>T	p.Asp251Tyr (Asp235Tyr)	

BS, Bleeding Score; *Median and range; **Ratio, VWF:RCo/ VWF:Ag; Plat., platelet; RIPA – ristocetin-induced platelet aggregation 0.5 mg/ml; NA - not available; E – enhanced; Mult., multimers; CL, complete loss and PL, partial loss of high-molecular-weight multimers; ℘ indicates mutation in *cis;* Dom., Domain

Among the six probands, four had thrombocytopenia (15, 50, 79 and 130 × 10^3 platelets/µL). Because the samples from three probands were provided by other centres, it was only possible to perform RIPA in the other three patients who showed an enhanced platelet aggregation in response to 0.5 mg/ml of ristocetin.

The frequently described mutations associated with VWD 2B, p.Arg1306Trp and p.Val1316Met, in the region coding for the A1 VWF domain, were found in five patients (Table 8 and Figure 7). One proband (P25) was referred to us with a suspected diagnosis of Bernard–Soulier syndrome because of the severe thrombocytopenia (15 × 10^3 platelets/µl) and the giant platelets observed in a peripheral blood smear. However, the coagulation multimer assays and molecular studies allowed a reclassification as type 2B VWD with a compound heterozygous *in cis* for mutations p.[Arg1306Trp;Arg854Gln]. The proband P50 was compound heterozygous in *trans* for p.Val1316Met with two other variants, the novel benign missense variant p.Pro1197Gln and c.5170+10C>T.

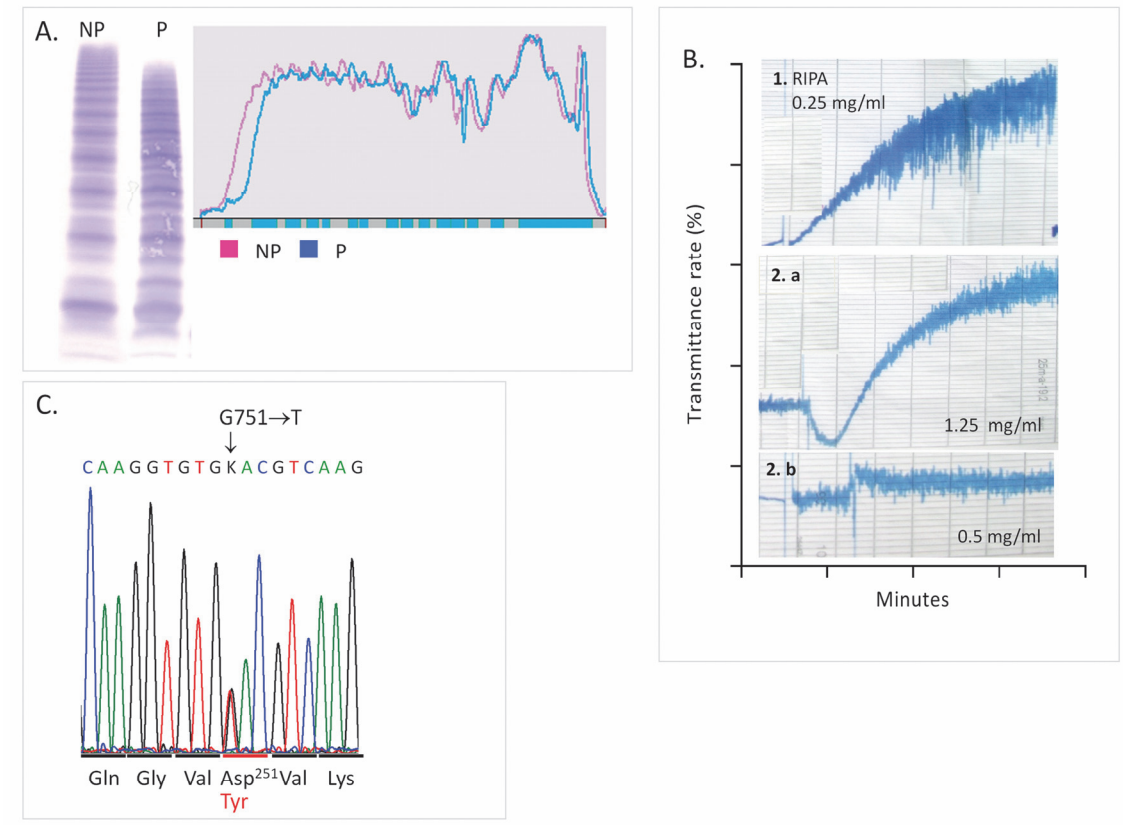

Figure 9 – Laboratory data of the PT-VWD patient. (A) Plasma VWF multimer analysis using 1.6% SDS-agarose gel electrophoresis and densitometry showing partial loss of the HMW multimers. NP = Normal plasma; P = Patient plasma; (B) 1. RIPA in the platelet-rich plasma of the patient showing aggregation with a low concentration of ristocetin (0.25 mg/ml); 2. RIPA mixing studies: a) patient plasma/control platelets showing a normal aggregation response at 1.25 mg/ml ristocetin and b) showing the lack of an aggregation response at 0.5 mg/ml; (C) *GP1BA* sequencing showing heterozygosity for c.751G>T, p.Asp251Tyr.

Finally, in proband P66, despite the VWF levels being consistent with type 2B VWD, no mutation was found in exon 28 *VWF.* The phenotype data included low VWF:RCo levels (47%), a VWF:RCo/VWF:Ag ratio of 0.4, partial loss of the HMW multimers and RIPA at 0.25 mg/ml. A differential diagnosis of probable platelet-type VWD (PT-VWD) was performed using simple RIPA mixing studies (control platelets in patient plasma), which revealed the absence of aggregation with 0.5 mg/ml of ristocetin. Therefore, a platelet-type phenocopy (pseudo-VWD) was considered. The sequencing of *GP1BA* revealed the presence of a previously described missense mutation, c.751G>T, p.Asp251Tyr in the heterozygous state in exon 2, confirming the reclassification of this patient as PT-VWD (Figure 9).

3.4.4.5. Type 2A and 2M VWD

The VWF:RCo and VWF:Ag levels corresponded to discrepant VWF:RCo/VWF:Ag ratios (0.39 ± 0.22) in these two groups of patients. The VWF:CB levels were similar to the VWF:RCo levels for type 2A VWD and to the VWF:Ag levels for 2M VWD. The VWF multimeric patterns in the type 2A VWD group showed the loss of the HMW multimers, and the pattern for the 2M VWD group showed the presence of all–size multimers (Table 9).

Probands with type 2A VWD had a significantly higher BS (median = 15) than those with type 2M VWD (median = 8.5) (P = 0.020, Mann–Whitney U test). In 20 probands, 10 different mutations were detected in exon 28, which encodes the VWF A1 and A2 domains (Figure 7). Among these five mutations (p.Arg1374Cys p.Arg1374His, p.Arg1399Cys, p.Ser1506Leu and p.Ile1628Thr) that occurred repetitively, p.Ile1628Thr was the most frequent mutation in 2A VWD (40% of cases) and p.Arg1374His was the most frequent in 2M VWD (37% of cases) (Table 9).

Of the nine probands with a 2A VWD phenotype, only one (P20) was heterozygous for the missense mutation in exon 26 (domain D3), c.3388T>C p.Cys1130Arg, with low levels of VWF:RCo (16%), a discrepant VWF:RCo/VWF:Ag ratio of < 0.7 and a characteristic 2A (IIE) VWF multimeric pattern (absence of large multimers and no

triplet structure of individual bands), which is indicative of a multimerisation defect (Table 9, Figure 10).

Table 9 – Phenotypic and molecular data of 20 probands with type 2A and 2M VWD.

Laboratory values										Molecular data			
Probands ID (n)	**Gender/age (M/F year)**	**BS**	**FVIII* (%)**	**VWF: Ag (%)***	**VWF: RCo (%)***	**Ratio**	**VWF: CB(%)**	**RIPA**	**Multimers/ subtype**	**Exon**	**Nucleotide change**	**Amino Acid Change**	**Dom.**
P20 (1)	F/36	19	27	22	16	0.7	10	D	LHMWM/ 2A/IIE	26	c.3388T>C	p.Cys1130Arg	D3
P23 (1)	F/58	15	22	31	7	0.23	8	Abs	LHMWM/ 2A	28	c.3814T>G	p.Cys1272Gly	A1
P24 (1)	M/22	10	23	22	3	0.14	4	Abs	LHMWM/ 2A	28	c.3815G>T	p.Cys1272Phe	A1
P42, P43 (2)	M/46; F/8	14;24	28; 37	21;14	12; 10	0.57; 0.71	4;10	D	LHMWM/ 2A/I	28	c.4517C>T	p.Ser1506Leu	A2
P44, P45, P46, P47 (4)	F/24; M/65 F/46; F/28	12;15 17;12	40 (34-49)	52 (42-65)	13 (10-23)	0.22 (0.15-0.35)	11 (6-19)	N	LHMWM/ 2A/II	28	c.4883T>C	p.Ile1628Thr	A2
P30 (1)	F/36	7	48	16	11	0.69	19	D	Normal/ 2M	28	c.3944G>A	p.Arg1315His	A1
										44	c.7464C>T‡	p.(=)	C3
P29 (1)	F/6	3	24	22	14	0.64	NA	NA	Smear/ 2M	28	c.3943C>T	p.Arg1315Cys	A1
P32, P33 (2)	F/48; F76	9;1	45; 87	70; 36	20; 13	0.17; 0.36	NA, 32	NA	Smear/ 2M	28	c.4120C>T	p.Arg1374Cys	A1
P34, P35 P36, P37 (4)	M/2; F/63;F/31	4; 9;3	37 (15-60)	26 (20-57)	8 (4-10)	0.3 (0.16-0.50)	20 (16-45)	NA	Normal/ 2M	28	c.4121G>A	p.Arg1374His	A1
P39, P40 (2)	F/42; M/9	12;7	100;82	85; 55	31; 32	0.36; 0.58	53; 37	N	Smear/ 2M	28	c.4195C>T	p.Arg1399Cys	A1
P38 (1)	M/32	7	51	31	19	0.61	40	N	Normal/ 2M	28	**c.4117G>T**	**p.Asp1373Tyr**	A1

*Median and range; Ratio VWF:RCo/VWF:Ag; RIPA – ristocetin-induced platelet aggregation1.25 mg/ml; Abs, absent; D, decreased; N, normal; NA - not available; LHMWM – loss of high molecular weight multimers; the mutation marked in **bold** was not previously reported; ‡ indicates mutation in *trans.* Dom., Domain

The probands P23 and P24 were heterozygous for two mutations affecting the same position and located in the first amino acid of the A1 loop domain: c.3814T>G, p.Cys1272Gly and c.3815G>T, p.Cys1272Phe. Interestingly, the mutation p.Cys1272Phe showed compound heterozygosity with a potential splice site variant (c.7288-68G>A) that could strongly activate a cryptic donor (Suppl. Table 1, Suppl. Table 3). Despite this mutation location being less common in type 2A VWD, the laboratory phenotype was consistent in these two probands: a marked decrease in VWF:RCo levels (5%–7%), a VWF:RCo/VWF:Ag ratio of < 0.18, a multimeric pattern showing a loss of high- and intermediate-molecular weight multimers (Figure 10) and RIPA at 1.25 mg/ml was absent.

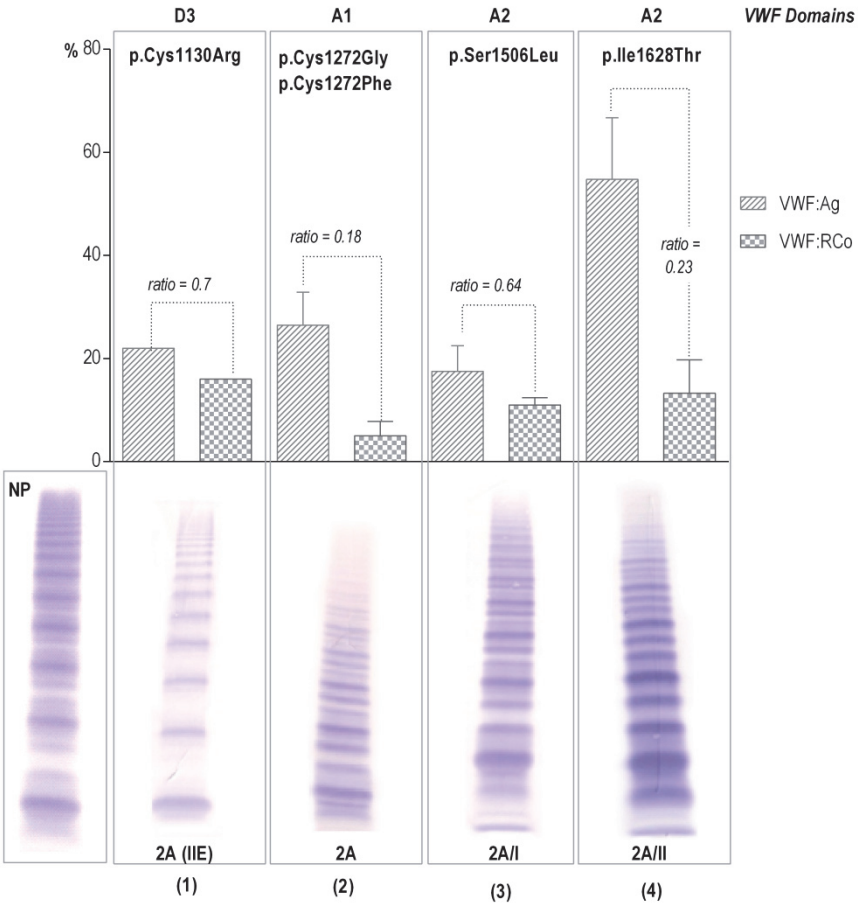

Figure 10 – Five missense mutations identified in probands with VWD 2A structural defects, their VWF:RCo/VWF:Ag ratios and the multimer VWF patterns. The VWF:RCo and VWF:Ag levels shows that p.Cys1130Arg and p.Ser1506Leu had a disproportionate VWF:RCo/VWF:Ag ratio that was close to 0.7; p.Cys1272Gly/Phe and p.Ile1628Thyr had lower values that were indicative of a higher extent of proteolysis. The VWF multimers analysed by SDS-agarose gel electrophoresis displayed a characteristic pattern in each mutation: (1) – multimerization defect (p.Cys1130Arg in domain D3) with the absence of large multimers and no triplet structure; (2) – change in protein folding (p.Cys1272Gly/Phe in loop of A1 domain) with a loss of large and intermediate multimers; (3) – intracellular proteolysis of large VWF multimers, (p.Ser1506Leu) with a loss of large multimers; (4) – hypersensitivity to ADAMTS13 (p.Ile1628Thr in A2 domain) with the absence of large VWF multimers and increased triplet structure. NP = Normal plasma

The remaining six probands were heterozygous for two frequently described missense mutations clustered in the A2 domain: c.4517C>T, p.Ser1506Leu (n = 2) and c.4883T>C, p.Ile1628Thr (n = 4). The two patients heterozygous for the p.Ser1506Leu mutation, described as a VWD 2A group I defect with intracellular proteolysis of large VWF multimers, showed a similar multimeric pattern (Figure 10). These probands had moderate/severe type 2A VWD with low levels of VWF:Ag (median 17.5%) and VWF:RCo (median 11%), a low VWF:RCo/VWF:Ag ratio (median 0.64) and no RIPA at 1.25 mg/ml. The remaining four probands were heterozygous for p.Ile1628Thr, which

has been described as a VWD 2A group II defect characterised by hypersensitivity to ADAMTS13 with increased proteolysis in plasma (Michiels & van Vliet, 2009). They had mild/moderate type 2A VWD with low levels of VWF:Ag (median 54.8%) and VWF:RCo (median 13.2%), a low VWF:RCo/VWF:Ag ratio (median 0.2) and normal RIPA at 1.25 mg/ml. These four patients showed a typical proteolytic pattern with a lack of large VWF multimers and the presence of a pronounced triplet structure, which is characteristic of group 2A/II.

Of the 11 probands with the type 2M VWD phenotype, eight (72%) were heterozygous for the frequent missense mutations involving two arginine residues in the A1 domain: p.Arg1315Cys, p.Arg1315His, p.Arg1374Cys and p.Arg1374His (Table 9).

The laboratory phenotype of the probands heterozygous for p.Arg1315Cys and p.Arg1315His mutations was similar with low levels of VWF:Ag (median 19%) and VWF:RCo (median 11.5%), a decreased VWF:RCo/VWF:Ag ratio (0.66) and a normal multimeric pattern. However, the proband with the mutation p.Arg1315His was compound heterozygous with the silent mutation c.7464C>T detected in exon 44, which may create a new donor splicing site (Table 9 and Suppl. Table 3). The proband's mother, who was also heterozygous for p.Arg1315His, had a type 1 VWD phenotype with higher levels of VWF:Ag (32%) and VWF:RCo (26%).

The missense mutations p.Arg1374Cys and p.Arg1374His were the most frequent cause of type 2M VWD in our cohort of patients. All the six probands showed a decreased VWF:RCo/VWF:Ag ratio (median 0.3), a high VWF:CB/VWF:Ag ratio and the presence of all–size multimers, albeit with a certain degree of smearing. Two probands had the missense mutation c.4195C>T; p.Arg1399Cys associated with a mild phenotype and pronounced smearing of the multimer pattern.

The last patient (P38) was compound heterozygous for four previously undescribed missense variants. However, a family study was not conducted; therefore, which of them were in *cis* or in *trans* was unknown: p.[Val343Leu(;)Ile482Met(;)Asp1373Tyr(;) His1419Gln]. Two variants (p.Asp1373Tyr and p.His1419Gln) were clustered in the A1

domain, and the other two (p.Val343Leu and p.Ile482Met) were in the VWFpp region (D1–D2 domain). Of these four variants, only one was pathogenic (p.Asp1373Tyr), whereas the other three were benign variants. The phenotype was mild with a normal multimeric pattern (Table 9).

The knowledge of all putative mutations for every patient of this cohort permitted a broad overview of the inheritance and the combination of mutant alleles that affected each VWD type. The recessive inheritance of a majority of the mutant alleles identified in types 1, 3 and 2N VWD matched the following combination: missense/null in 5/7 for severe type 1; null/null in 13/15 for type 3 and missense/missense in 11/12 for type 2N. As expected, type 2 VWD (2A, 2B and 2M) showed a dominantly inherited trait with the allelic combination wild type/missense in 23/25 for type 2 (Table 5–9; Figure 11).

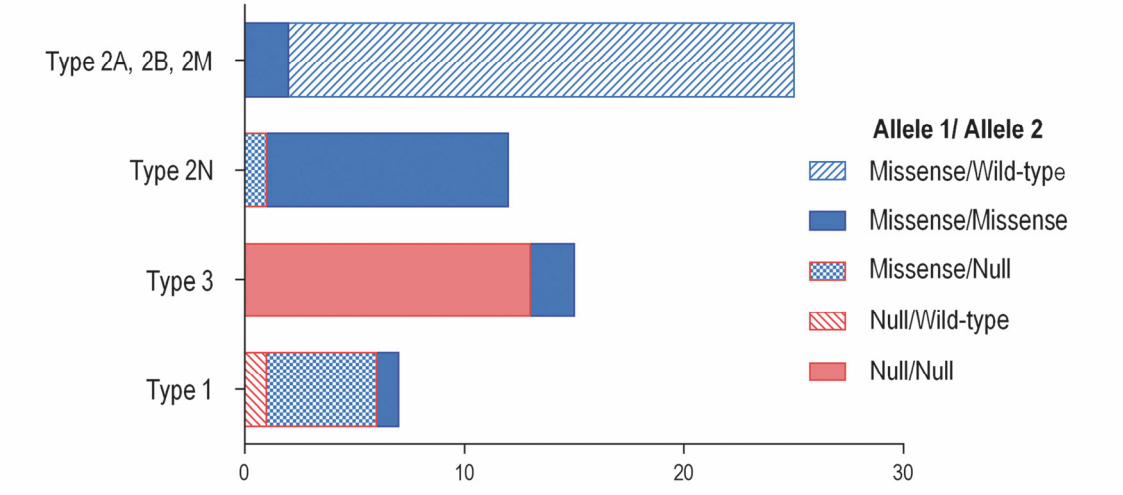

Figure 11. Mutation combination in mutated alleles in different VWD types. The main type of mutation detected in each VWD type was as follows: severe type 1 VWD, missense/null; type 3 VWD, null/null; 2N VWD, missense/missense; and type 2 VWD (2A, 2B and 2M), missense/wild type.

3.5. Discussion

This study comprised a genotype–phenotype correlation in a cohort of 60 unrelated Portuguese families (92 individuals) diagnosed with types 1, 2 and 3 VWD. The VWD diagnosis based on the results of a battery of laboratory tests is often difficult, and therefore, establishing patients' disease severity and risk of bleeding becomes challenging (Budde U, Favaloro E, 2011; Castaman *et al*, 2014). Thus, in our diagnosis

laboratory, a phenotype and genotype analysis in patients with VWD has become crucial for understanding the VWF function and great inter-individual variability.

As shown in Figure 7, besides the additional mutations identified by NGS, the characteristic mutation distribution across *VWF* was preserved in each VWD type: in type 3 VWD, the mutations were scattered throughout *VWF*; in severe type 1 VWD, the profile was similar, but the absence of mutations in the regions coding for the A1, A2, D' and D3 domains was evident; in type 2N VWD, a mutation cluster in the region coding for the D1, D' and D3 domains was detected; in types 2A, 2B and 2M VWD, the mutation spreading was notable, but a mutation cluster in the regions coding for the A1 domain emerged. These findings are in line with previous reports (Gadisseur *et al*, 2009a; Michiels *et al*, 2009; Bowman *et al*, 2013; Federici *et al*, 2011; Michiels & van Vliet, 2009).

The group of seven probands with severe type 1 VWD showed a high degree of genetic heterogeneity with 10 different mutations, of which five (three missense mutations, one nonsense and one large deletion) have not been previously described (35%). The *in silico* analysis for all three missense mutations showed that they were expected to be deleterious and disease causing (Suppl. Table 1). Five out of seven probands with mutations in regions coding for the VWF D1, D2, D4 and C2-C4 domains showed an increased FVIII:C/VWF:Ag ratio, which reflected impaired VWF secretion, possibly due to misfolding, as previously described (Eikenboom *et al*, 2009).

The missense mutation p.Ala631Val has only been described in a large study of healthy controls (Bellissimo *et al*, 2012) as an outlier owing to a mild type 1 VWD phenotype being presented. Nevertheless, in the present study, this mutation was found in compound heterozygosity with c.7730-4C>G, and the inheritance of both mutations was responsible for a severe phenotype (Figure 12). In addition, the compound heterozygosity of the novel missense mutation p.Ala1714Pro with c.5170+10C>T was associated with severe type 1 VWD. Moreover, when inherited alone, this mutation exhibited a type 2M VWD phenotype (Figure 12). The p.Ala1714Pro mutation as well

as others in the A3 domain showed an accentuated defective collagen binding with a normal multimer pattern (Riddell *et al*, 2009).

The variants c.7730-4C>G and c.5170+10C>T, although the low probability of a deleterious effect predicted by *in silico* algorithms, they seem to have had some influence on VWD phenotype in these families: the first one showed effect in autosomal recessive pattern and the second one showed a cumulative effect in a compound heterozygous state on decrease of VWF levels (Figure 12). However, the variant c.5170+10C>T is frequent in the studied populations, despite the variable frequencies found in different databases (ExAC Aggregated_Populations: MAF = 0.0036; EVS_EA: MAF = 0.0046 and dbSNP: MAF = 0.0089) and it may be controversial to consider this a pathogenic variant.

Nevertheless, it should be noted that other deep intronic changes that can only be detected by whole gene sequencing could be present and may also influenced these phenotypes. These findings corroborate the concept that the variants' pathogenicity should be determined by the entire body of evidence in each family (Richards *et al*, 2015). Moreover, these likely pathogenic variants could not explain the disease; however, they seem to influence the phenotype and could behave as a pathogenic in one case and non-pathogenic in another, depending on the other co-inherited changes (Batlle *et al*, 2015).

These data have proven the added value of practice guidelines for the evaluation of the pathogenicity of potential splice site variants. Among the six variants that were predicted as potential splice sites by *in silico* analysis, two were considered pathogenic, two likely pathogenic and the other two with uncertain significance (Suppl. Table 1).

Therefore, these classification criteria did not replace the functional mRNA studies, the only method that can elucidate the true effect of the variation detected (Corrales *et al*, 2011); however, this classification provided a more precise genotype–phenotype correlation.

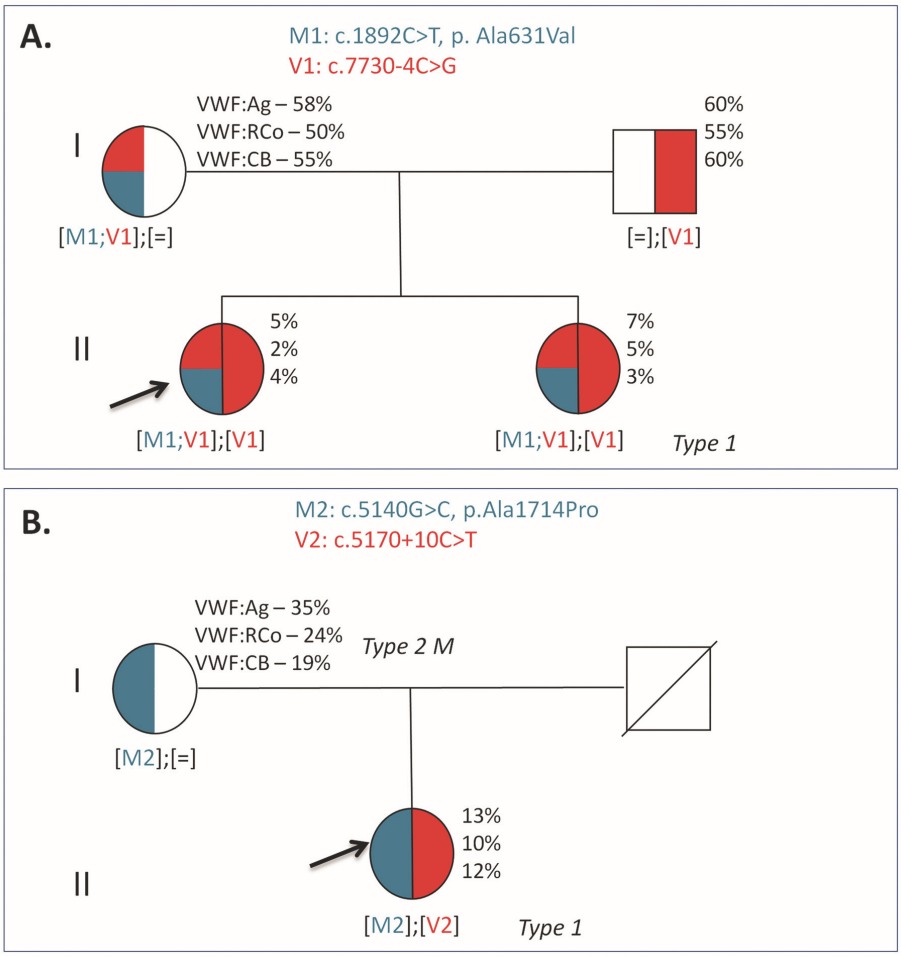

Figure 12 - Pedigree and laboratory features associated with c.7730-4C>G and c.5170+10C>T variants. The study of the parents of these probands allowed the tracing of the mutation inheritance on each allele. The variants c.7730-4C>G in the homozygous state (A) and c.5170+10C>T (B) in the compound heterozygous state appear to have contributed to the changes in VWD phenotype in each proband. The arrow indicates the *propositus*; M, mutation; V, variant; and [=],'no change' in the other allele.

Our cohort of type 3 VWD evidenced, as usual, two patterns of mutations: homozygous for null mutations and compound heterozygous/homozygous for missense mutations of *VWF* (Figure 11). Two nonsense mutations (p.Gln1311*, p.Gln1556*) and a novel small deletion (p.Val1805Glyfs*8) were expected to have the obvious deleterious mechanisms, i.e., premature termination codons and a frameshift leading to a truncated VWF protein. The homozygous p.Gln1311* mutation associated with a gene conversion and initially reported in Spanish Romani families (Casaña *et al*, 2000) was the most frequent mutation in 5/15 families, of which one was also Romani. Among these five patients, one had developed an inhibitor that has also been described in homozygous gene conversions (Surdhar, 2001). In three families studied

in this cohort, obligate carriers of p.Gln1556* and p.Gln1311* were not phenotypically silent and manifested mild VWF levels and mucocutaneous bleeding symptoms, showing an inheritance pattern that is co-dominant (Bowman *et al*, 2013) and not recessive, as described in other studies. One carrier of p.Gln1556* had the variant p.Tyr1584Cys in *trans* without any worsening of phenotype. The small duplication p.Cys2235Argfs*8 previously described in Spanish patients (Corrales *et al*, 2012) and also repetitively found in this Portuguese study could share a common ancestral origin.

The molecular mechanisms of missense mutations in compound heterozygous state in the D3 domain (p.Tyr1146Cys) could be related to ineffective multimerisation and the subsequent defective intracellular transport and secretion with intracellular retention (Schneppenheim & Budde, 2011).

Finally, the p.Pro1162Leu in the homozygous state detected in a type 3 VWD proband was also found in another proband with type 2N VWD (Table 7). This controversial finding implied that p.Pro1162Leu was unlikely to be a pathogenic variant for type 3 VWD. However, the same genotype was also the only one found in a type 3 VWD Spanish family (Batlle *et al*, 2015); therefore, it seems reasonable to expect that the p.Pro1162Leu (c.3485_3486delinsTG) variant is a part of a compound genotype that causes type 3 VWD, which has not yet been entirely explained. This possibility is in accordance with the lack of detection of molecular mechanism of type 3 VWD despite NGS improvements. The explanation for this includes deep intronic mutations that may only be identified through whole-gene sequencing and distant regulatory elements outside *VWF* (Bowman *et al*, 2013).

The functional VWF:FVIIIB defect (type 2N) was mainly caused by the missense mutation p.Arg854Gln in the homozygous state in our cohort, similarly to previous studies. This is a frequent deleterious variant in Caucasian populations (Goodeve, 2010; Jacquemin, 2009). Nevertheless, genetic variability was introduced by compound heterozygous with novel mutations: p.Arg854Gln with the deleterious novel missense mutation p.Asp879Glu and p.Arg816Trp with the novel small deletion c.100delT (p.Arg34Aspfs*49) (Table 7, Suppl. Table 1). The ethnic variability was noted in an

African proband who showed heterozygosity for the frequent polymorphism p.His817Gln in association with p.Pro1162Leu in the homozygous state. Recent multiethnic studies found that *VWF* missense variants in the D' and D3 domains, previously identified in European ancestry VWD probands, are more frequent in African Americans (AAs) (Bellissimo *et al*, 2012; Johnsen *et al*, 2013; Wang *et al*, 2013). These variants include p.His817Gln, which was strongly associated with diminished FVIII:C levels (Johnsen *et al*, 2013). The p.Pro1162Leu variant (caused by c.3485_3486delinsTG) was previously only reported in one healthy AA in the heterozygous state (Bellissimo *et al*, 2012). Nevertheless, the same variant caused by a different nucleotide change (c.3485C>T) was described with polymorphic frequency in AAs (MAF = 0.262) (The 1000 Genomes Project Consortium, 2015; National Center for Biotechnology Information dbSNP (build 137)). Taken together these data, it could be reasonable to interpret that such different variants at the genomic level, although theoretically lead to identical amino acid change, could result in unlike consequences at the transcriptional level and to distinct deleterious effects.

This pleiotropic effect on the VWF molecule may be explained by the ethnic diversity of the *VWF* coding polymorphisms recently documented in large cohorts (Wang *et al*, 2013; Johnsen *et al*, 2013; Bellissimo *et al*, 2012). As previously reported, these mutations that result in low VWF antigen levels could be partially compensated by the high baseline VWF antigen levels found in Africans. They could be VWD causing only when they are confined in ethnic-specific haplotypes; in fact, only a study of large Caucasian and African cohorts could link specific *VWF* variants to a bleeding phenotype (Wang *et al*, 2013).

In agreement with previous studies, the genotype–phenotype correlation in the 25 patients with types 2A, 2B and 2M VWD was explained by the VWF domain affected and was therefore divided into structural and functional defects (Schneppenheim & Budde, 2011; Meyer *et al*, 2011).

Our type 2B VWD cohort of patients with gain-of-function mutations in the A1 domain showed two common mutations, p.Arg1306Trp and p.Val1316Met (Gadisseur *et al*,

2009b). In addition, we observed a highly variable platelet count with a large range (15–242 × 10^3 platelets/µl), which is consistent with previous studies (Table 8), although only four patients had thrombocytopenia. This variability was explained by several mutations and their coding positions within the VWF A1 domain, e.g., the altered VWF GPIb-α–binding conformation (Federici *et al*, 2011). Although p.Arg1306Trp and p.Val1316Met are frequently correlated with thrombocytopenia, we observed some heterogeneity even in individuals with the same mutation. These data were also in agreement with the wide degree of heterogeneity of the clinical and laboratory features reported for affected members of families that have type 2B VWD (Federici *et al*, 2011).

Herein, we report a case of PT-VWD misdiagnosed as type 2B VWD. The functional studies allowed a differential diagnosis, but molecular studies confirmed the recently described missense mutation p.Asp251Tyr (Asp235Tyr) in *GP1BA* (Enayat *et al*, 2012). PT-VWD is certainly an underdiagnosed deficiency, where molecular study is a valuable diagnostic tool.

We found mutations related to all previously described type 2A structural defects (Woods *et al*, 2012; Budde *et al*, 2008; Michiels & van Vliet, 2009; Gadisseur *et al*, 2009a): i) a multimerisation defect (p.Cys1130Arg in domain D3) with the absence of large multimers and no triplet structure; ii) a change in protein folding (p.Cys1272Gly/Phe in loop of A1 domain) that causes of loss of large and intermediate multimers; iii) intracellular proteolysis of large VWF multimers (p.Ser1506Leu) with a loss of large multimers; and iv) hypersensitivity to ADAMTS13 (p.Ile1628Thr in A2 domain) with the absence of large VWF multimers and increased triplet structure (Table 9, Figure 10).

The mutations p.Cys1130Arg and p.Ser1506Leu showed disproportionate VWF:RCo/VWF:Ag ratios, but near 0.7; the mutations p.Cys1272Gly/Phe and p.Ile1628Thr had very low ratio values, indicative of enhanced proteolysis, as previously described (Woods *et al*, 2012; Michiels & van Vliet, 2009) (Figure 10).

Our findings in type 2M VWD patients support the well-characterised profile: mutations in the A1 domain, which are typically associated with decreased or absent RIPA and a low VWF:RCo/VWF:Ag ratio, combined with a normal VWF:CB/VWF:Ag ratio. We identified the usual A1 domain mutation clusters, p.Arg1315Cys/His and p.Arg1374Cys/His, with the addition of the novel mutations p.Asp1373Tyr and p.His1419Gln. The VWD subtype classification of the p.Arg1374His mutation has been controversial. This mutation was described as type 2A (Budde *et al*, 2008) or type 2M (Castaman *et al*, 2012a). In fact, some authors argue that the mutation is difficult to classify as type 2M (because of the possibly relative decrease in large VWF multimers) or any other type 2 (because of the normal banding pattern of each multimer) VWD; therefore, a classification of type 2U VWD (unclassifiable) has been suggested (Gadisseur *et al*, 2009c). In our study, three probands showed a multimer pattern, with the full complement of multimers, and one showed a slight decrease in the largest forms. Given the criterion of a low VWF:RCo/VWF:Ag ratio and normal VWF:CB/VWF:Ag ratio, which was evident in each of the four probands, they were classified as having type 2M VWD.

The same criterion was applied to the p.Arg1315Cys, p.Arg1374Cys and p.Arg1399Cys mutations that showed a smeary multimer pattern (no clear separation between individual oligomer triplets), which was particularly pronounced in two probands and two relatives with p.Arg1399Cys. This structural VWF alteration usually occurs when the responsible mutation involves cysteine residues, which may affect the disulphide bonding of VWF dimers (Goodeve, 2010; Budde *et al*, 2008). A recent study analysed the impact of cysteine mutations that affect the carboxyl-terminal domains of VWF (Yadegari *et al*, 2013), it clearly demonstrated the importance of cysteine residues for the structural conformation and consequently multimerisation, even in patients with type 2A VWD with a smeary multimer pattern. Therefore, the smeary appearance could be associated with the alterations in VWF domains that determine whether VWD is type 2A or 2M; however, the cysteine mutations seem to be the hallmark of this multimer pattern (Budde *et al*, 2008; Yadegari *et al*, 2013; Goodeve, 2010).

This study described, for the first time, the mutational spectrum in a cohort of Portuguese VWD patients. Overall, VWD studies highlight the high degree of variability in clinical presentation and the considerable heterogeneity of the molecular basis, which supports the great interest shown in reports from different geographic regions (Corrales *et al*, 2009; Castaman *et al*, 2012a; Hampshire *et al*, 2013; Yadegari *et al*, 2012; Bowman *et al*, 2013). Moreover, large studies have highlighted the ethnic variability in the phenotype of many *VWF* missense variants (Bellissimo *et al*, 2012; Johnsen *et al*, 2013; Wang *et al*, 2013). We applied these insights to the context of familial studies to determine their usefulness for predicting individual bleeding risk. Accordingly, the genotype–phenotype correlation in each proband family with diverse VWD types was assessed to establish family overviews and elucidate phenotypic discrepancies.

This accurate analysis was only possible because NGS has made it easier to study *VWF* ROIs. The NGS methodology applied in this study was the same as that used in the recently published 'Molecular and clinical profile of von Willebrand disease in Spain (PCM–EVW–ES)' project. NGS has proven to be an excellent technology that enables more rapid diagnosis with a huge economic advantage (only 70€/sample, which is even cheaper than most phenotypic tests) (Batlle *et al*, 2015). Similarly, these findings support the adjustment of our VWD diagnosis algorithm, introducing the complete sequencing of *VWF* (NGS) when the VWF:RCo level is < 30% or the FVIII/VWF:Ag ratio is < 0.5.

Because of the geographical proximity of the two populations, we expected to detect the same common mutations in the patients of our cohort as for the cohort of PCM–EVW–ES (Batlle *et al*, 2015). In fact, in type 3 VWD patients, the following were observed: the nonsense mutation p.Gln1311* was the most prevalent, and the missense mutation p.Pro1162Leu was also present in homozygous state. The p.Cys2235Argfs*8 was only detected in Spanish and Portuguese patients, and interestingly, the variant p.His817Gln was also noted in association with diminished FVIII:C levels and the type 2N phenotype.

Our new molecular study approach permitted the identification of 27 novel *VWF* mutations, with some occurring repetitively, illustrating the advantages of identifying the most prevalent mutations in a region and their correlation with the severity of bleeding phenotypes. The approach allowed us to distinguish between clinical situations that have the same symptoms with different genetic causes such as mild Haemophilia A and type 2N VWD, Bernard–Soulier syndrome and 2B VWD as well as 2B VWD and PT-VWD. In contrast, despite being a restricted study, we identified some pleiotropic mutation effects. In both situations, it was evinced that molecular studies are indispensable for an accurate diagnosis.

The molecular characterisation of VWD patients allows precise classification into the correct VWD type and the identification of carriers in familial genetic studies. This classification is particularly relevant and even mandatory for genetic counselling for type 3 VWD and, in general, for patients with higher bleeding risk. Moreover, it facilitates the evaluation of prophylactic requirements and clinical orientation, particularly in risky situations.

In conclusion, this study of 60 VWD Portuguese families will contribute to the better understanding of the molecular genetics of *VWF*-related phenotypes. NGS, in our experience, provides an effective laboratory workflow for the analysis of a single large gene, such as *VWF*. These findings support the idea that improving VWD diagnosis strategies will enhance clinical and laboratory approaches; thus, the most appropriate treatment for each patient can be determined.

What is known about this topic?

- VWD is an inherited bleeding disorder characterised by a variable bleeding tendency and heterogeneous laboratory phenotype.
- A genotype–phenotype correlation in patients with VWD has become crucial for understanding the function and great inter-individual variability of VWF.

- With the advent of NGS, this correlation is easier to obtain because of the possibility of sequencing the entire *VWF* coding region for a very reasonable cost.

What does this paper add?

- Twenty-seven novel VWD mutations or potential mutations, including seven missense mutations, two nonsense mutations, two small deletions and a large deletion of exon 31, extend the mutational spectrum of VWF.
- The genotype–phenotype correlation analysis in our cohort of patients enables the unravelling of several diagnostic discrepancies and the identification of potential pleiotropic effects of mutations.
- The present study showed that NGS provides an accurate molecular analysis; however, its interplay with a detailed clinical data registry and familial studies is crucial.

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Conflicts of interest

None declared.

CHAPTER

4

VWF COLLAGEN (TYPES III AND VI)-BINDING DEFECTS IN A COHORT OF TYPE 2M VWD PATIENTS – A STRATEGY FOR IMPROVEMENT OF A CHALLENGING DIAGNOSIS

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CHAPTER 4. VWF COLLAGEN (TYPES III AND VI)-BINDING DEFECTS IN A COHORT OF TYPE 2M VWD PATIENTS – A STRATEGY FOR IMPROVEMENT OF A CHALLENGING DIAGNOSIS

4.1. Introduction

The diagnosis of type 2 von Willebrand disease (VWD) includes qualitative defects in von Willebrand factor (VWF) related to ineffective interactions with platelets, collagen or FVIII. Type 2M VWD fits a well-characterised profile with a normal multimer distribution but a reduction in VWF-platelet interactions or the existence of collagen-binding defects (Sadler *et al*, 2006). VWF binds fibrillar types I and III collagen via the A3 and A1 domains but binds subendothelial microfibrillar type VI collagen exclusively via the A1 domain (Flood *et al*, 2012a). The standard functional assays for VWD detection do not always evaluate the collagen-binding function of VWF (VWF:CB), and assays for diverse collagen types are even less common, which may have contributed to the scarcity of reported VWF:CB deficiencies (Flood *et al*, 2012b). Functional and molecular studies focused on this VWF dysfunction have increasingly reported *VWF* sequence variations that affect the ability of VWF to bind type I, type III and type VI collagen (Larsen *et al*, 2013; Flood *et al*, 2012b; Riddell *et al*, 2009; Legendre *et al*, 2013). However, some of these mutations may not interfere with the screening assays, which show normal values, and can only be detected using type VI collagen. Hence, this VWF dysfunction could be underdiagnosed even though it is typically reported in patients with a mild bleeding history (Flood *et al*, 2012b).

4.2. Materials and Methods

As recently reported, we studied genotype–phenotype correlations in a cohort of Portuguese patients comprising the entire spectrum of VWD types (Fidalgo *et al*, 2016). The VWF functional analysis was performed according to an algorithm based on established recommendations; however, the specific tests evaluating VWF:CB used only collagen type III (VWF:C^{III}B). Molecular analysis of the entire VWF coding region was performed by next-generation sequencing (NGS). Then, we re-evaluated VWF:CB with collagen type VI (VWF:C^{VI}B) in some patients to analyse the value added by the incorporation of this assay into the initial approach for VWD studies.

Herein, we reported eight out of 11 patients (seven adults and one child) previously diagnosed with type 2M VWD and a 9-year-old boy with a history of epistaxis requiring cautery, normal VWF values and decreased VWF:C^{III}B (35%). VWF:C^{III}B and VWF:C^{VI}B were measured in these nine patients.

In addition to VWF:C^{III}B, VWF:C^{VI}B was also determined using an ELISA kit (TECHNOZYM® VWF:CBA ELISA, Technoclone, Vienna, Austria) in a Triturus Immunoassay System (Diagnostic Grifols, Barcelona, Spain). The VWF:RCo was determined based on the VWF-platelet GPIb binding activity (Innovance, Siemens Healthcare, Marburg, Germany); VWF:RCo and VWF:Ag levels were measured on a BCS XP coagulation analyser according to the manufacturer's instructions (Siemens Healthcare, Marburg, Germany). Forty-two healthy volunteers acted as the control group for the VWF antigen level (VWF:Ag), ristocetin cofactor activity (VWF:RCo) and VWF collagen binding (VWF:C^{III}B and VWF:C^{VI}B) measurements.

4.3. Results and Discussion

The phenotypic and molecular data from the patients are summarised in Table 10. Among the nine patients, seven had bleeding scores > 3 (median = 7). The recommended cut-off of 0.7 for the lower limit of the normal ratios was used to analyse VWF:RCo/VWF:Ag, VWF:C^{III}B/VWF:Ag and VWF:C^{VI}B/VWF:Ag (Sadler *et al*, 2006). Overall, eight patients had VWF:RCo/VWF:Ag ratios lower than 0.7, and all nine patients exhibited VWF multimeric patterns with the presence of all-size multimers. However, we also found patients with discrepant low ratios (< 0.7) for VWF:C^{III}B/VWF:Ag (n = 4) and VWF:C^{VI}B/VWF:Ag (n = 6). The patient who had a normal VWF:RCo/VWF:Ag ratio exhibited reduced binding to both types of collagen (type III and type VI).

The mutational analysis of samples from these nine patients identified eight different VWF variants. Six of these variants were detected in exon 28, one in exon 29, and one in exon 44; these regions encoded the VWF A1, A3 and C3 domains, respectively (Table 10).

Table 10 – Phenotypic and molecular data of 9 patients with type 2M VWD

Patients' demographics			Laboratory values*							Molecular data			
ID	(M/F year)	BS	VWF:Ag (%)	VWF:RCo (%)	VWF:RCo/VWF:Ag R	VWF:$C^{III}B$ (%)	VWF:$C^{III}B$/VWF:Ag R	VWF:$C^{VI}B$ (%)	VWF:$C^{VI}B$/VWF:Ag R	Exon	Nucleotide change	Amino Acid Change	Dom.
P1	F/36	7	22	11	0.50	19	0.86	13	0.59	28	c.3944G>A	p.Arg1315His	A1
										44	c.7464C>T‡	p.(=)	C3
P2	M/32	7	32	19	0.59	35	1.09	32	1.00	28	**c.4117G>T**	**p.Asp1373Tyr**	A1
P3, P4	F/48; F/76	9; 1	35; 36	19; 16	0.54; 0.44	25;32	0.71; 0.89	31; 26	0.89; 0.72	28	c.4120C>T	p.Arg1374Cys	A1
P5, P6	M/73; F/31	8; 3	46; 23	8.5; 4	0.18; 0.17	19.5; 9.4	0.4; 0.4	15; 4	0.33; 0.17	28	c.4121G>A	p.Arg1374His	A1
P7	M/9	7	56	32	0.58	37	0.67	17.4	0.31	28	c.4195C>T	p.Arg1399Cys	A1
P8	M/9	3	57	43	0.75	35	0.61	18	0.32	28	c.4196G>A	p.Arg1399His	A1
P9	F/28	7	35	24	0.69	11	0.31	19.2	0.54	29	**c.5140G>C**	**p.Ala1714Pro**	A3
Normal Range			50-160	48-173	> 0.70	60-130	> 0.70	59-138	> 0.70				

Bleeding scores (BS) were calculated using a validated questionnaire; * median observed on two or more occasions. R, ratio; the mutations marked in **bold** were not previously reported; ‡ indicates mutation in *trans;* Dom., Domain.

All identified variants except one (p.Arg1399His) were classified as pathogenic based on the available evidence according to the practice guidelines for the evaluation of pathogenicity (Fidalgo *et al*, 2016). The benign variant p.Arg1399His was frequent in some populations studied despite the variable frequencies found in different population databases (ExAC: MAF = 0.012, EVS_EA: MAF = 0.014 and dbSNP: MAF = 0.0020).

The genotype and laboratory phenotype correlation was demonstrated by a comparison of the different VWF:RCo, VWF:$C^{III}B$ and VWF:$C^{VI}B$ levels and the respective ratios (VWF:RCo/VWF:Ag, VWF:$C^{III}B$/VWF:Ag and VWF:$C^{VI}B$/VWF:Ag) for each mutation (Figure 13 A, B).

One patient was heterozygous for the novel p.Asp1373Tyr mutation (P2) and two patients (P3 and P4) were heterozygous for the frequently occurring p.Arg1374Cys mutation despite having a VWF:RCo/VWF:Ag ratio < 0.7 (range 0.44–0.59); the collagen binding values were normal, the VWF:$C^{III}B$/VWF:Ag ratio ranged from 0.71–1.09, and the VWF:$C^{VI}B$/VWF:Ag ratio ranged from 0.72–1. In these patients, these mutations only appeared to be associated with a specific VWF-platelet defect. Similar findings were reported for the p.Arg1374Cys mutation in a familial study, albeit only relative to collagen type III (Penas *et al*, 2005).

Patient P1, who exhibited compound heterozygosity for the p.Arg1315His mutation and a donor splicing site mutation c.7464C>T in exon 44, had similar reduced VWF:RCo/VWF:Ag and VWF:C^{VI}B/VWF:Ag ratios (0.50 and 0.59, respectively) but a normal VWF:C^{III}B/VWF:Ag ratio (0.86).

Patients P5 and P6, who were heterozygous for the frequently occurring p.Arg1374His mutation, exhibited significant and parallel reductions in binding to platelets and to both collagens as follows: VWF:RCo/VWF:Ag, 0.18 and 0.17 for patients P5 and P6, respectively; VWF:C^{III}B/VWF:Ag, 0.4 and 0.4 for patients P5 and P6, respectively; and VWF:C^{VI}B/VWF:Ag, 0.33 and 0.17 for patients P5 and P6, respectively. These findings were consistent with studies that described this mutation with very low or undetectable VWF:RCo levels (Gadisseur *et al*, 2009c).

Patients P7 and P8, who were heterozygous for p.Arg1399Cys and the benign variant p.Arg1399His, had the highest VWF:Ag and VWF:RCo levels (56–57% and 32–43%, respectively). Nevertheless, a significant reduction was observed in binding to collagen type VI (VWF:C^{VI}B; 17.4–18%). The VWF:C^{VI}B/VWF:Ag ratio was lower than the VWF:C^{III}B/VWF:Ag ratio, as shown in Figure 13B, indicating a markedly severe defect in type VI collagen binding. The p.Arg1399Cys mutation was associated with a decrease in VWF:RCo compared to p.Arg1399His possibly due to the cysteine mutation, which affected disulphide bonding of the VWF dimers and resulted in a smeared appearance of the multimer (Figure 13C). These findings were in line with previous reports that described variability in platelet and collagen-binding defects, but the results clearly connected these mutations to defects in type VI collagen (Flood *et al*, 2012a, 2012b).

The VWD subtype classifications of the p.Arg1315His, p.Arg1374Cys, p.Arg1374His and p.Arg1399Cys mutations are controversial and are described as type 1 severe, type 2A, type 2M and unclassifiable, respectively (Gadisseur *et al*, 2009c). Some authors have argued that classifying these mutations as type 2M VWD (due to the possible relative decrease in large VWF multimers) or as any other type 2 VWD (due to the normal banding pattern of each multimer) is difficult. Therefore, a classification of type 2U VWD (unclassifiable) has been suggested. In our study, the presence of all-size VWF

multimers and VWF-platelet and/or VWF collagen-binding defects were the criteria used to classify these patients as type 2M VWD.

Finally, patient P9, who was heterozygous for the novel missense mutation p.Ala1714Pro, showed a significant defect in binding to type III collagen (VWF:C^{III}B: 11.3%), similar to patients with other mutations in the A3 domain (Riddell *et al*, 2009), and the lowest VWF:C^{III}B/VWF:Ag ratio value (0.31) (Figure 13B). The VWF:RCo/VWF:Ag and VWF:C^{VI}B/VWF:Ag ratios were more similar (0.69 and 0.54, respectively). Indeed, this novel A3 mutation appeared to be associated with a profile of a reduced VWF:Ag level and a more pronounced collagen-binding type III defect (i.e., a quantitative and a qualitative defect). Although not yet demonstrated *in vitro*, these data were consistent with those from other reported studies. First, the well-characterised mutation that involved the same hydrophobic linear amino acids (p.Ala1716Pro) was associated with type 1 VWD, although this association was based solely on the VWF:Ag and VWF:RCo levels (Castaman *et al*, 2012b). Second, one study described two mutations in the A3 domain that led to a combined qualitative and quantitative defect in VWF that exhibited decreased binding to collagen (types I and III) and the platelet glycoproteins Ib and IIb-IIIa (Legendre *et al*, 2013).

In conclusion, our study identified a group of 2M *VWF* mutations with a variable range of functional defects, including the classical VWF-platelet defect and the VWF collagen-binding defect or both. This variability has been increasingly observed in several recent studies (Larsen *et al*, 2013; Legendre *et al*, 2013), and the application of these insights in the context of genotype-phenotype correlations can improve the approach used by diagnostic laboratories.

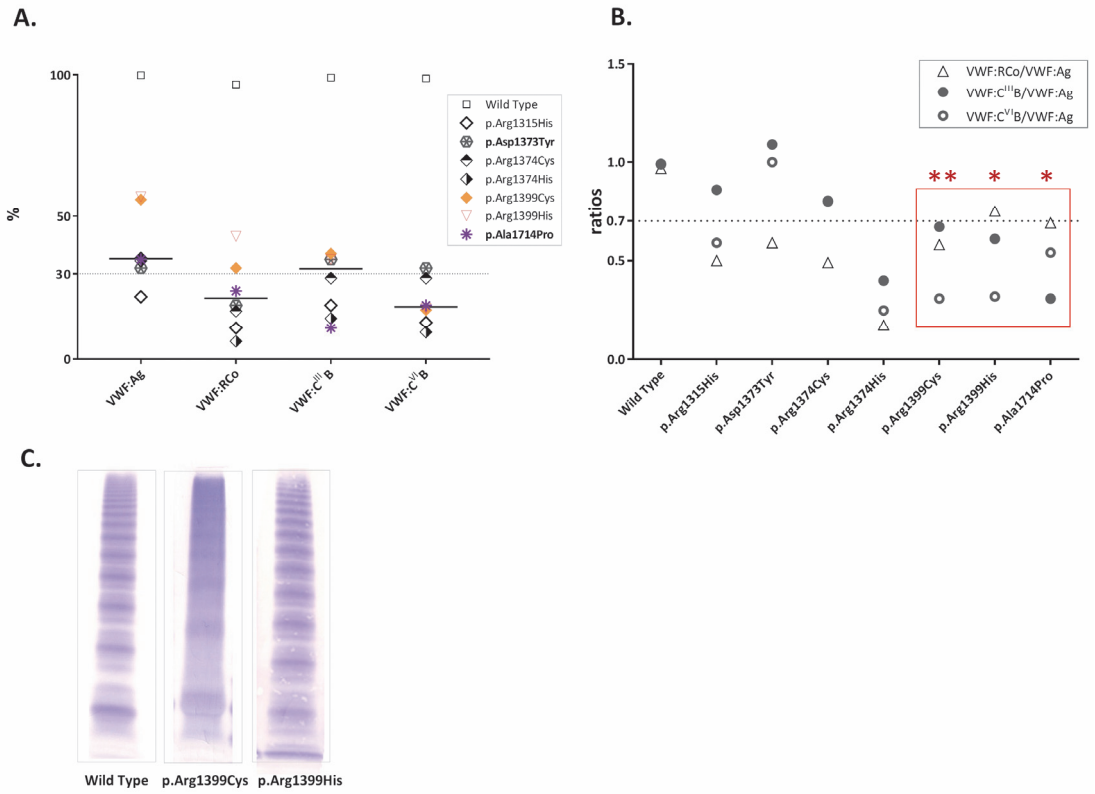

Figure 13 – Comparison distribution of VWF levels for the missense mutations in the A1 (n = 6) and A3 (n = 1) domains identified in 9 patients with type 2M VWD.

A. Median VWF level values for each mutation. The dot plots show the VWF:Ag (antigen), VWF:RCo (ristocetin cofactor), VWF:C^{III}B (collagen binding type III) and VWF:C^{VI}B (collagen binding type VI) levels for all missense mutations, with each dot representing a single case. The dotted line marks the level that corresponds to the selection criterion for VWF deficiency (VWF:RCo ≤ 30%). The p.Arg1399Cys (red dot) and p.Arg1399His mutations (orange dot) exhibited a specific type VI collagen-binding defect; the novel p.Ala1714Pro mutation (purple dot) exhibited a more pronounced type III binding defect.

B. Dot plots of the VWF:RCo/VWF:Ag, VWF:CIIIB/VWF:Ag and VWF:CVIB/VWF:Ag ratios for each mutation. The dotted line marks ratios ≤ 0.7 that correspond to the discrepant value that suggests type 2 VWD. When analysed together, the values of the three ratios revealed different information for each missense mutation: (i) p.Arg1315His had reduced VWF:RCo/VWF:Ag and VWF:C^{VI}B/VWF:Ag ratios but a normal VWF:C^{III}B/VWF:Ag ratio, indicating a more pronounced type VI collagen-binding defect; (ii) p.Arg1374His showed parallel reductions in binding to platelets and to both collagens; (iii) the novel p.Arg1373Tyr and p.Arg1374Cys mutations had lower values for VWF:RCo/VWF:Ag, but both ratios VWF:C^{III}B/VWF:Ag and VWF:C^{VI}B/VWF:Ag were normal, indicating a specific VWF-platelet defect; and (iv) the last three mutations had higher VWF:RCo/VWF:Ag ratio values, and the comparison between VWF:C^{III}B and VWF:C^{VI}B revealed that they were the only values that remained significant. p.Arg1399Cys (**P = 0.004) and p.Arg1399His (*P = 0.01) exhibited specific type VI collagen binding defects, whereas the novel p.Ala1714Pro (*P = 0.03) exhibited a specific type III collagen binding defect. Differences were analysed using Fisher's exact test (GraphPad Prism 6.0, GraphPad Software, La Jolla, CA, USA; www.graphpad.com) and were assumed to be significant when the two-tailed p-value was < 0.05. The red rectangle highlights the significant values.

C. The multimer VWF patterns of p.Arg1399Cys and p.Arg1399His. The VWF multimers analysed by SDS-agarose gel electrophoresis displayed a distinct pattern: p.Arg1399Cys showed a smeary multimer pattern (no clear separation between individual oligomer triplets) associated with mutations that involved cysteine residues, which might affect the disulphide bonding of the VWF dimers.

However, the phenotypic variability in 2M VWD associated with the same mutations often remains unclear, even across families. Similar to other genetic diseases, expecting the involvement of modifier genes seems reasonable; therefore, NGS with targeted gene panels could help clarify this challenging diagnosis.

This analysis in our small cohort of patients evinced that a laboratory approach based on the correlation of type III and type VI collagen-binding assays and molecular studies is indispensable for a more accurate diagnosis of type 2M VWD. Moreover, the detection of the specific type VI collagen-binding defect may contribute to the correct diagnosis of patients with a mild bleeding disorder who are often classified as having an undefined cause (Flood *et al*, 2012a). An example was the child (P8, Table 10) with a history of epistaxis who was heterozygous for p.Arg1399His. This patient would have remained undiagnosed if studied with a minimalist laboratory analysis (VWF:Ag and VWF:RCo).

The inclusion of both collagen-binding assays (type III and type VI) in our diagnostic algorithm enhanced the efficacy of the VWD diagnosis and consequently the identification of the most appropriate management for each patient.

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Author contribution

TF was responsible for the study design and wrote the manuscript. TF, ACO, CSP and PM performed the laboratory studies and analysed the data. RS, GF, TS and CC provided clinical support. MLR revised the manuscript.

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CHAPTER

5

ACQUIRED VON WILLEBRAND SYNDROME (AVWS) IN HEMATOLOGIC MALIGNANCIES – HOW THE CLINICAL-LABORATORY CORRELATION IMPROVES A CHALLENGING DIAGNOSIS

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CHAPTER 5. ACQUIRED VON WILLEBRAND SYNDROME (AVWS) IN HEMATOLOGIC MALIGNANCIES – HOW THE CLINICAL-LABORATORY CORRELATION IMPROVES A CHALLENGING DIAGNOSIS

5.1. Introduction

Acquired von Willebrand syndrome (AVWS) is a rare acquired bleeding disorder with clinical and laboratory characteristics similar to congenital Von Willebrand disease (VWD) (Veyradier *et al*, 2000). AVWS is a relatively uncommon and under-diagnosed acquired bleeding disorder with an estimated prevalence of approximately 0.04 to 0.13%, which occurs in individuals with no personal or familial history of bleeding diathesis. It is usually associated with an underlying disorder, namely lymphoproliferative disorders (48%), cardiovascular diseases (21%), myeloproliferative neoplasms (15%), other cancers (5%) and autoimmune diseases (2%). More rarely, it can arise in the context of hypothyroidism, uraemia and viral infections, or in relation to drugs, such as ciprofloxacin (Sucker *et al*, 2009; Perez-Rodriguez *et al*, 2011; Federici, 2008).

A variety of pathogenic mechanisms have been proposed as causing structural or functional deficiencies of von Willebrand factor (VWF). These include autoantibodies, either by interfering with platelets or collagen binding, or by increasing VWF clearance from the plasma. The loss of high-molecular-weight multimers (HMWM) was demonstrated in patients with hematologic disorders due to their adsorption onto clonal plasma cells or platelets. Therefore, the disease is the result of the occurrence of various phenomena, namely: (i) the presence of specific or nonspecific autoantibodies that inactivate VWF; (ii) adsorption of VWF onto neoplastic cell clones; (iii) loss of HMWM after exposure to high shear stress; and (iv) increased proteolytic degradation of VWF by circulating proteases (Veyradier *et al*, 2000; Federici *et al*, 2001; Tiede *et al*, 2011).

The diagnosis is challenging because it greatly depends on clinical suspicion. On the other hand, the tests used to assess AVWS are the same as those for VWD, and the differential diagnosis between both can sometimes be difficult. Therefore, AVWS must

be suspected whenever laboratory findings show low levels of VWF in a patient with no previous bleeding history, particularly in the context of an underlying disorder. However, it should be noted that VWD is the most frequent hereditary bleeding disorder; thus, there is a chance of presenting the two bleeding disorders simultaneously (Tiede *et al*, 2011; Federici, 2008).

There is no consensual approach to the treatment of AVWS, except that it should always comprise two major goals: (i) treatment of symptomatic bleeding and prevention of bleeding during surgery or a *major* invasive procedure; and (ii) identification and treatment of the underlying disease. The therapeutic options for control/prophylaxis of bleeding manifestations include desmopressin (DDAVP), concentrates containing FVIII/VWF, recombinant Factor VIIa (rFVIIa), intravenous gamma globulin (IVIG) and plasmapheresis (Tiede *et al*, 2011; Federici *et al*, 2001; Tefferi *et al*, 2001; Eikenboom *et al*, 2007).

5.2. Materials and Methods

Herein, we report six patients with AVWS and the different underlying diseases, and describe the diagnostic workup, management and respective follow-ups. These six individuals, four women and two men, with a mean age at diagnosis of 73.8 years (36–86 years), were diagnosed between 2008 and 2015 in our Department of Haematology at Centro Hospitalar Universitário de Coimbra.

VWF analysis was performed according to the ISTH algorithm (Sadler *et al*, 2006): the VWF:RCo was determined based on the VWF-platelet GPIb binding activity (Innovance); FVIII:C, VWF:RCo and VWF:Ag levels were measured on a BCS XP coagulation analyser according to the manufacturer's instructions (Siemens Healthcare, Marburg, Germany); VWFpp was measured using the Sanquin ELISA Kit (Amsterdam, Netherlands). Forty-two healthy volunteers acted as a control group for measurements of the VWF antigen level (VWF:Ag), ristocetin cofactor activity (VWF:RCo), VWF collagen binding (VWF:CB) and propeptide (VWFpp).

Details of the bleeding episodes, laboratory data and treatment are provided in Table 11; the different multimer patterns observed are represented in Figure 14.

5.3. Results and Discussion

Waldenstrom's Macroglobulinaemia (WM)

Case 1. A 71-year-old man (P1), previously healthy, was referred for severe bleeding after tooth extraction. Bleeding gums in the last year were mentioned. Based on VWF assays (prolonged APTT ratio 1.48; FVIII:C, 55%; VWF:Ag, 35%; VWF:RCo, 30%; ratio VWF:RCo/VWF:Ag = 0.85), a diagnosis of type 1 AVWS was made. Interestingly, the VWF multimer pattern showed a blurred structure usually present in WM. The management of the bleeding episode was made with sutures, local haemostatic measures and antifibrinolytics. Indeed, WM was later diagnosed and treated with multidrug chemotherapy according to the R-CVP protocol.

Table 11 – Description of bleeding episodes and laboratory profile at diagnosis in 6 patients with AVWS, and respective assessment of response to treatment.

Patient	Diagnosis	Bleeding/ Treatment	APTT ratio	FVIII (%)	VWF: Ag (%)	VWF: RCo (%)	VWF: CB (%)	VWF: RCo/Ag Ratio	VWF: CB/Ag Ratio	VWF pp (%)	VWF pp/Ag Ratio	Multimeric profile
1	WM	After tooth extraction	**1.5**	**55**	**35**	**30**	NA	0.85	-	NA	-	Blurred structure
		Chemotherapy	1.2	152	133	109	NA	-	-	-	-	Normal
2	WM	Menorrhagias, epistaxis, fundus haemorrhage	**1.8**	**26**	**32**	**40**	NA	1.25	-	100	**3.1**	NA
		Chemotherapy	1.1	72	92	80	NA	-	-	NA	NA	Normal
3	MZL	After tooth extraction	**2.0**	**14**	**23**	**4**	NA	**0.17**	-	98.6	**4.3**	Loss HMWM
		Chemotherapy	1.2	96	147	116	NA	-	-	100	1.0	Normal
4	CML	Haemarthrosis	**1.3**	92	86	**43**	**42**	**0.5**	**0.48**	100	1.1	Decreased HMWM
		Chemotherapy	1.2	87	101	85	87	0.84	0.86	100	1.0	Normal
5	ET	Spontaneous bruising	**1.3**	105	83	**37**	51	**0.44**	**0.61**	69	0.8	Decreased HMWM
		Hydroxyurea	0.9	115	99	65	81	0.70	0.82	83	0.8	Normal
6	ET	Spontaneous bruising	1.2	103	79	**44**	54	**0.56**	**0.68**	100	0.97	Decreased HMWM
		Hydroxyurea	1.1	95	93	66	88	0.70	0.94	81	0.87	Normal
		Normal range	0.9-1.2	50-150	50-160	48-173	60-130	<0.7	<0.7	60-140	0.9-1.45	

WM, Waldenstron's Macroglobulinemia; MZL, Marginal Zone Lymphoma; CML, Chronic Myeloid Leukemia; ET, Essential Thrombocythemia; Decreased values are marked in **bold**. NA, Not available; HMWM, high-molecular-weight multimers.

Case 2. A 52-year-old woman (P2) was referred for menorrhagia, epistaxis, headache, rash, visual changes and paraesthesia with three weeks of evolution. She was transferred with a diagnosis of WM. During hospitalisation, a fundus haemorrhage was detected. The patient underwent a laboratory evaluation that disclosed a prolonged APTT ratio (1.80), associated with reduced VWF assays (FVIII:C, 26%; VWF:Ag, 32 %; VWF:RCo, 40%; ratio VWF:RCo/VWF:Ag = 1.25), revealing a type 1 VWF deficiency. She was treated with therapeutic plasmapheresis for hyperviscosity syndrome and multidrug chemotherapy according to the R-CVP protocol. She did not require treatment for haemorrhagic manifestations.

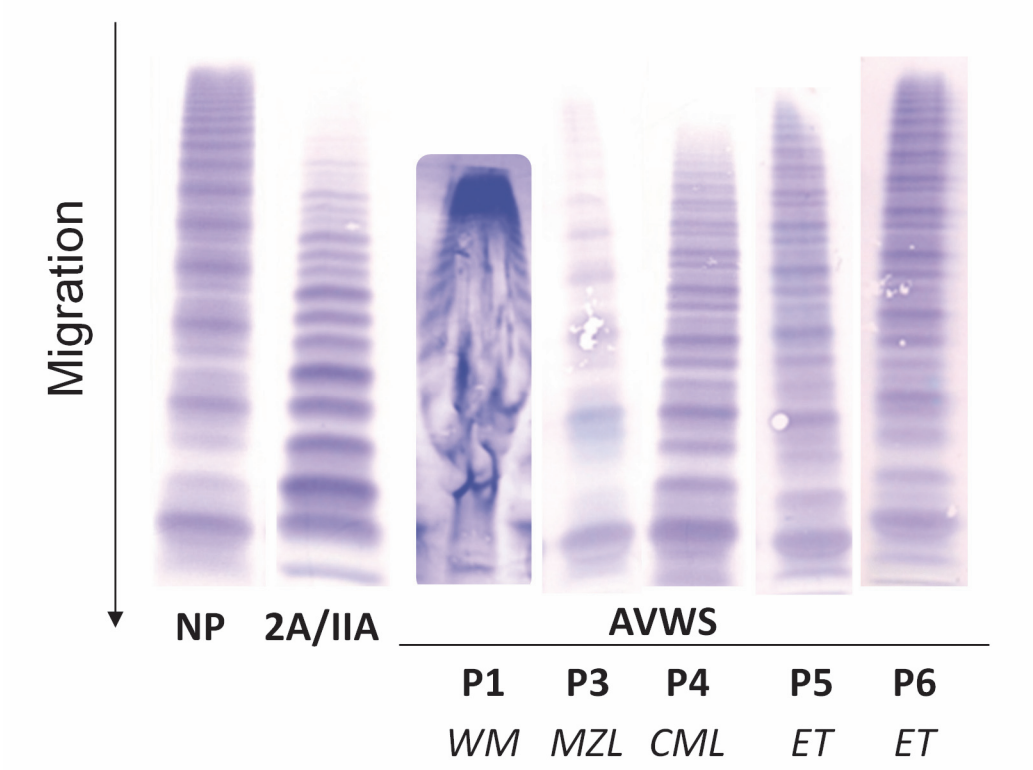

Figure 14 - Multimeric VWF patterns in SDS-agarose gel electrophoresis (1.6%) observed in five patients with AVWS. NP, normal plasma; 2A/IIA congenital VWD control. Lymphoproliferative disorders: P1, deformed pattern with blurred structure of triplets usually described in WM; P3, pattern with loss of HMW multimers and relative increase of the lower satellite band within triplets. Myeloproliferative disorders: P4, P5 and P6 show patterns with variable decrease of HMW multimers and disturbed triplet structure. WM, Waldenstrom's Macroglobulinaemia; MZL, Marginal Zone Lymphoma; CML, Chronic Myeloid Leukaemia; ET, Essential Thrombocythemia. The multimeric patterns are from different gels.

These two cases illustrate how the therapeutic approaches to AVWS not only depend on the underlying disease, but also the kind of trauma. In the P1 case, the bleeding

history at a late stage of life made us suspect AVWS, the laboratory profile confirmed a mild type 1 VWF deficiency, and local haemostatic measures and antifibrinolytics were enough to restore correct haemostasis. The multimer pattern pointed to the underlying disease.

Conversely, in the P2 case, a severe clinical manifestation of hyperviscosity was already evident with severe mucocutaneous bleeding, and an urgent plasmapheresis was carried out. This technique is effective in lowering the amount of IgM paraprotein in the bloodstream, thereby reducing the viscosity symptoms responsible for a high shear stress that causes platelet activation and subsequent VWF proteolysis.

Both patients had specific treatments directed towards WM, which normalised VWF levels and multimer patterns.

Marginal Zone Lymphoma (MZL)

***Case 3*.** A 53-year-old woman (P3), previously healthy, was referred for profuse bleeding after tooth extraction bruises. She referred to spontaneous bleeding gums and a weight loss of approximately 12 kg in one year prior to diagnosis. The VWF analysis revealed a severe type 2 VWF deficiency (prolonged APTT ratio 2.0; FVIII:C, 14%; VWF:Ag, 23%; VWF:RCo, 4%; ratio VWF:RCo/VWF:Ag = 0.17; loss of HMWM). In addition, the elevated ratio of VWFpp/VWF:Ag = 4.3 was consistent with rapid VWF clearance and the multimeric pattern. As the presence of an underlying disease was unknown, the molecular study of exon 28 of the *VWF* gene was performed, which revealed a missense variant c.3797C>A, p.Pro1266Gln (Malmö/New York variant, resulting from gene conversion). However, this variant is characterised by enhanced RIPA – consistent with type 2B VWD - but normal levels of VWF, a normal multimeric pattern and normal platelet count (Federici *et al*, 2011). Patients with this phenotype rarely have significant bleeding, suggesting this variant is an extremely mild form of VWD type 2B. Meanwhile, a cervical lymphadenopathy was detected in the patient. After a lymph node biopsy, the diagnosis of MZL was made and, subsequently, AVWS. The management of bleeding episodes and prophylaxis for excision of adenopathy

were made with FVIII/VWF concentrate and intravenous antifibrinolytics. The patient received multidrug therapy directed towards MZL that resulted in phenotypic remission with normalisation of the VWF levels and multimer pattern.

Chronic Myeloid Leukaemia (CML)

Case 4. A 36-year-old man (P4) with splenomegaly 10 cm below the rib cage, leucocytosis (279 x 10^3/μL) and thrombocytosis (1334 x 10^3 platelets/μL) was diagnosed with a chronic phase of CML. He started treatment with hydroxyurea plus anti-aggregation with aspirin. However, seven days after diagnosis, he had a traumatic haemarthrosis of the right knee. Subsequently, aspirin treatment was suspended, and arthroscopy and drainage were performed. Due to a slight prolongation of APTT, a haemostasis study was performed, which allowed for the AVWS diagnosis based on the APTT ratio 1.3; FVIII:C, 92%; VWF:Ag, 86%; VWF:RCo, 43%; VWF:CB 42%; ratio VWF:RCo/VWF:Ag = 0.5; and decrease of HMWM. The haemorrhagic event was controlled with local measures, and treatment with the tyrosine kinase inhibitor resulted in prompt resolution of his symptoms and normalisation of the VWF levels and multimer pattern.

Essential Thrombocythemia (ET)

Case 5. An 82-year-old woman (P5) with thrombocytosis (965 x 10^3 platelets/μL) was diagnosed with ET. She was under antiplatelet therapy, which she suspended after the appearance of spontaneous bruising in the upper limbs. There were no other bleeding symptoms. She kept a slight prolongation of APTT; thus, a haemostasis study was conducted, which revealed a profile consistent with AVWS: prolonged APTT ratio 1.3; FVIII:C, 105%; VWF:Ag, 83%; VWF:RCo, 37%; VWF:CB 51%; ratio VWF:RCo/VWF:Ag = 0.44; decrease of HMWM. She did not need treatment for the haemorrhagic symptoms.

Case 6. A 75-year-old woman (P6) with thrombocytosis (1043 x 10^3 platelets/μL) was diagnosed with ET. She had petechial haemorrhages and spontaneous bruising. The VWF analysis was performed, which revealed a normal APTT ratio 1.1; FVIII:C, 103%;

VWF:Ag, 79%; VWF:RCo, 44%; VWF:CB 54%; ratio VWF:RCo/VWF:Ag = 0.55; decrease of HMWM.

The cytoreduction with hydroxyurea in both cases resulted in phenotypic remission with normalisation of VWF levels and multimer patterns.

In conclusion, the clinical-laboratory correlation in the six cases led to the diagnosis of AVWS. The detection of low VWF levels in P1 and P3 came before the diagnosis of the underlying lymphoproliferative disease. In P3, the detection of the *VWF* variant, p.Pro1266Gln, could lead to a misdiagnosis in this case as congenital VWD. However, the discordant genotype-phenotype correlation and late haemorrhagic manifestation led to the suspicion of simultaneous AVWS.

We observed different degrees of VWF deficiency, even within the same underlying haematologic disease, e.g., WM (P1 and P2) and ET (P5 and P6). The propeptide indicates normal VWF synthesis and propeptide/VWF:Ag ratios indicate accelerated plasmatic clearance of VWF that was more accentuated in P2 and P3. On the other hand, the reduced function/antigen ratio (VWF:RCo/Ag or VWF:CB/Ag) has proven to be a sensitive parameter indicating structural or functional disorders, even in patients who had absolute activity within a normal range (P5 and P6).
Including the evaluation of VWF analysis in late haemorrhagic episodes and/or in AVWS-associated disorders enhanced the efficacy of AVWS diagnosis, leading to a more tailored management approach in each patient.

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Author contribution

TF was responsible for the study design and wrote the manuscript with GF. TF, ACO, CSP and PM performed the laboratory studies and analyzed the data. GF, RS, MD, AP, and MJM provided clinical support. MLR revised the manuscript.

Conflict-of-interest disclosure:

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CHAPTER

6

COMBINED STUDY OF ADAMTS13 ACTIVITY AND GENETIC ANALYSIS OF COMPLEMENT GENES IN THE DIAGNOSIS OF THROMBOTIC MICROANGIOPATHIES – THE ADD-VALUE OF NEXT-GENERATION SEQUENCING

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CHAPTER 6. COMBINED STUDY OF ADAMTS13 ACTIVITY AND GENETIC ANALYSIS OF COMPLEMENT GENES IN THE DIAGNOSIS OF THROMBOTIC MICROANGIOPATHIES – THE ADD-VALUE OF NEXT-GENERATION SEQUENCING.

6.1. Summary

The two main forms of thrombotic microangiopathy (TMA) are thrombotic thrombocytopenic purpura (TTP) and atypical haemolytic uraemic syndrome (aHUS). ADAMTS13 deficiency and dysregulation of the complement pathway result in TTP and aHUS, respectively, but the overlap of their clinical characteristics makes the differential diagnosis challenging. Severe ADAMTS13 deficiency (<10%) is an important indicator in the differential diagnosis of TTP/aHUS. Advances in next-generation sequencing (NGS) have improved the molecular study of a large number of genes implicated in TMA.

We aimed to develop a TMA diagnosis workflow based on ADAMTS13 activity and screening of mutations using a custom NGS gene panel. To this end, we analysed genotype–phenotype correlations in 45 patients (11 TTP and 34 aHUS) based on the impact of the predicted pathogenicity of each variant found and the co-inherited known genetic risk factors for TMA.

In total, 33 different rare variants, eight of which novel (in *ADAMTS13, CFH,* and *CD46*), were identified across seven genes. The eleven TTP patients were homozygous (n = 6), compound heterozygous (n = 2) and heterozygous (n = 3) for 10 *ADAMTS13* variants (six pathogenic mutations). Among the 34 aHUS patients, 17 were heterozygous for 23 variants in the different complement genes with distinct consequences, ranging from single pathogenic mutations associated with complete disease penetrance to benign variants that cause aHUS only when combined with other variants and/or *CFH* and *CD46* risk haplotypes or *CFHR1-3* deletion.

Our study provides evidence of the usefulness of the NGS panel as an excellent technology that enables more rapid diagnosis of TMA, and is a valuable asset in clinical practice to discriminate between TTP and aHUS.

Keywords: Thrombotic thrombocytopenic purpura; Atypical haemolytic uraemic syndrome; ADAMTS13; Complement; Next-generation sequencing

6.2. Introduction

Thrombotic microangiopathies (TMAs) are characterized by microvascular occlusion, thrombocytopenia and non-immune haemolytic anaemia. The two main forms of TMAs are thrombotic thrombocytopenic purpura (TTP) and atypical haemolytic uraemic syndrome (aHUS). These two types of TMA have overlapping clinical phenotypes. Conceptually, TTP has been distinguished from aHUS by more common neurological manifestations, whereas, in aHUS, the renal involvement is more pronounced. However, this rule is not always reliable; some aHUS patients have neurological complications and some TTP patients have renal failure (George & Nester, 2014; Mannucci & Cugno, 2015; Kremer Hovinga & Lämmle, 2012; Crawley & Scully, 2013). Over the last decade, a noteworthy progress has been made to improve the clinical and laboratory approaches to predict the expected signs and symptoms, patient outcome and genotype-phenotype correlations. Despite this, the differential diagnosis of TTP/aHUS remains challenging.

TTP is mainly caused by a severe plasma deficiency (i.e., <10%) of the cleaving protease of von Willebrand factor (VWF) - ADAMTS13 (A Disintegrin And Metalloproteinase with a ThromboSpondin type 1 motif, member 13). A severe ADAMTS13 deficiency is due, more often, to anti-ADAMTS13 autoantibodies (acquired) or, rarely, to homozygous or compound heterozygous *ADAMTS13* gene mutations (hereditary, also called Upshaw-Schulman syndrome) (Levy *et al*, 2001; Sadler, 2008). However, heterozygosity for *ADAMTS13* mutations was observed among patients diagnosed with acute acquired TTP and severe ADAMTS13 deficiency (Meyer *et al*, 2007; Camilleri *et al*, 2008). In all forms, the predominant pathogenic factor of TTP are the increased number of ultra-large multimers of VWF resulting from the lack of proteolytic regulation by ADAMTS13 and leading to platelet clumping in the microcirculation of various organs (Sadler, 2008).

While TTP is characterized by the severe deficiency of ADAMTS13, aHUS is characterized by hyperactivation of the alternative complement pathway resulting from either a loss-of-function mutation in a regulatory gene (*CFH, CFI, CD46 (MCP) or*

THBD) or a gain-of-function mutation in an effector gene (*CFB* or *C3*) (George & Nester, 2014; Marina Noris & Remuzzi, 2009). The mutations were found mainly in the heterozygous state, and approximately 5% of patients have combined mutations, usually in *CFH* with either *CD46* or *CFI*. Homozygosity for risk haplotypes of *CFH* (*CFH-H3*) and *MCP* (*MCPggaac*) have been shown to significantly increase disease penetrance and severity (Caprioli *et al*, 2003; Esparza-Gordillo *et al*, 2005; Bresin *et al*, 2013). Additional genetic risk factors include the *CFHR1–3* deletion in homozygous state caused by non-allelic homologous recombination of *CFHR3* and *CFHR1* (Moore *et al*, 2010). Finally, recessive mutations in *DGKE*, which encodes diacylglycerol kinase-ε and is expressed in endothelial cells, platelets and podocytes, were identified in children with the onset of aHUS in the first year of life (Loirat *et al*, 2015).

Poor penetrance is a common clinical feature, and adult onset occurs in patients with severe deficiencies. In addition, both deficiencies predispose patients to a TMA after a triggering event, such as pregnancy, bacterial and viral infections, neoplasia, autoimmune disorders and exposure to certain drugs (Lämmle *et al*, 2008; Scully *et al*, 2014; Auer Von *et al*, 2015).

Severe ADAMTS13 deficiency (<10%) is an important indicator in the differential diagnosis of TTP/aHUS. However, several studies in aHUS patients have shown reduced ADAMTS13 activity that may predispose patients to the TMA phenotype (Remuzzi *et al*, 2002; Feng *et al*, 2013b). Indeed, *ADAMTS13* polymorphisms associated with partial deficiency of ADAMTS13 in the presence of a primary trigger or when co-inherited with a mutation in a complement gene have been described in these patients (Plaimauer *et al*, 2006; Feng *et al*, 2013b; Noris *et al*, 2005). These findings suggest that TTP and aHUS could exhibit overlap not only in their clinical characteristics but also in their pathophysiological mechanisms.

To better understand this clinical variability, which is found even within families, it is necessary to characterize the mutational profile. Nevertheless, up to now the molecular analysis of genes implicated in TMA was no affordable by diagnostic laboratories since the high cost that suppose the study of multiple genes by

conventional Sanger DNA sequencing. The advent of next-generation sequencing (NGS) is changing this paradigm since NGS allows simultaneously sequence large gene panels and generates competitive results at a lower cost and in a shorter amount of time.

From this perspective, we conducted a study with two main objectives. First, to better understand the genotype–phenotype correlations we detailed phenotypic characterization in our cohort of patients with TMA who had mutations in ADAMTS13 and/or in complement genes. Second, we designed and validated an NGS-based gene panel to facilitate genetic testing in TTP and aHUS. This approach allowed the implementation, within our Department of Haematology, of an efficient methodology to establish the diagnosis and prognosis of these two rare diseases that exhibit phenotypic similarities.

6.3. Materials and Methods

6.3.1. Patients and controls

We reviewed 154 Caucasian patients of Portuguese origin with TMA investigated from January 2007 to January 2016 in the Department of Haematology at Centro Hospitalar Universitário de Coimbra. The selection diagnostic criteria based on international guidelines (Scully *et al*, 2012) were thrombocytopenia, anaemia and morphological evidence of red cell fragmentation. Sixty-three patients were diagnosed in our department and the remaining 91 patients were referred from external centres in different regions of Portugal. These hospitals diagnosed and treated patients with TMA and sent samples to our centre to perform ADAMTS13 activity assay and/or genetic screening of *ADAMTS13* and complement genes. Eight patients were excluded as their ADAMTS13 assay sample was sent after the administration of plasma or initiation of plasma exchange (PEX).

The 146 individuals in this study included both adults and children with a median age of 36 years (1 month –89 years) and a sex distribution of 88 females and 59 males. The adult patients (n = 110) had a median age of 44 years with a range of 20–89 years. The

child probands (n = 30) had a median age of 9 years with a range of 1–18 years, and infants included in this study (n = 7) ranged in age from one week to 11 months.

We also investigated 10 asymptomatic relatives of five patients in whom an *ADAMTS13* mutation had been identified. In accordance with the Declaration of Helsinki, informed consent was obtained from all patients or from their family members.

Forty-two healthy volunteers acted as a control group for ADAMTS13 assay measurements. The control group included unrelated individuals (32 females and 10 males) without an individual or family history of excessive bleeding, thrombosis or haemolytic anaemia, with a mean age of 33.5 ± 11.12 yrs.

6.3.2. Samples and sample processing

ADAMTS13 assays were performed on blood collected into vacuum tubes containing 3.2% sodium citrate and centrifuged within 15 min at room temperature for 20 min at 2500 g. The obtained platelet-poor plasma was then separated into aliquots and kept frozen at −80 °C until further use. The ADAMTS13 assays (activity, antigen and presence of anti-ADAMTS13 autoantibodies of IgG class) were performed using an ELISA kit (TECHNOZYM® ADAMTS13 ELISA, Technoclone, Vienna, Austria) according to the manufacturer's recommendations on a Triturus Immunoassay System. The ADAMTS13 antigen concentration in plasma was measured using anti-ADAMTS13 mAbs directed against the CUB domain (normal range: 0.60 – 1.60 µg/mL). The ADAMTS13 activity was measured using a 73 amino acid peptide substrate (GST-vWF73-His) based on the cleavage site of the VWF A2 domain (normal range: 40–130%). According to the manufacturers' specifications, the presence of anti-ADAMTS13 autoantibodies of IgG class was positive for a titre greater than 15.1 arbitrary units (AU)/mL.

Genomic DNA was extracted from EDTA whole blood by automatic isolation on an iPrep™ instrument using a gDNA Blood Kit (Thermo Fisher Scientific, Waltham, MA, USA). The DNA concentration was adjusted to a range of 25–50 ng/µl.

6.3.3. Strategy for mutation analysis

Two different direct sequencing methodologies were used for the molecular analysis in this study:

Sanger direct sequencing – Until March 2015, Sanger sequencing was performed according to the following strategy: (i) all of the coding sequences of *ADAMTS13* [regions of interest (ROIs)] in patients with repeated measurements of ADAMTS13 activity < 10% after resolution of the acute episode and no evidence of anti-ADAMTS13 IgG antibodies; (ii) all of the coding sequences of *CFH, CFI, CFB, C3, CD46, THBD and DGKE* (ROIs) in patients with suspected aHUS.

Next-Generation Sequencing (*NGS)* panel – In the beginning of last year, we developed a custom gene panel that includes *ADAMTS13, CFH, CFHR1, CFHR3, CFHR4, CFHR5, CFI, CFB, C3, THBD and DGKE* to facilitate genetic testing in TTP and aHUS patients. Using Ion AmpliSeq™ Designer (Thermo Fisher Scientific, Waltham, MA, USA), we selected the regions of interest (ROIs) to be sequenced, including all exons, promoter regions and the intronic flanking regions (at least 20 bp).

Before its implementation, the gene panel was validated using samples from eight patients (TTP and aHUS) previously studied by Sanger sequencing and in which *ADAMTS13*, *CFH* and *CFI* variants have been identified. In addition, the coding *VWF* [regions of interest (ROIs)] were analysed, as previously reported (Fidalgo *et al*, 2016) in all TTP samples (DNA samples were sent to Unitat de Diagnòstic i Teràpia Molecular, Banc de Sang i Teixits (BST, Barcelona)). We analysed *VWF* because TTP phenotype could theoretically be influenced by an intrinsic resistance of VWF to proteolysis by ADAMTS13. Regarding to comparison between laboratories on the basis of coverage and diagnostic yield of NGS, these samples were analysed to *ADAMTS13* ROIs using other NGS panel, as described in the Suppl. Materials.

6.3.4. Sequencing using NGS and identification of genetic variants

Sample preparation, target genomic enrichment, and sequencing – For each sample, the DNA concentration was determined using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and then diluted to 4 ng/μl. Library preparation was performed using the AmpliSeq™Library Kit 2.0 (Thermo Fisher Scientific) following the manufacturer's protocol. For each sample, 14 ng of DNA was mixed with each of two pools of primers and polymerase to generate a multiplexed PCR. After that, Ion Xpress™-specific index paired-end barcode adapters (Thermo Fisher Scientific) were ligated to the 5' and 3' ends of DNA fragments by incubation with DNA ligase for 30 min at 22 °C and 10 min at 72 °C. The adapter-ligated DNA fragments were purified by a reaction clean-up and removal of adapter-dimers with Agencourt® AMPure® XP Reagent (Beckman Coulter). Library quality and concentration were evaluated using a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). The pooled barcoded libraries were clonally amplified using the Ion OneTouch2™ (Thermo Fisher Scientific) system according to the manufacturer's instructions. Ion sphere particles were enriched using the E/S module, and, subsequently, the enriched template-positive particles were loaded onto an Ion 316™ or 318™ chip and sequenced using Ion Hi-Q™ Sequencing 200 Kit chemistry on an Ion Torrent™ PGM sequencing system (Thermo Fisher Scientific).

The NGS pipeline output, after QC/QA filtering, was analysed using Torrent Suite (v3.6; Thermo Fisher Scientific, S.L.), and sequences were aligned to the human genome version 19 (hg19) using T-MAP (version 3.6.58977). Variants were called using the Torrent Variant Caller (version 3.6.59049, with Germ Line – Low Stringency settings) and annotated using Ion Reporter (Thermo Fisher Scientific). The minimum depth of coverage required for each nucleotide in the ROI to be identified/considered a candidate mutation was ≥10X. However, the mean coverage for all the ROIs (although highly variable) was at least 500-fold in all patients. This workflow allows the alignment of the resulting sequences against hg19 and concurrent *in silico* analysis, permitting the identification of potential pathogenic variants and filtering the known polymorphisms described to date in the dbSNP (National Center for Biotechnology Information dbSNP

(build 137)) and 1000 Genomes databases (The 1000 Genomes Project Consortium, 2015).

For confirmation of the variants identified by NGS, the corresponding region was amplified using PCR and sequenced by Sanger direct sequencing, as described in the Suppl. Materials.

6.3.5. Multiplex ligation-dependent probe amplification (MLPA)

The *CFHR3-1* copy number variation (a tandem deletion), was screened using SALSA MLPA P236-A3 kit (MRC-Holland). Fragment size analysis was performed using an ABI 3130 Genetic Analyzer (Thermo Fisher Scientific). The output file (.fsa) from Fragment Analysis software (Thermo Fisher Scientific) was used as input for Coffalyser.Net, specific software for the analysis of MLPA data (MRC-Holland). Data normalisation was performed using four healthy controls.

6.3.6. In silico analysis

The impact of missense changes was further analysed according to the recommended criteria that included evolutionary conservation of an amino acid or nucleotide, location and context within the protein sequence and the biochemical consequence of the amino acid substitutions (Richards *et al*, 2015; Gonzalez-Garay, 2014). This analysis was assessed using five different *in silico* algorithms: PROVEAN, SIFT, PolyPhen-2, MutationAssessor and MutPred (Suppl. References) (last accessed April 3rd, 2016). Missense variants were considered deleterious if at least three of the five prediction programmes suggested a pathogenic effect.

6.3.7. Genetic databases

As recommended by the guidelines for the interpretation of sequence variants (Richards *et al*, 2015), we defined those variants that are not reported in the international databases (population- and disease-specific) or in the published literature as ‘novel’. Therefore, in addition to using the polymorphism databases (dbSNP and 1000 Genomes databases) mentioned above, we also checked the frequency of

variants found in the Exome Aggregation Consortium (ExAC), Exome Variant Server (EVS), Human Gene Mutation Database and FH aHUS Mutation Database (Suppl. References) (last accessed April 10th, 2016).

6.3.8. Assessment of the pathogenicity of variants

The following criteria were used to evaluate the pathogenicity of the variants: 1) whether the variant was a stop/frameshift variant, which was considered to most likely be disease causing, 2) co-segregation in the family, 3) whether the variation had been previously identified in international databases, 4) *in silico* evaluation and 5) presence of the second mutant allele in the case of autosomal recessive inheritance.

Variants were classified as pathogenic, likely pathogenic, uncertain significance, likely benign or benign based on the available evidence, according to the practice guidelines for the evaluation of pathogenicity recently published by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (Richards *et al*, 2015).

The amino acid numbering and nomenclature used is in accordance with the international recommendations for the description of sequence variants of the Human Genome Variation Society (http://www.HGVS.org).

6.4. Results

6.4.1. Characteristics of the patients studied

The present study reviewed 146 patients from different regions of Portugal for whom ADAMTS13 activity levels were measured; 60 patients had a level of ≤10% (severe deficiency), and 86 patients had a level of >10% (Figure 15). The majority of patients 53 out of 60 (88%) patients with severe deficiency had a positive anti-ADAMTS13 IgG antibodies level (acquired TTP) with a median titre of 62 UA/mL (range: 15-121); the remaining seven patients had no evidence of anti-ADAMTS13 IgG antibodies (hereditary TTP). The group with an ADAMTS13 activity >10% included 34 (39.5%) patients with suspected aHUS and 52 (60.5%) patients with a range of underlying

diseases (malignant hypertension, HELLP syndrome, solid organ transplantation and systemic lupus erythematosus) that were excluded.

Based on these findings, three groups of patients were selected for this analysis (n = 45) and underwent a detailed phenotypic study and mutational analysis: (i) seven hereditary TTP patients; (ii) four acquired TTP patients who have had ADAMTS13 activity of ≈ 40% after an acute episode, but no detected anti-ADAMTS13 IgG antibodies and (iii) 34 aHUS patients (Figure 15). The demographic, laboratory and clinical data are summarized in Table 12.

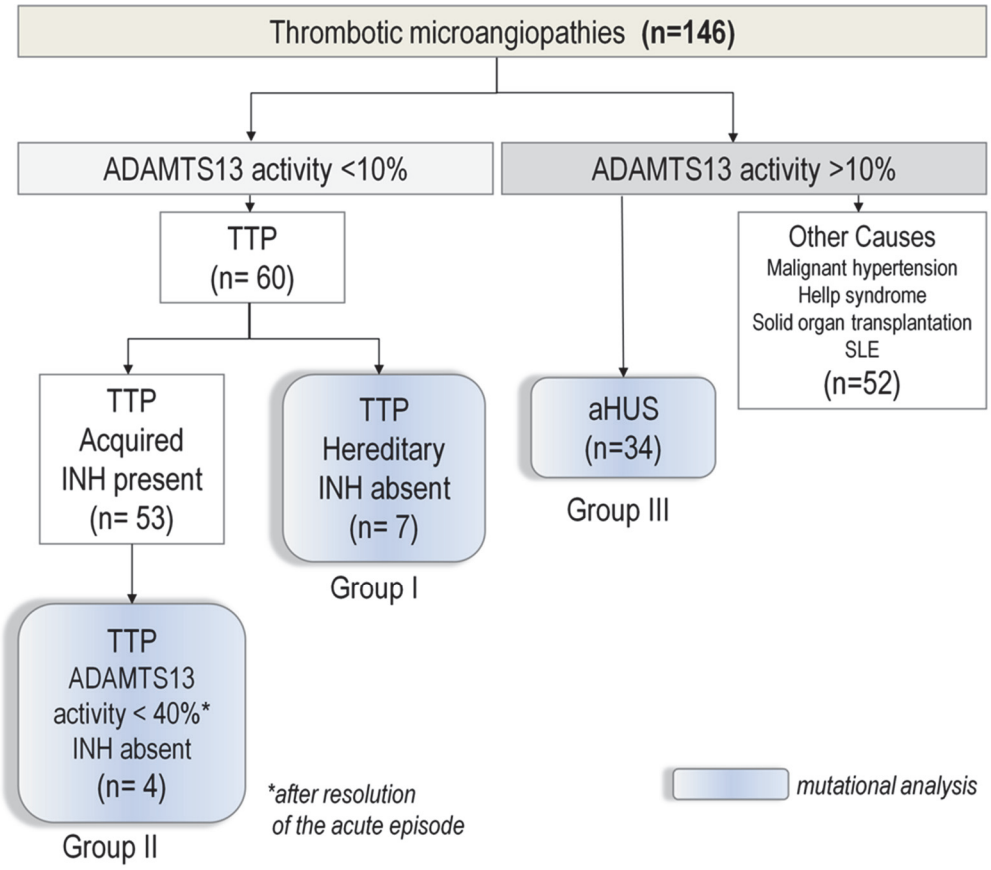

Figure 15 – Flow chart of patients' enrolment based on ADAMST13 activity and clinical characteristics.

Table 12 – Demographic, laboratory and clinical data of the 45 patients at the time of the first acute episode.

	Group I Hereditary TTP (n = 7)	Group II Acquired TTP* (n = 4)	Group III aHUS (n =34)
Male/Female	3/4	2/2	13/21
Age onset	5 yr (7 month – 26 yr)	22.5 yr (13 – 28 yr)	30 yr (3 month – 68 yr)
Laboratory findings			
Haemoglobin (g/L)	6.6 (5.6 – 10.9)	9 (8.1 – 9)	7.5 (7 – 11.4)
Platelet count (×10^3 platelets/μL)	15 (5 – 44)	35 (14 – 50)	58 (5– 140)
Creatinine (μmol/L)	52 (17 – 130)	73 (50 – 140)	415 (34 – 1872)
LDH (U/L)	1946.5 (617 – 4350)	2015 (1667 – 2430)	2895 (436 – 11430)
ADAMTS13 activity (%)	1 (0 –3)	3 (0-5)	73 (43-128)
ADAMTS13 activity (%) after acute episode	1 (0 –2)	37.5 (35-38)	ND
Clinical findings			
Neurological symptoms	2	1	10
Fever symptoms	3	3	14
Renal failure	0	1	24
Triggers			
Pregnancy	2 (4 Losses; No live pregnancies)	0	0
Gastroenteritis, other infections	7	1	24
No triggers	2	3	10
Treatment of the first episode			
Plasma, infusion or exchange	PI (n = 5); PEX (n = 2)	PI (n = 4); PEX (n = 4)	PEX, PI (n = 6); PEX (n = 12); PI (n = 12)
Prednisolone	2	4	9
Outcome at first episode			
Plasma, infusion every 1–3 weeks	2	0	0
Human factor VIII concentrate BPL 8Y	1	0	0
Anti-C5 antibody (Eculizumab)	NA	NA	3
Complete remission	8	4	31
Relapses (n)	4 (3 – 12)	0	3

*With ADAMTS13 activity diminished after acute episode and no detectable anti-ADAMTS13 IgG;
ND, not determined; PI, plasma infusion; PEX, plasma exchange; NA, not applicable; BPL 8Y, BioProducts Laboratory, Elstree, Herts, UK

6.4.2. Identification of gene defects in TMA patients

The NGS method confirmed all variants previously detected by Sanger sequencing method in *ADAMTS13*, *CFH* and *CFI* (100% sensitivity) used to NGS panel validation. The *VWF* analysis by NGS did not reveal any rare variant in TTP samples. Concerning to the variants found in *ADAMT13,* the results were completely concordant between those obtained in our laboratory and by the laboratory from the Banc de Sang i Teixits of Barcelona (Spain). In total, 33 different variants scattered across seven genes (*ADAMTS13, CFH, CD46 , C3, CFI, CFB* and *CFHR5*), whose frequencies in the different populations studied in the 1000 Genomes were below 1%, are summarized in Suppl. Table 4 and include: 30 missense variants (91%), two small deletions (6%) and one splice site mutation at the 3'-splice-site consensus AG (3%). The frequencies of those variants were also checked in the ExAC and EVS_EA population databases. In total, eight variants had never been reported in the population databases and international disease databases, and four had only been reported in the population databases. No rare variants were identified in *CFHR3, CFHR4, THBD and DGKE* in this cohort.

6.4.3. Prediction of pathogenic variants

Sixteen out of 30 missense variants were predicted to be deleterious (Suppl. Table 4). According to the practice guidelines (Richards *et al*, 2015), these *in silico* analyses combined with other evidence (population, functional and reported studies) permitted the following classification of the variants: pathogenic (n=14; 47%), likely pathogenic (n=4; 13%), uncertain significance (n=2; 7%), likely benign (n=6; 20%) and benign (n=4; 13%) (Suppl. Table 5). Accordingly, we used the term ‘mutation’ only to refer to pathogenic variants.

6.4.4. Potential functional impact of novel variants

Seven of the eight novel variants (89%) were pathogenic: five missense changes (*CFH:* c.240T>G p.Cys80Trp; c.335A>G, p.Tyr112Cys; c.493G>T, p.Asp165Tyr; c.3562A>G, p.Lys1188Glu; and c.3644G>T, p.Arg1215Leu) and two small deletions (*ADAMTS13*: c.762_774del12pb, p.Pro256Serfs*12; and *CD46* c.800-821del, Thr267Ilefs*24) (Suppl.

Table 5). The remaining variant was indicated to be a likely benign variant (*CFH* c.1864A>G, p.Ile622Val). Of the four missense changes found only in the two population databases, one was considered to be likely pathogenic (*CFHR5*:c.329T>C, p.Val110Ala); the remaining three, each of them were inherited in presence of a second variant and, therefore, considered with uncertain significance (*ADAMTS13*: c.1874G>A, p.Arg625His; c.2218G>A, p.Glu740Lys and; *CD46*: c.686G>A, p.Arg229Gln) (Suppl. Table 5).

6.4.5. Phenotype–genotype analysis in TTP patients

ADAMTS13 variants were identified in all 11 TTP patients of this cohort (100%): two patients had more than one variant, six patients were homozygous and three patients presented an *ADAMTS13* variant in heterozygous state (Table 13 and Figure 16). The *ADAMTS13* analysis revealed ten distinct rare variants (one novel). Six were pathogenic, one likely pathogenic, one was of uncertain significance and another two were likely benign (Suppl. Table 5). Of 11 complement genes studied, only a rare benign variant in *CFI* was identified in one TTP patient (P10). The family pedigrees of patients 1-11 are shown in Figure 17A and B.

The seven hereditary TTP patients (**group I**) comprised four females and three males from six unrelated families. Two of them had pregnancy-associated TTP, and five presented with TTP in childhood (two were siblings). The ages of onset in the two adults were 25 and 26 years, and the mean age for childhood onset was 4 years (seven months to 6 years). All seven patients had persistently low/undetectable levels of ADAMTS13 activity (< 1%) and ADAMTS13 antigen (< 0.1 μg/mL) confirmed at presentation and again after an acute episode. The median platelet count was 15 x 10^3/μL (range 5–44), and the serum creatinine level was 52 μmol L^{-1} (range 34–130) during the acute phase (Table 12).

Patient P1, a 20-year-old male who first developed TTP at the age of 4 years, was found to be homozygous for the *ADAMTS13* c.2074C>T, p.Arg692Cys mutation and for four single-nucleotide polymorphisms (SNPs) involved in ADAMTS13 deficiency:

p.Arg7Trp, p.Gln448Glu, p.Pro618Ala and p.Ala732Val. The patient's mother was heterozygous for the p.Arg692Cys mutation and for the four SNPs, which showed that all were co-inherited in the same allele (Table 13 and Figure 17A).

The P2, P3, P4 and P5 patients were all homozygous for the same missense mutation, *ADAMTS13* c.2260T>C, p.Cys754Arg, and for the SNP p.Gln448Glu. The parents of P2, and the parents of P3 and P4 (both non-consanguineous), were heterozygous for the p.Cys754Arg mutation and the SNP p.Gln448Glu, and, accordingly, they all had ADAMTS13 activities within the expected range (range 37-51%) (Table 13 and Figure 17A).

Patients P2 (a 25-year-old female), P3 and P4 (a sister and brother, aged 15 and 13 years old, respectively) and P5 (a 29-year-old male), who developed their first TTP at a similar age, all had infections as a trigger but with presented different degrees of severity. Indeed, patients P2 and P3 had experienced numerous crises with and without infections that required prophylaxis treatment; P2 was treated with plasma infusion (PI) and P3 with intermediate-purity human factor VIII concentrate BPL 8Y (BioProducts Laboratory, Elstree, Herts, UK) which has some ADAMTS13 activity (Table 13).

Patients P6 and P7 had adult-onset TTP precipitated by pregnancy. P6, a 45-year-old woman who had experienced two pregnancy losses at the ages of 26 and 28, was compound heterozygous for two missense mutations and a novel small deletion [c.762_774del12pb, p.Pro256Ser*fs**12; c.1874G>A, p.Arg625His]+[c.2260T>C, p.Cys754Arg] and heterozygous for the SNP p.Gln448Glu. This woman suffered subsequent episodes of TTP outside of pregnancy, all of them successfully treated with PI. Interestingly, her brother had the same genotype with undetectable ADAMTS13 levels and had no TTP events (Table 13 and Figure 17A).

Table 13 – Phenotypic and genotype characteristics from group I (P1 - P7) and group II (P8 - P11) with thrombotic thrombocytopenic purpura carrying rare variants in ADAMTS13.

							ADAMTS13							
Patient ID	**Sex**	**Age onset (yrs)**	**Triggers events**	**Treatment**	**Relapses**	**Prophylaxis**	**Nucleotide Change**	**Amino Acid Change**	**VC****	***Zygosity***	***ADAMTS13 SNPs mod.***	***MCPggaac alleles***	***CFH- H3 alleles***	***CFHR1-3 alleles***
P1†	M	4	Recurrence with infections	PI	3	No	c.2074C>T	p.Arg692Cys	P	Homozygous	p.Arg7Trp* p.Gln448Glu* p.Pro618Ala* p.Ala732Val*	0	0	0
P2†	F	5	Recurrence with infections	PI	12	PI, 2U 3/3 weeks	c.2260T>C	p.Cys754Arg	P	Homozygous	p. Gln448Glu*	0	1	0
P3†	F	5	Recurrence with infections	PI; BPL 8Y	8	BPL 8Y§ 3/3 weeks	c.2260T>C	p.Cys754Arg	P	Homozygous	p.Gln448Glu*	0	0	0
P4 † brother P3	M	3	3 episodes-with acute infection	PI; BPL 8Y	3	No	c.2260T>C	p.Cys754Arg	P	Homozygous	p.Gln448Glu*	0	0	0
P5†	M	7 m	Recurrence with infections	PI	4	No	c.2260T>C	p.Cys754Arg	P	Homozygous	p.Gln448Glu*	0	1	0
P6‡	F	26	Pregnancy	PI	4	No	c.2260T>C c.1874G>A **c.762_774del12pb**	p.Cys754Arg p.Arg625His	P UVS P	Compound heterozygous	p.Gln448Glu	1	1	0
P7‡	F	26	Pregnancy	PEX;PI	4	PI, 2U 3/3 weeks	c.3368G>A	p.Arg1123His	P	Homozygous	—	0	1	0
P8ζ	M	20	No	PEX;PI	0	No	c.1370C>T	p.Pro457Leu	P	Heterozygous	p.Gln448Glu p.Ala900Val	0	2	0
P9ζ	F	25	1 episode-with acute infection	PEX;PI	0	No	c.2914C>T	p.Arg972Trp	P	Heterozygous	p.Ala900Val	0	0	0
P10ζ	M	13	No	PEX;PI	0	No	c.1368G>T c.2218G>A *CFI*, c.1642G>C	p.Gln456His p.Glu740Lys p.Glu548Gln	LP LB B	Compound heterozygous	—	0	0	0
P11ζ	F	28	1 episode-with acute infection	PEX;PI	0	No	c.3287G>A	p.Arg1096His	LB	Heterozygous	p.Gln448Glu* p.Arg7Trp	0	0	0

†indicates childhood TTP; ‡ Indicates pregnancy-associated TTP; ζ Indicates acquired TTP with mutation detected; M, male; F, female; m, months; PI, plasma infusion; BPL 8Y, BioProducts Laboratory, Elstree, Herts, UK; §Patient received 1000 IU *per dose*; PEX, plasma exchange; **VC, variant classification: P = pathogenic, LP = likely pathogenic, LB = likely benign, and B = benign; UVS = unknown significance; *SNPs in homozygosity; SNPs, single nucleotide polymorphisms; mod, modulators. Novel variant is marked in **bold**.

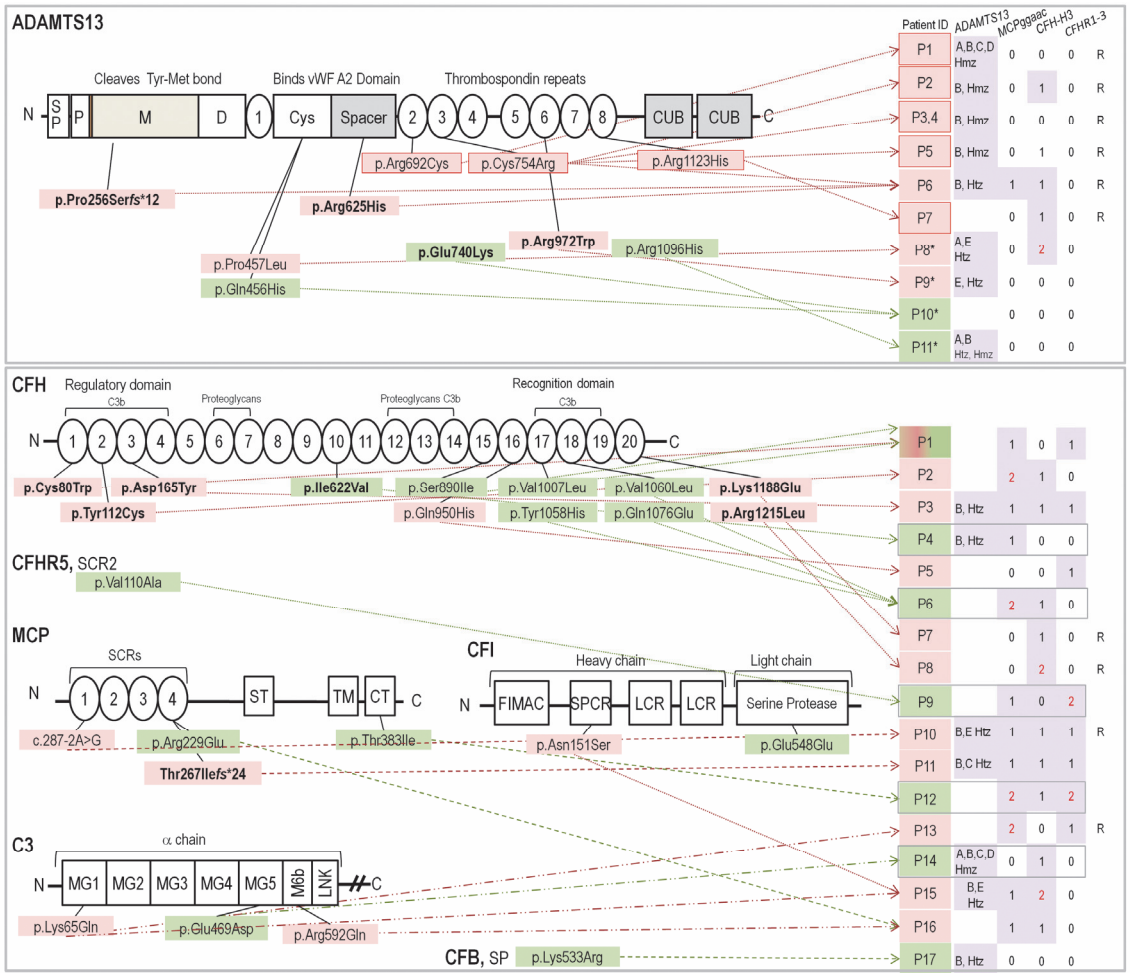

Figure 16 - Location of variants in the protein domain structures of ADAMTS13, CFH, CFHR5, MCP, CFI, C3 and CFB. The scheme represents a rare gene variant(s) inherited for each patient (indicated by an arrow) as well as the co-inherited known genetic risk factors for TMA. The four *ADAMST13* SNPs, A (p.Arg7Trp), B (p.Gln448Glu), C (p.Pro618Ala) and D (p.Ala732Val), the *CFH* and *MCP* risk haplotypes, and the *CFHR1-3* deletion are represented. The TTP patients were homozygous (n = 6), compound heterozygous (n =1) and heterozygous (n = 4) for 10 *ADAMTS13* variants: six pathogenic and four benign/likely benign variants. The seventeen aHUS patients were heterozygous for 23 variants in the different complement genes ranging from single pathogenic mutations (n = 11) to benign variants combined with other variants and/or *CFH* and *MCPggaac* risk haplotypes or the *CFHR1-3* deletion. This scheme shows the autosomal recessive inheritance of TTP with biallelic pathogenic variants in *ADAMTS13* and the autosomal dominant inheritance of aHUS with polygenic variants (mainly *CFH*).

CFH, complement factor H; MCP, membrane cofactor protein; CFI, complement factor I; C3, Complement C3; P, patient; red border, homozygous mutations; pink boxes, pathogenic mutations; green boxes, other variants (likely pathogenic, benign); light purple boxes, *ADAMST13* SNPs and alleles carrying *CFH*, *MCPggaac* risk haplotypes and the *CFHR1-3* deletion.

Patient P7, a 37-year-old woman who developed TTP during her first pregnancy at 25 years of age, was treated with plasma exchange (PEX) at presentation and had a foetal death. Three years later she had a second pregnancy loss. The hereditary TTP diagnosis was made after a new investigation because she suffered recurrent stroke and was

found to be homozygous for the previously reported missense pathogenic mutation *ADAMTS13* c.3368G>A p.Arg1123His. This patient now receives prophylaxis treatment with PI (Table 13 and Figure 17A).

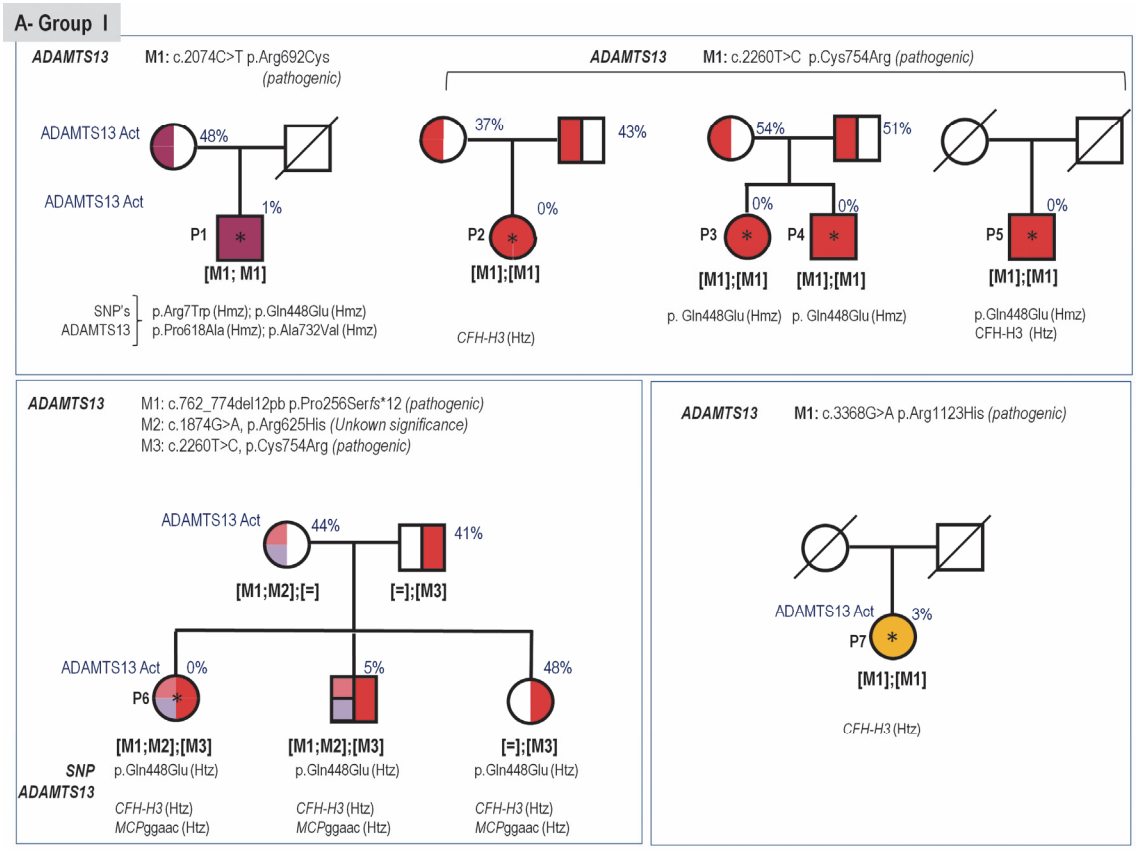

Figure 17: Pedigree and laboratory features associated with ADAMTS13 variants. A - Group I, seven hereditary TTP patients; Six patients were homozygous and one was compound heterozygous for *ADAMTS13* pathogenic mutations; p.Cys754Arg occurred repetitively (66%).

Among four acquired TTP patients (**group II**) who had a median ADAMTS13 activity of 37.5% (range 35-38) after an acute episode, but no detectable anti-ADAMTS13 IgG antibodies, three patients presented TTP in adulthood and one presented TTP at 13 years. The median age of onset was 22.5 years (range 13–28 years). The median platelet count was 35 x 10^3/μL (range 14–50), and the serum creatinine level was 100 μmol L^{-1} (range 73–200) (Table 12).

Patients P8, P9, and P11 were heterozygous for a single missense mutation: *ADAMTS13* c.1370C>T, p.Pro457Leu; c.2914C>T, p.Arg972Trp and c.3287G>A p.Arg1096His, respectively. Patient P10 developed TTP at 13 years old without triggers

and was compound heterozygous for two missense variants in *ADAMTS13* (c.1368G>T, p.Gln456His and c.2218G>A, p.Glu740Lys) and a benign missense variant in *CFI* (c.1642G>C, p.Glu548Gln) (Table 13 and Figure 17B).

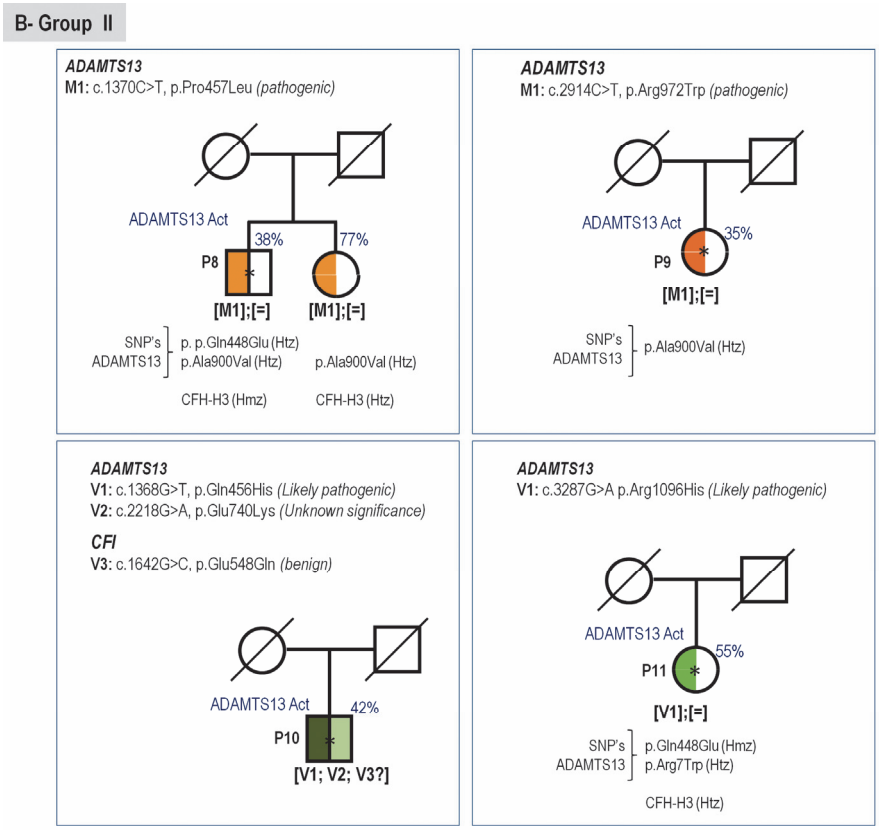

Figure 17: B – Group II, four acquired TTP patients who have ADAMTS13 activity of < 40% after an acute episode but no detected anti-ADAMTS13 IgG antibodies. All patients were heterozygous for *ADAMTS13* variants. The * indicates the patients who have had TTP episode(s); M, mutation; V, variant; and [=],'no change' in the other allele.

Among the 11 patients who carried *ADAMTS13* variants, only one patient (P6) was double heterozygous for the risk haplotypes *CFH-H3/MCPggaac*, and other patient was homozygous for *CFH-H3*. No patients were homozygous for the *CFHR3-1* deletion (Table 13).

6.4.6. Phenotype–genotype analysis in aHUS patients

Sporadic aHUS was found in 34 patients (**group III**), 21 women and 13 men, including 23 adults with a median age of onset of 43 years (range 17-67), 9 children with a median age of onset of 4 years (range: 1-13), and two infants aged three and six

months. All aHUS patients had ADAMTS13 activity levels > 40% (median 73%, range 43–128%). The median platelet count was 58 x 10^3/μL (range 5–150), and the serum creatinine level was 415 μmol L^{-1} (range 34–1872) during the acute phase (Table 12).

In 17 out of 34 (50%) aHUS patient's mutations were identified, 13 patients had mutations in heterozygous state and four patients were compound heterozygous. Two patients presented three variants in a single gene (*CFH*) and the remaining two patients had combined variants in two different complement genes *(C3/CFI* and *C3*/*CD46).* In total, 23 rare variants (seven non-previously described) were identified in six complement genes that were located mostly in *CFH* (12), with four in *CD46,* four in *C3*, one in *CFI*, one in *CFB* and one in *CFHR5* (Table 14 and Figure 16).

Six aHUS patients carried single *CFH* variants (Table 14). Among these, the patients P2, P3, P5, P7 and P8, four women and one child, with ages of onset between 2 and 45 years old, carried a single pathogenic missense mutation. Four of them had not been previously described: c.335A>G, p.Tyr112Cys; c.493G>T, p.Asp165Tyr; c.3562A>G, p.Lys1188Glu and c.3644G>T, p.Arg1215Leu. However, they were in well-established mutational regions of *CFH*. The other mutation, *CFH* c.2850G>T, p.Gln950His, had previously been associated with aHUS. Patient P4, a woman with a late onset of 67 years, carried a novel likely benign missense variant. Patient P1, with an early onset of 3 months, carried the *CFH* variants [c.240T>Gp.Cys80Trp(;)c.2669G>T,p.Ser890Ile(;) c.3019G>Tp.Val1007Leu], only the first was a pathogenic mutation. Patient P6, with an onset of 46 years, carried the *CFH* variants [c.3172T>C,p.Tyr1058His(;) c.3178G>C,p.Val1060Leu(;)c.3226C>G,p.Gln1076Glu]; the first two were likely benign and the last was a likely pathogenic variant. In both cases, the family study was not possible and, therefore, which variants were in *cis* or in *trans* was unknown. Three out of eight patients who carried single *CFH* variants were heterozygous/homozygous for both risk haplotypes *MCPggaac* and *CFH-H3:* P3 was double heterozygous, while P2 and P6 were homozygous for *MCPggaac* and heterozygous for *CFH-H3.* None of these patients were homozygous for the *CFHR1–3* deletion (Table 14).

Patients P7 and P8, who carried the novel pathogenic *CFH* mutations p.Lys1188Glu and p.Arg1215Leu, affecting the C-terminal short-consensus-repeat (SCR20), had no identified event triggers but both have had one relapse. The p.Arg1215Leu mutation affects the same residue as the previously published mutations. Patient P8, with an onset of 2 years of age, was successfully treated on each occasion with plasma exchange (PEX). Patient P7, with an onset of 30 years of age, was initially treated with PEX and, after an aHUS relapse, was given Eculizumab (anti-C5 antibody) treatment. The other six patients that carried *CFH* variants, had identified triggering conditions, and were all successfully treated with plasma infusion or PEX with no reported relapses (Table 14).

P9, a woman with a late onset of 64 years, had no identified triggers, carried a single likely pathogenic missense *CFHR5* variant, c.329T>C, p.Val110Ala, and was found to be homozygous for the *CFHR1–3* deletion.

Three aHUS patients carried single *CD46* variants; curiously, all of them were at least double heterozygous for the risk haplotypes *MCPggaac* and *CFH-H3*. Patient P10, with an onset of 30 years of age, who carried the previously described splice variant c.287-2A>G, has had three aHUS relapses after upper respiratory tract infection episodes, but all were successfully treated with plasma infusion. Curiously, the onset of the disease in this patient preceded the availability of ADAMTS13 activity measurement, and, based on normal renal function and relapsing episodes, the patient was initially diagnosed with hereditary TTP. In the last relapsing episode, the normal ADAMTS13

Table 14 – Phenotypic and genotype characteristics of 17 patients from group III with atypical haemolytic uremic syndrome carrying rare variants in complement genes.

Patient ID	Sex	Age onset (yrs)	Triggers*	Treatment	Relapses	*Gene*	Variants identified: Nucleotide Change	Variants identified: Amino Acid Change	VC**	*Zygosity*	*ADAMTS13 SNPs mod.*	*MCPggaac alleles*	*CFH- H3 alleles*	*CFHR1-3 alleles*
P1	F	3 months	No	PI	0	*CFH*	**c.240 T>G** c.2669G>T c.3019 G>T	**p.Cys80Trp** p.Ser890Ile p.Val1007Leu	P LP LB	Compound heterozygous	—	1	0	1
P2	F	39	G	PEX	0	*CFH*	**c.335A>G**	**p.Tyr112Cys**	P	Heterozygous	—	2	1	0
P3	F	45		PI	0	*CFH*	**c.493G>T**	**p.Asp165Tyr**	P	Heterozygous	—	1	1	1
P4	F	67	post surgery	PEX	0	*CFH*	**c.1864A>G**	**p.Ile622Val**	LB	Heterozygous	p.Gln448Glu	1	0	0
P5	F	35		PI	0	*CFH*	c.2850G>T	p.Gln950His	P	Heterozygous	—	0	0	1
P6	F	46	post surgery	PEX	0	*CFH*	c.3172T>C c.3178G>C c.3226C>G	p.Tyr1058His p.Val1060Leu p.Gln1076Glu	LB LB LP	Compound heterozygous	—	2	1	0
P7	F	30	No	PEX, PI Eculizumab	1	*CFH*	**c.3562A>G**	**p.Lys1188Glu**	P	Heterozygous	—	0	1	0
P8	M	2	No	PI	1	*CFH*	**c.3644G>T**	**p.Arg1215Leu**	P	Heterozygous	0	0	2	0
P9	F	64	No	PI	0	*CFHR5*	c.329T>C	p.Val110Ala	LP	Heterozygous	—	1	0	2
P10	M	35	T	PI, prednisolone	3	*CD46 (MCP)*	c.287-2A>G		P	Heterozygous	p.Gln448Glu p.Ala900Val	1	1	1
P11	M	4	G	PI	0	*CD46 (MCP)*	**c.800_821del**	**Thr267Ilefs*24**	P	Heterozygous	—	1	1	1
P12	M	37	No	PI, Eculizumab	0	*CD46 (MCP)*	c.1148C>T	p.Thr383Ile	B	Heterozygous	—	2	1	2
P13	M	4	No	PI	2	*C3*	c.193A>C	p.Lys65Gln	P	Heterozygous	—	2	0	1
P14	M	50	No	PEX	0	*C3*	c.1407G>C	p.Glu469Asp	B	Heterozygous	p.Arg7Trp p.Gln448Glu p.Pro618Ala p.Ala732Val	0	1	0
P15	F	33	G	PEX, prednisolone Eculizumab	0	*C3* *CFI*	c.193A>C c.452A>G	p.Lys65Gln p.Asn151Ser	P P	Compound heterozygous	p.Gln448Glu p.Ala900Val	1	2	0
P16	F	20	No	PEX, PI	0	*C3* *CD46 (MCP)*	c.1775G>A c.686G>A	p.Arg592Gln p.Arg229Gln	P UVS	Compound heterozygous	0	1	1	0
P17	F	6 months	G	PI	0	*CFB*	c.1598A>G	p.Lys533Arg	B	Heterozygous	p.Gln448Glu	0	0	0

*Triggers: No = none identified, G = gastroenteritis (Shiga-like toxin E. coli negative), T= Tonsillitis; M, male; F, female; m, months; PI, plasma infusion; PEX, plasma exchange; **VC, variant classification: P = pathogenic, LP = likely pathogenic, LB = likely benign, and B = benign; UVS = unknown significance; SNPs, single nucleotide polymorphisms; mod, modulators. Novel variants are marked in **bold**.

activity induced a suspicion of aHUS. Patient P11, with an age of onset of 4 years, who carried the novel small deletion *CD46* c.800_821del, Thr267Ilefs*24 was also successfully treated with plasma infusion. Patient P12, with an onset of 37 years of age and with no identified triggering events, carried a single predicted benign missense variant c.1148C>T, p.Thr383Ile and was homozygous for the risk haplotype *MCPggaac* and homozygous for the *CFHR1/3* deletion. This patient had a bad outcome and is on Eculizumab treatment (Table 14).

Two patients carried single *C3* variants: P13, aHUS onset of 4 years of age, carried the pathogenic missense mutation *C3* c.193A>C, p.Lys65Gln, while P14, with a late onset of 50 years of age, carried the benign missense variant *C3* c.1407G>C, p.Glu469Asp. In both cases no triggers were identified. The patient P14, heterozygous for a benign variant, was also found to be homozygous for the *ADAMTS13* SNPs: p.Arg7Trp, p.Gln448Glu, p.Pro618Ala and p.Ala732Val. Two patients carried *C3*-combined variants: P15, aHUS onset of 33 years after a gastroenteritis episode, carried two pathogenic missense mutations, *C3,* c.193A>C, p.Lys65Gln and *CFI*, c.452A>G, p.Asn151Ser; P16, aHUS onset of 20 years of age, with no identified triggers, carried a pathogenic missense mutation in *C3*, c.1775G>A, p.Arg592Gln, and a missense variant of *CD46,* c.686G>A, p.Arg229Gln (unknown significance). These two patients carrying *C3*-combined variants also had the risk haplotype *MCPggaac* and *CFH-H3* in the heterozygous state. All of these 17 aHUS patients except for one (P15) were treated successfully with PEX or plasma infusion. P15 was initially treated with PEX but a bad outcome led to the use of Eculizumab treatment (Table 14).

Patient P17, a six-month-old girl, died during the presenting aHUS event; the triggering condition identified was a gastroenteritis episode. This patient carried only a single benign missense *CFB* variant, c.1598A>G p.Lys533Arg, and neither risk haplotypes nor the *CFHR1-3* deletion was identified.

Screening for the CFH autoantibody was not performed in the two patients (P9 and P12) who were homozygous for the *CFHR1-3* deletion because they were retrospectively studied in clinical remission.

We noticed that, considering all the aHUS patients carrying single or combined complement gene variants, we found that 11/17 (65%) were heterozygous for at least one pathogenic mutation and the three patients who have had relapses were all carrying pathogenic mutations (*CFH, CD46* and *C3*). On the other hand, however, our data suggest that patients carrying non-pathogenic variants co-inherited more genetic risk factors: patient P12 carrying a single benign *CD46* variant and the patient P9 with a single likely pathogenic *CFHR5* variant were homozygous for the *CFHR1–3* deletion, and the patient P14 carrying a single benign *C3* variant was homozygous for a block of four *ADAMTS13* SNPs. The additional genetic risk factors could have a cumulative or synergistic effect with more impact on benign variants, i.e., they appear to be a requisite to disease manifestation in carriers of benign variants.

6.5. Discussion

The deficiency of ADAMTS13 and dysregulation complement causes the pathogenesis of TTP and aHUS, respectively, but the overlap of their clinical characteristics makes the differential diagnosis challenging. Several diagnostic tools to differentiate these two pathologies have been proposed, such as a platelet count ≤ 30 X 10^3/μL and a creatinine level ≤200 μmol L^{-1}, to have positive predictive value for severe ADAMTS13 deficiency (Coppo *et al*, 2010); however, this approach could misdiagnose some aHUS patients with normal renal function as TTP. In parallel, the ADAMTS13 levels of <10% were described as 100% sensitive and 100% specific for TTP (Cataland & Wu, 2014; Phillips *et al*, 2016), and this ADAMTS13 activity cut-off has been accepted as a screening tool in acute TMA (Phillips *et al*, 2016). Therefore, as there is no rapid diagnostic test to assess the complement pathway activity, ADAMTS13 deficiency has been an important indicator in the differential diagnosis of TTP/aHUS. However, aHUS patients have been reported with mild ADAMTS13 deficiency leading to an overlapping phenotype in these patients (Feng *et al*, 2013b).

We developed a workflow based on ADAMTS13 activity and screening for genetic TMA susceptibility factors using a NGS-based targeted gene panel. This study comprised a

genotype–phenotype correlation in a cohort of 45 Portuguese patients diagnosed with TMA episodes comprising 11 TTP patients and 34 aHUS patients.

Our data were in agreement with two findings in previously reported studies (Lotta *et al*, 2012): first, homozygous variants in the N-terminal ADAMTS13 region (p.Arg692Cys and p.Cys754Arg) were associated with lower age at the onset of the first TTP episode compared to homozygous mutations in the C-terminal region (p.Arg1123His) and, for instance, p.Arg1123His variant was found in a pregnancy-associated TTP patient, with an age of onset of 26 years; second, siblings and unrelated patients with the same genotype had a similar age of disease onset (Camilleri *et al*, 2008).

Indeed, homozygosity for the previously described p.Arg692Cys and p.Cys754Arg variants, for which *in vitro* functional analyses have shown reduced ADAMTS13 secretion, have always been found in childhood TTP patients (Camilleri *et al*, 2012; Kim *et al*, 2014). The p.Arg692Cys variant was described in two Salvadoran children who, as in our patient, were homozygous for four SNPs involved in ADAMTS13 deficiency: *ADAMTS13* p.Arg7Trp, p.Gln448Glu, p.Pro618Ala and p.Ala732Val (Plaimauer *et al*, 2006; Kim *et al*, 2014).

The p.Cys754Arg variant was previously described only in two sibling (Camilleri *et al*, 2012), and, curiously, this variant was the most frequent in 4/6 families (66%) of our hereditary TTP cohort (Fidalgo *et al*, 2009) (Table 13, Figure 17). In homozygous state in four childhood TTP patients that despite having similar ages of onset, presented varying TTP phenotypes, i.e., the two girls had more relapses and required regular prophylactic plasma infusions/BPLY8. The remaining pregnancy-associated TTP patient, was compound heterozygous for p.Cys754Arg and a novel small deletion (p.Pro256Ser*fs**12) with obvious deleterious consequences. However, a TTP triggered by pregnancy suggested that this *ADAMTS13* compound genotype [p.Cys754Arg]+[p.Pro256Ser*fs**12] resulted in a less severe form of TTP than the homozygous genotype [p.Cys754Arg]+[p.Cys754Arg] with childhood onset. The familial study of this patient revealed that her brother had the same genotype with undetectable ADAMTS13 levels and had no TTP events (Figure 17A). These data

confirmed that, in this family, as described in similar reports (Camilleri *et al*, 2012; Scully *et al*, 2014), the addition of triggering conditions with increase of VWF levels (as pregnancy) to the genetic background was crucial to induce a TTP episode.

Of note, two variants could have had a Portuguese ancestral origin, the recurrent p.Cys754Arg (the most frequent in our TTP cohort) and; p.Arg1123His, since it was previously described in a compound heterozygous state, also associated with an adult onset, in a 26-year-old man who was the son of a Danish mother and a Portuguese father (Rank *et al*, 2014).

After acute episodes in the four TTP patients who had an *ADAMTS13* rare variant and anti-ADAMTS13 IgG antibodies, antibody levels returned to normal and ADAMTS13 activity increased to median 37.5% (range 35-38), and this could be explained by their heterozygosity. The variants p.Pro457Leu and p.Arg1096His were previously found to be associated with acquired ADAMTS13 deficiency (Feng *et al*, 2013b; Hing *et al*, 2013; Fidalgo *et al*, 2009; Meyer *et al*, 2007). Expression studies revealed reduced ADAMTS13 secretion, and it was suggested that increased intracellular degradation could promote autoantibody production (Meyer *et al*, 2007; Feng *et al*, 2013b). One study reported the association of anti-ADAMTS13 IgG antibodies with the missense variant p.Arg1060Trp (Camilleri *et al*, 2008). Another study described a case of familial acquired TTP of identical twins with anti-ADAMTS13 IgG antibodies suggesting that genetic determinants could play a role in inhibitor formation (Studt *et al*, 2004). Two were carriers of pathogenic missense mutations and the other two of predicted benign, likely benign and likely pathogenic variants, but no significant difference in the age of onset of episodes was observed between both patient pairs (Figure 17B). As occurs with other carriers of heterozygous variants, it seemed that the loss of ADAMTS13 activity by itself was not sufficient to initiate an acute episode of TTP. However, no other underlying clinical conditions were identified at the time of detection of anti-ADAMTS13 IgG antibodies in these four patients. Therefore, these cases are very interesting to study and understand whether certain missense variants

along with other genetic determinants can act as risk factors (Camilleri *et al*, 2008; Meyer *et al*, 2007; Studt *et al*, 2004).

In agreement with previous studies, only 50% of patients diagnosed with aHUS have rare variants in the complement genes (Loirat & Frémeaux-Bacchi, 2011; Córdoba *et al*, 2014). These were predominantly of type loss-of-function variants in the complement regulator genes *CFH, CFHR5, CD46* and *CFI* (78%) along with gain-of-function variants in the complement inductor genes *C3* (18%) and *CFB* (4%). *CFH* variants (12/23, 52%) will be the most prevalent in aHUS patients, as described in large studies in other populations (Córdoba *et al*, 2014; Loirat & Frémeaux-Bacchi, 2011; Vieira-Martins *et al*, 2016).

Likewise, the well-documented cumulative effect of different genetic risk factors for the aHUS phenotype (Moore *et al*, 2010; Caprioli *et al*, 2006; Bresin *et al*, 2013) was also observed in our patients: compound heterozygous in *CFH* (2/12, 17%); combined variants in more than one gene (2/17, 12%); the combination of variants with the concomitant presence of both risk haplotypes, *CFH-H3* and *MCPggaac* (compound heterozygous/homozygous) (8/17, 47%); and homozygous for the *CFHR1-3* deletion (2/17, 12%). The coexistence of excessive complement activation and partially decreased ADAMTS13 activity was described as a risk factor for the aHUS phenotype (Feng *et al*, 2013b). The analyses of *ADAMTS13* in our aHUS cohort revealed only common variants (SNPs): Gln448Glu, the most prevalent (20%), and p.Arg7Trp, p.Gln448Glu, p.Pro618Ala and p.Ala732Val co-inherited in one patient. The Gln448Glu variant alone had little impact but, conversely, the block of these four SNPs has been shown to have a higher impact on ADAMTS13 activity (Plaimauer *et al*, 2006).

Eight patients had 12 *CFH* variants across the 20 SCR (short consensus repeat) domains that compose the principal regulator of the complement system. Eight out of twelve *CFH* variants were located at the C–terminal SCR15-SCR20 domains that mediate surface binding and target recognition; the SCR19-20 domains, in particular, are crucial to differentiating self and non-self (Córdoba *et al*, 2014; Vieira-Martins *et al*, 2016). Two novel single pathogenic mutations were found in the SCR20 domain, which

represents a hot spot in *CFH* variants associated to a higher penetrance in aHUS patients (Bresin *et al*, 2013; Córdoba *et al*, 2014). The other three novel pathogenic mutations were located in SCR1-SCR3, which mediate the binding of C3 and heparin-like oligosaccharides (Vieira-Martins *et al*, 2016). Each of eight patients carried at least one pathogenic mutation, and none of them had other combined complement gene variants suggesting that these *CFH* variants alone may be sufficient to cause aHUS.

One patient carried a novel likely pathogenic single *CFHR5* variant combined with homozygous *CFHR1-3* deletion. *CFHR5* competes with factor H for binding to C3b, and genetic variants of this gene were reported in aHUS patients (Maga *et al*, 2010). This genotype seemed to have a synergist effect on phenotype, and was, nevertheless, associated with a late onset of aHUS (64 years).

Four patients had *CD46* variants, with three variants located across the four extracellular SCRs (binding sites for C3b) and one variant in the cytoplasmic region. One out four patients carrying *CD46* variants have a second variant in other gene (*C3*) of the complement (Bresin *et al*, 2013). All four patients were concomitantly carrying both risk haplotypes, *MCPggaac* and *CFH-H3,* and one was homozygous for the *CFHR1-3* deletion (Bresin *et al*, 2013; Provaznikova *et al*, 2012; Caprioli *et al*, 2006). Therefore, as described, this combination could be reinforced by the defective protection of cellular surfaces caused by *CD46* mutations and fluid phase dysregulation of the complement system caused by *CFH* polymorphisms (Córdoba *et al*, 2014). These data suggest that the concomitant presence of a benign *CD46* variant and risk haplotypes may be required for full manifestation of the disease, as described in previous studies (Esparza-Gordillo *et al*, 2005; Caprioli *et al*, 2003; Bresin *et al*, 2013).

Two patients carried single *C3* variants (one pathogenic and one predicted as benign), and another two carried a combination of variants in *C3/CFI* (both pathogenic) and in C3/*CD46* (pathogenic/unknown significance). The two pathogenic *C3* mutations were located near the C3/CFH binding sites, CFH-SCR3 and CFH-SCR4, affecting interactions with CFH; conversely, the benign variant was distant from these binding sites (Schramm *et al*, 2015). The pathogenic mutation *C3* c.193A>C, p.Lys65Gln was

recurrent and was found both as a single and combined variant. The aHUS patients with *C3* variants usually develop severe disease, with a degree of severity similar to the reported for patients with *CFH* variants (Schramm *et al*, 2015). In these patients, this severity appears to be associated with the age of onset; 2-33 years for patients carrying pathogenic mutations and 50 years for patient carrying a predicted benign variant.

The patient with the worst outcome of the aHUS cohort, a child who died at the first episode, carried a single predicted benign *CFB* variant, p.Lys533Arg, without concomitant risk haplotypes. *CFB* variants should be of gain-of-function type when they result in C3 convertase overactivity. Nevertheless, this phenotype is not always shared between all of the identified *CFB* variants, and not all variants were causative factors for aHUS. The *CFB* p.Lys533Arg variant, which is located in the serine–protease (SP) domain but distant from the catalytic triad, was reported in a detailed functional study as having an incomplete phenotype, i.e., the functional consequence was mild and was less likely to result directly in aHUS pathogenesis (Marinozzi *et al*, 2014). However, this finding is controversial because the same variant was also previously described in a 7-year-old girl of South Asian ethnicity who did not survive to a severe aHUS episode (Tawadrous *et al*, 2010). On the basis of the similar severe clinical phenotype, we hypothesize that these two children had the same genetic abnormality, but it is likely that the p.Lys533Arg predicted benign variant could segregate in a haplotype block comprising the responsible mutation that has not been identified. These cases highlight the complexity of aHUS genetics.

In summary, we have successfully designed, validated and applied our NGS gene panel to identify: i) a cohort of 11 TTP patients carrying 10 different *ADAMTS13* variants; one of them, p.Cys754Arg, occurred repetitively. We found that the childhood onset and adult onset of TTP patients was correlated with pathogenic variants located in specific ADAMTS13 regions; however, patients carrying the same *ADAMTS13* pathogenic variant showed heterogeneous clinical phenotypes of TTP with a wide range of severity with some patients requiring regular therapy. These findings are in line with previous

reports (Lotta *et al*, 2012; Camilleri *et al*, 2012). ii) In a cohort of 34 aHUS patients, 17 patients were heterozygous for 23 variants in the different complement genes with distinct consequences, ranging from a single pathogenic mutation associated with complete disease penetrance to benign variants that cause aHUS only when combined with other variants and/or *CFH* and *MCP* risk haplotypes or the *CFHR1-3* deletion.

In total, were identified seven novel pathogenic mutations (*ADAMTS13, CFH,* and *CD46*) and their correlation with the severity of phenotypes.

In most of these patients, there were associations with triggering/precipitating events (mainly infections) confirming that environmental factors are critical determinants of aHUS development. Nevertheless, this NGS study ended with 17 (50%) genetically unresolved aHUS patients, as described in others aHUS studies (Bresin *et al*, 2013; Córdoba *et al*, 2014). Therefore, the expectation that the whole-exome sequencing approaches could identify other genes involved in aHUS is high; however, these studies still remain under investigation (Bu *et al*, 2015). Conversely, NGS-targeted gene panels are being introduced into clinical practice provide substantial benefits for definitive diagnoses in haematological diseases as demonstrated several recent reports (Simeoni *et al*, 2016; Del Orbe Barreto *et al*, 2016; Fidalgo *et al*, 2016).

This study described for the first time the mutational spectrum in a cohort of Portuguese TMA patients. Overall, studies of hereditary TTP and aHUS patients emphasize the high degree of variability in clinical presentation and the considerable heterogeneity of the molecular basis of these diseases, which supports the great interest shown in reports from different geographic regions (Camilleri *et al*, 2012; Phillips *et al*, 2016; Feng *et al*, 2013b; Maga *et al*, 2010; Bresin *et al*, 2013; Córdoba *et al*, 2014).

The prediction of pathogenicity of the genetic variation according to the recommendations of the American College of Medical Genetics and Genomics (Richards *et al*, 2015) has become crucial for understanding the great inter-individual variability. Herein, we report in detail the genotype–phenotype correlations in a cohort

of TMA patients, based on the impact of the predicted pathogenicity of each rare variant and also the co-inherited known genetic risk factors for TMA. The knowledge of all putative variants for every patient permitted a broad overview of the pathogenicity and the combination of alleles that affected each patient. This accurate analysis was only possible because our custom NGS panel has made it easier to simultaneously study *ADAMTS13* and the complement genes. Moreover, this technology has changed the paradigm of routine molecular studies: in the face of the multiple genetic changes found in every patient, the critical challenge became discriminating disease-associated variants from the broader background of variants present in all patients' genomes. This analysis has led us to a well-established bioinformatics pipeline according to NGS guidelines (Matthijs *et al*, 2015; Richards *et al*, 2015); and evidences that a clinical-laboratory approach for each patient's genotypic data must be evaluated considering their specific and differential clinical manifestations.

In conclusion, the study of these 45 TMA Portuguese patients contributes to the better understanding of the molecular genetics of *ADAMTS13/*complement gene-related phenotypes. Moreover, provide evidence of the usefulness of the NGS panel as an excellent advantageous technology that enables more rapid and economic diagnosis of TMA. These findings show that this is a valuable asset in clinical practice given that a correct diagnosis is essential for determining the most effective treatment for each patient with this complex disease.

What is known about this topic?

- TTP and aHUS could exhibit overlapping in clinical characteristics (sign and symptoms) and in pathophysiological mechanisms making challenging the differential diagnosis.
- Several genes were implicated with the clinical variability and the cost of their analyses by Sanger sequencing is not affordable by diagnostic laboratories.
- With the advent of NGS, the complete and simultaneous characterization of all these genes at a very reasonable cost becomes a possibility for the study of these diseases.

What does this paper add?

- A NGS-based targeted gene panel was designed and validated for the complete and simultaneous study of the eleven genes related with TMA in a rapid and economic manner.
- This technology was applied to 45 TMA Portuguese patients identifying thirty three different variants and eight of them were new.
- The application of this panel allows the identification of all putative variants and known genetic susceptibility factors for a better genotype-phenotype correlation in each patient.

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Author contributions

TF was responsible for study design and wrote the manuscript. TF, PM, CSP and ACO performed the functional and molecular studies and analyzed the data. FV, NB, IC, MC and RB contributed to NGS study. RS, TM, and MJM provided clinical support. FV and MLR revised the manuscript.

Conflicts of interest: None declared.

CHAPTER

7

GENERAL DISCUSSION & CONCLUDING REMARKS

CHAPTER 7. GENERAL DISCUSSION & CONCLUDING REMARKS

7.1. General Discussion

VWF is a glycoprotein circulating in plasma as large multimers. When activated upon vascular damage, VWF plays a key role in platelet-dependent primary haemostasis. VWF is also the carrier protein of coagulation FVIII.

VWF illustrates the duality between haemorrhagic and thrombotic mechanisms because is a key player in both processes (Sadler, 1998; Sadler *et al*, 2006).

Abnormalities in VWF secretion, intravascular clearance of VWF, the assembly of VWF multimers, or exaggerated proteolytic degradation by ADAMTS13 can cause diverse forms of von Willebrand disease (VWD) (Batlle J *et al*, 2011; Castaman *et al*, 2013).

VWD is considered the most common inherited bleeding disorder and is characterised mainly by mucosal bleeding manifestations. Patients with severe forms of VWD may also suffer from joint, muscle and central nervous system bleeding. Moreover, VWD is associated with a variable bleeding tendency and heterogeneous laboratory phenotype. A genotype-phenotype correlation in patients with VWD has become crucial for understanding the function and great inter-individual variability of VWF (Goodeve *et al*, 2007; Lillicrap, 2013).

Conversely, defects in proteolysis of VWF by ADAMTS13 can cause TTP, a disease caused by clumping of platelets by ultra-large VWF and defined clinically by microangiopathic hemolytic anaemia and thrombocytopenia (George & Vesely, 2003; Lämmle & George, 2004). However, the two main forms of TMAs are TTP and aHUS. The deficiency of ADAMTS13 and dysregulation of the complement pathway cause the pathogenesis of TTP and aHUS, respectively. TTP and aHUS could exhibit overlapping in clinical characteristics (signs and symptoms), and in pathophysiological mechanisms, making the differential diagnosis challenging (Mannucci & Cugno, 2015; Marina Noris & Remuzzi, 2009; Kremer Hovinga & Lämmle, 2012).

The molecular analysis of *VWF, ADAMTS13* and complement genes are very useful in the diagnosis of VWD and the differential diagnosis of TTP/aHUS. Studying the large-size *VWF* and the several genes implicated in TTP/aHUS using conventional Sanger DNA sequencing in diagnostic laboratories have been at a disadvantage due to its high costs. Nevertheless, the prospects for molecular diagnosis will be improved with the introduction of next-generation sequencing (NGS) platforms; massive parallel sequencing has reduced the cost and increased the throughput of DNA sequencing (Batlle *et al*, 2015).

Based on these findings, we analyzed the VWF-related diseases from two different perspectives: haemorrhagic and thrombotic disease. Therefore, the present study investigated two cohorts of patients, one with different types of VWD (Chapter 3-5) and the other with TMAs (TTP/aHUS) (Chapter 6). For both studies, we used the same algorithm: first, functional studies of VWF or ADAMTS13 followed by molecular studies with custom NGS-based targeted genes.

This study described, for the first time, the mutational spectrum in a cohort of Portuguese VWD patients, comprising a genotype-phenotype correlation in 60 unrelated families (92 individuals) diagnosed with types 1, 2 and 3 VWD (Chapter 3). The VWD diagnosis based on the results of a battery of laboratory tests is often difficult and, therefore, establishing patients' disease severity and risk of bleeding becomes challenging (Budde U & Favaloro E, 2011; Castaman *et al*, 2014). Thus, in our diagnosis laboratory, a phenotype and genotype analysis in patients with VWD has become crucial for understanding the VWF function and great inter-individual variability.

In our study, besides the additional mutations identified by NGS, the characteristic mutation distribution across *VWF* was preserved in each VWD type: in type 3 VWD, the mutations were scattered throughout *VWF*; in severe type 1 VWD, the profile was similar, but the absence of mutations in the regions coding for the A1, A2, D' and D3 domains was evident; in type 2N VWD, a mutation cluster in the region coding for the D1, D' and D3 domains was detected; in types 2A, 2B and 2M VWD, the mutation

spreading was notable, but a mutation cluster in the regions coding for the A1 domain emerged. These findings are in line with previous reports (Gadisseur *et al*, 2009a; Michiels *et al*, 2009; Bowman *et al*, 2013; Federici *et al*, 2011; Michiels & van Vliet, 2009).

In patients with severe type 1 VWD, a high degree of genetic heterogeneity was found with 10 different mutations, of which five (three missense mutations, one nonsense and one large deletion) have not been previously described (35%). In this group, it is also important to highlight two missense mutations that showed a pleiotropic effect: p.Ala631Val described as a mild type 1 VWD phenotype (Bellissimo *et al*., 2012), but in compound heterozygosity with c.7730-4C>G, was responsible for a severe phenotype; and p.Ala1714Pro that when inherited alone exhibited a type 2M VWD phenotype, and when co-inherited with c.5170+10C>T was associated with severe type 1 VWD. These behaviours were unexpected given that the variants, c.7730-4C>G and c.5170+10C>T, were not associated themselves with a predicted deleterious effect, but when co-inherited *in trans*, they seem to aggravate the VWD phenotype.

In patients with severe type 3 VWD, as usual, two patterns of mutations were evident: homozygous for null mutations and compound heterozygous/homozygous for missense mutations of *VWF*. Two nonsense mutations (p.Gln1311*, p.Gln1556*) and a novel small deletion (p.Val1805Glyfs*8) were expected to have the obvious deleterious mechanisms, i.e. premature termination codons and a frameshift leading to a truncated VWF protein. The homozygous p.Gln1311* mutation associated with a gene conversion and initially reported in Spanish Romani families (Casaña *et al*, 2000) was the most frequent mutation in 5 out of 15 families, of which one was also Romani (Surdhar, 2001).

An unexpected genotype/phenotype was observed with the missense mutation p.Pro1162Leu in the homozygous state detected in a type 3 VWD proband that was also found in another proband with type 2N VWD. This controversial finding implied that p.Pro1162Leu was unlikely to be a pathogenic variant for type 3 VWD. However, the same genotype was also the only one found in a type 3 VWD Spanish family (Batlle

et al, 2015) therefore, it seems reasonable to expect that the p.Pro1162Leu (c.3485_3486delinsTG) variant is part of a compound genotype that causes type 3 VWD, which has not yet been entirely explained.

In patients with type 2N VWD, the cause was mainly the missense mutation p.Arg854Gln in the homozygous state, similar to previous studies. This is a frequent deleterious variant in Caucasian populations (Goodeve, 2010; Jacquemin, 2009). Nevertheless, genetic variability was introduced by compound heterozygosity with novel mutations: p.Arg854Gln with the deleterious novel missense mutation p.Asp879Glu and p.Arg816Trp with the novel small deletion c.100delT (p.Arg34Aspfs*49). The ethnic variability was noted in an African proband who showed heterozygosity for the frequent polymorphism, p.His817Gln, in association with p.Pro1162Leu in the homozygous state. Recent multi-ethnic studies found that *VWF* missense variants in the D' and D3 domains, previously identified in European ancestry VWD probands, have been reported to be common in African-Americans (AAs) (Bellissimo *et al*, 2012; Johnsen *et al*, 2013; Wang *et al*, 2013; Goodeve, 2013). These variants include p.His817Gln, which was strongly associated with diminished FVIII:C levels (Johnsen *et al*, 2013).

Patients with type 2B VWD, with gain-of-function mutations in the A1 domain, showed two common mutations, p.Arg1306Trp and p.Val1316Met (Gadisseur *et al*, 2009b). In addition, we observed a highly variable platelet count with a large range (15–242 × 10^3 platelets/μl), which is consistent with previous studies, although only four patients had thrombocytopenia. This variability was explained by several mutations and their coding positions within the VWF A1 domain, e.g. the altered VWF GPIb-α–binding conformation (Federici *et al*, 2011). Although p.Arg1306Trp and p.Val1316Met are frequently correlated with thrombocytopenia, we observed some heterogeneity even in individuals with the same mutation. These data were also in agreement with the wide degree of heterogeneity of the clinical and laboratory features reported for affected members of families that have type 2B VWD (Federici *et al*, 2011).

We report a case of PT-VWD misdiagnosed as type 2B VWD. The functional studies allowed a differential diagnosis, but molecular studies confirmed the recently described missense mutation p.Asp251Tyr (Asp235Tyr) in *GP1BA* (Enayat *et al*, 2012). PT-VWD is certainly an underdiagnosed deficiency where molecular study is a valuable diagnostic tool.

In patients with type 2A VWD, we found mutations related to all previously described type 2A structural defects (Woods *et al*, 2012; Budde *et al*, 2008; Michiels & van Vliet, 2009; Gadisseur *et al*, 2009a): i) a multimerisation defect (p.Cys1130Arg in the D3 domain) with the absence of large multimers and no triplet structure; ii) a change in protein folding (p.Cys1272Gly/Phe in the loop of the A1 domain) that causes the loss of large and intermediate multimers; iii) intracellular proteolysis of large VWF multimers (p.Ser1506Leu) with the loss of large multimers; and iv) hypersensitivity to ADAMTS13 (p.Ile1628Thr in A2 domain) with the absence of large VWF multimers and increased triplet structure.

Our findings in type 2M VWD patients support the well-characterised profile: mutations in the A1 domain, which are typically associated with decreased or absent RIPA and a low VWF:RCo/VWF:Ag ratio, combined with a normal VWF:CB/VWF:Ag ratio. We identified the usual A1 domain mutation clusters, p.Arg1315Cys/His and p.Arg1374Cys/His, with the addition of the novel mutations, p.Asp1373Tyr and p.His1419Gln. The VWD subtype classification of the p.Arg1374His mutation has been controversial. This mutation was described as type 2A (Budde *et al*, 2008) or type 2M (Castaman *et al*, 2012a). In fact, some authors argue that the mutation is difficult to classify as type 2M (because of the possibly relative decrease in large VWF multimers) or any other type 2 (because of the normal banding pattern of each multimer) VWD; therefore, a classification of type 2U VWD (unclassifiable) has been suggested (Gadisseur *et al*, 2009c). In our study, three probands showed a multimer pattern with the full complement of multimers, and one showed a slight decrease in the largest forms. Given the criterion of a low VWF:RCo/VWF:Ag ratio and normal

VWF:CB/VWF:Ag ratio, which was evident in each of the four probands, they were classified as having type 2M VWD.

Overall, VWD studies highlight the high degree of variability in clinical presentation and the considerable heterogeneity of the molecular basis, which supports the great interest shown in reports from different geographic regions (Corrales *et al*, 2009; Castaman *et al*, 2012a; Hampshire *et al*, 2013; Yadegari *et al*, 2012; Bowman *et al*, 2013). Moreover, large studies have highlighted the ethnic variability in the phenotype of many *VWF* missense variants (Bellissimo *et al*, 2012; Johnsen *et al*, 2013; Wang *et al*, 2013). We applied these insights to the context of familial studies to determine their usefulness for predicting individual bleeding risk. Accordingly, the genotype–phenotype correlation in each proband family with diverse VWD types was assessed to establish family overviews and elucidate phenotypic discrepancies.

This accurate analysis was only possible because NGS has made it easier to study *VWF* ROIs. NGS has proven to be an excellent technology that enables more rapid diagnosis with a huge economic advantage (only €70/sample, which is even cheaper than most phenotypic tests) (Batlle *et al*, 2015). These findings support the adjustment of our VWD diagnosis algorithm, which introduces the complete sequencing of *VWF* (NGS) when the VWF:RCo level is < 30% or the FVIII/VWF:Ag ratio is < 0.5.

Our new molecular study approach permitted the identification of 27 novel *VWF* mutations, with some occurring repetitively, illustrating the advantages of identifying the most prevalent mutations in a region and their correlation with the severity of bleeding phenotypes. The approach allowed us to distinguish between clinical situations that have the same symptoms but different genetic causes, such as mild Haemophilia A and type 2N VWD, Bernard-Soulier syndrome and 2B VWD, as well as 2B VWD and PT-VWD. In contrast, despite being a restricted study, we identified some pleiotropic mutation effects. In both situations, it was indicated that molecular studies are indispensable for an accurate diagnosis.

The molecular characterisation of VWD patients allows for precise classification into the correct VWD type and the identification of carriers in familial genetic studies. This classification is particularly relevant and even mandatory for genetic counselling for type 3 VWD and, in general, for patients with higher bleeding risk. Moreover, it facilitates the evaluation of prophylactic requirements and clinical orientation, particularly in risky situations.

In conclusion, this study of 60 VWD Portuguese families will contribute to the better understanding of the molecular genetics of VWF-related phenotypes. NGS, in our experience, provides an effective laboratory workflow for the analysis of a single large gene, such as VWF.

We extend our study to other type 2M VWD caused by VWF collagen-binding (types III and VI) defects (Chapter 4). It is well known that some *VWF* sequence variations affect the ability of VWF to bind type I, type III and type VI collagen but may not interfere with the screening assays, which show normal values, and can only be detected using type VI collagen (Larsen *et al*, 2013; Flood *et al*, 2012b; Riddell *et al*, 2009; Legendre *et al*, 2013). Hence, this VWF dysfunction could be underdiagnosed, despite it being typically reported in patients with a mild bleeding history (Flood *et al*, 2012b). Our study in a cohort of nine patients identified a group of 2M VWF mutations with a variable range of functional defects, including the classical VWF-platelet defect, the VWF collagen-binding defect or both. This variability has been increasingly observed by several recent studies (Larsen *et al*, 2013; Legendre *et al*, 2013), and the application of these insights in the context of genotype-phenotype correlations can improve the approach used by diagnostic laboratories. This analysis in our cohort of patients evinced that a laboratory approach based on the correlation of type III and type VI collagen-binding assays and molecular studies is indispensable for a more accurate diagnosis of type 2M VWD. Moreover, the detection of the specific type VI collagen-binding defect may contribute to the correct diagnosis of patients with mild bleeding disorders that are often classified as having an undefined cause (Flood *et al*, 2012b). An example was a child in this cohort of patients with a history of epistaxis who was

heterozygous for p.Arg1399His. If this patient had been studied with a minimal laboratory analysis (VWF:Ag and VWF:RCo) he would have remained undiagnosed. The inclusion of both collagen-binding assays (type III and type VI) in our diagnostic algorithm enhanced the efficacy of VWD diagnosis and, consequently, the identification of the most appropriate management for each patient.

Another VWF deficiency potentially underdiagnosed was illustrated in this study by six patients with AVWS and different underlying hematologic malignancies: Waldenstrom's Macroglobulinemia (WM); Marginal Zone Lymphoma (MZL); Chronic Myeloid Leukaemia (CML) and Essential Thrombocythemia (ET) (Chapter 5). The detection of low VWF levels in two patients came before the diagnosis of the underlying lymphoproliferative disease. In one patient, a *VWF* variant was identified simultaneously with AVWS. In all patients, treatment of the underlying disease resulted in normalisation of VWF levels and multimer patterns. As described, we also found that VWFpp confirm a normal biosynthesis of VWF in these patients (Eikenboom *et al*, 2007); a reduced function/antigen ratio (VWF:RCo/Ag or VWF:CB/Ag) is a sensitive parameter for indicating structural or functional disorders, even in patients who had absolute activity within a normal range (Tiede *et al*, 2011).

The clinical-laboratory correlation in the six cases led to the diagnosis of AVWS. Including the evaluation of VWF analysis in late haemorrhagic episodes and/or in AVWS-associated disorders enhanced the efficacy of AVWS diagnosis, leading to a more tailored management approach for each patient.

In the second part of study we developed a workflow based on ADAMTS13 activity and screening for genetic TMA susceptibility factors using a NGS-based targeted gene panel (Chapter 6). This custom gene panel includes ADAMTS13, CFH, CFHR1, CFHR3, CFHR4, CFHR5, CFI, CFB, C3, THBD and DGKE to facilitate genetic testing in TTP and aHUS patients. This approach allowed the implementation of an efficient methodology to establish the differential diagnosis and prognosis of these two rare diseases that exhibit phenotypic similarities.

This study described for the first time the mutational spectrum in a cohort of Portuguese TMA patients. Herein, we described the impact of mutations in severity of 45 patients diagnosed with TMA episodes, comprising 11 TTP patients and 34 aHUS patients.

Our cohort of hereditary TTP showed, as expected, two patterns of variants: homozygosity and compound heterozygosity for pathogenic variants of ADAMTS13. Three missense pathogenic variants (p.Arg692Cys, p.Cys754Arg and p.Arg1123His) were located in the TSP1-2, the TSP1-3 and the distal TSP1-8 domains. The homozygous p.Arg692Cys and p.Cys754Arg variants were found in five childhood TTP patients belonging to four apparently unrelated families, and the homozygous p.Arg1123His variant was found in a pregnancy-associated TTP patient with an onset age of 26 years. These data were in agreement with two findings in previously reported studies (Lotta *et al*, 2012): first, homozygous variants in the N-terminal ADAMTS13 region (p.Arg692Cys and p.Cys754Arg) were associated with a lower onset age of the first TTP episode compared to homozygous mutations in the C-terminal region (p.Arg1123His) and, for instance, the p.Arg1123His variant was found in a pregnancy-associated TTP patient with an onset age of 26 years; second, siblings and unrelated patients with the same genotype had a similar age of disease onset (Camilleri *et al*, 2008).

After acute episodes in four TTP patients who had an *ADAMTS13* rare variant and anti-ADAMTS13 IgG antibodies, antibody levels returned to normal and ADAMTS13 activity increased to median 37.5% (range 35–38); this could be explained by their heterozygosity. The variants p.Pro457Leu and p.Arg1096His were previously found to be associated with acquired ADAMTS13 deficiency (Feng *et al*, 2013b; Hing *et al*, 2013; Fidalgo *et al*, 2009; Meyer *et al*, 2007). Expression studies revealed reduced ADAMTS13 secretion, and it was suggested that increased intracellular degradation could promote autoantibody production (Meyer *et al*, 2007; Feng *et al*, 2013b). One study reported the association of anti-ADAMTS13 IgG antibodies with the missense variant p.Arg1060Trp (Camilleri *et al*, 2008). Another study described a case of familial

acquired TTP of identical twins with anti-ADAMTS13 IgG antibodies, suggesting that genetic determinants could play a role in inhibitor formation (Studt *et al*, 2004).

Two patients were carriers of pathogenic missense mutations and the other two of predicted benign, likely benign and likely pathogenic variants, but no significant differences in the onset age of episodes were observed between the two patient pairs. As occurs with other carriers of heterozygous variants, it seemed that the loss of ADAMTS13 activity by itself was not sufficient to initiate an acute episode of TTP. However, no other underlying clinical conditions were identified at the time of detection of anti-ADAMTS13 IgG antibodies in these four patients. Therefore, these cases are very interesting to study and to understand whether certain missense variants along with other genetic determinants can act as risk factors (Camilleri *et al*, 2008; Meyer *et al*, 2007; Studt *et al*, 2004).

In agreement with previous studies, only 50% of the patients diagnosed with aHUS had rare variants in the complement genes (Loirat & Frémeaux-Bacchi, 2011; Córdoba *et al*, 2014). These were predominantly loss-of-function variants in the complement regulator genes, *CFH, CFHR5, CD46* and *CFI* (78%), along with gain-of-function variants in the complement inductor genes *C3* (18%) and *CFB* (4%). *CFH* variants (12/23, 52%) were the most prevalent in aHUS patients, as described in large studies in other populations (Córdoba *et al*, 2014; Loirat & Frémeaux-Bacchi, 2011; Vieira-Martins *et al*, 2016).

Likewise, the well-documented cumulative effect of different genetic risk factors for the aHUS phenotype (Bresin *et al*., 2013; Jessica Caprioli *et al*., 2006; Moore *et al*., 2010) was also observed in our patients: compound heterozygosity in *CFH* (2/12, 17%); combined variants in more than one gene (2/17, 12%); the combination of variants with the concomitant presence of both risk haplotypes, *CFH-H3* and *MCPggaac* (compound heterozygous/homozygous) (8/17, 47%); and homozygous for the *CFHR1-3* deletion (2/17, 12%). The coexistence of excessive complement activation and partially decreased ADAMTS13 activity was described as a risk factor for the aHUS phenotype (Feng *et al*, 2013b). The analyses of *ADAMTS13* in our aHUS cohort revealed only

common variants (SNPs): Gln448Glu, the most prevalent (20%), and p.Arg7Trp, p.Gln448Glu, p.Pro618Ala and p.Ala732Val co-inherited in one patient. The Gln448Glu variant alone had little impact but, conversely, the block of these four SNPs has been shown to have a higher impact on ADAMTS13 activity (Plaimauer *et al*, 2006).

Eight patients had 12 *CFH* variants across the 20 short consensus repeat (SCR) domains that compose the principal regulator of the complement system. Each of eight patients carried at least one pathogenic mutation, and none of them had other combined complement gene variants, suggesting that these *CFH* variants alone may be sufficient to cause aHUS.

One patient carried a novel, likely pathogenic, single *CFHR5* variant combined with homozygous *CFHR1-3* deletion. *CFHR5* competes with factor H for binding to C3b, and genetic variants of this gene were reported in aHUS patients (Maga *et al*, 2010). This genotype seemed to have a synergistic effect on phenotype, and was, nevertheless, associated with a late onset of aHUS.

Four patients had *CD46* variants, with three variants located across the four extracellular SCRs (binding sites for C3b) and one variant in the cytoplasmic region. One out of four patients carrying *CD46* variants have a second variant in another gene (*C3*) of the complement (Bresin *et al*, 2013). All four patients were concomitantly carrying both risk haplotypes, *MCPggaac* and *CFH-H3,* and one was homozygous for the *CFHR1-3* deletion (Bresin *et al*, 2013; Provaznikova *et al*, 2012; Caprioli *et al*, 2006). Therefore, as described, this combination could be reinforced by the defective protection of cellular surfaces caused by *CD46* mutations and fluid phase dysregulation of the complement system caused by *CFH* polymorphisms (Córdoba *et al*, 2014). These data suggest that the concomitant presence of a benign *CD46* variant and risk haplotypes may be required for full manifestation of the disease, as described in previous studies (Esparza-Gordillo *et al*, 2005; Caprioli *et al*, 2003; Bresin *et al*, 2013).

Two patients carried single *C3* variants (one pathogenic and one predicted as benign), and another two carried a combination of variants in *C3/CFI* (both pathogenic) and in

C3/*CD46* (pathogenic/unknown significance). The two pathogenic *C3* mutations were located near the C3/CFH binding sites, CFH-SCR3 and CFH-SCR4, affecting interactions with CFH; conversely, the benign variant was distant from these binding sites (Schramm *et al*, 2015). The pathogenic mutation *C3* c.193A>C, p.Lys65Gln was recurrent and was found both as a single and a combined variant. The aHUS patients with *C3* variants usually develop severe disease, with a degree of severity similar to those reported for patients with *CFH* variants (Schramm *et al*, 2015). In these patients, this severity appears to be associated with the age of onset: 2–33 years for patients carrying pathogenic mutations and 50 years for patients carrying a predicted benign variant.

The patient with the worst outcome of the aHUS cohort, a child who died at the first episode, carried a single predicted benign *CFB* variant, p.Lys533Arg, without concomitant risk haplotypes. *CFB* variants should be of the gain-of-function type when they result in C3 convertase overactivity. Nevertheless, this phenotype is not always shared between all of the identified *CFB* variants, and not all variants were causative factors for aHUS. The *CFB* p.Lys533Arg variant, which is located in the serine–protease (SP) domain but distant from the catalytic triad, was reported in a detailed functional study as having an incomplete phenotype, i.e. the functional consequence was mild and was less likely to result directly in aHUS pathogenesis (Marinozzi *et al*, 2014). However, this finding is controversial because the same variant was also previously described in a 7-year-old girl of South Asian ethnicity who did not survive due to a severe aHUS episode (Tawadrous *et al*, 2010). On the basis of the similar severe clinical phenotype, we hypothesise that these two children had the same genetic abnormality, but it is likely that the p.Lys533Arg predicted benign variant could segregate in a haplotype block comprising the responsible mutation that has not been identified. These cases highlight the complexity of aHUS genetics.

In summary, we have successfully designed, validated and applied our NGS gene panel to characterize 45 TMA patients: i) a cohort of 11 TTP patients carrying 10 different *ADAMTS13* variants; one of them, p.Cys754Arg, occurred repetitively. We found that

childhood onset and adult onset of TTP patients were correlated with pathogenic variants located in specific ADAMTS13 regions; however, patients carrying the same *ADAMTS13* pathogenic variant showed heterogeneous clinical phenotypes of TTP with a wide range of severity with some patients requiring regular therapy. These findings are in line with previous reports (Lotta *et al*, 2012; Camilleri *et al*, 2012); ii) in a cohort of 34 aHUS patients, 17 patients were heterozygous for 23 variants in the different complement genes with distinct consequences, ranging from a single pathogenic mutation associated with complete disease penetrance to benign variants that cause aHUS only when combined with other variants and/or *CFH* and *MCP* risk haplotypes or the *CFHR1-3* deletion.

In total, seven novel pathogenic mutations (*ADAMTS13, CFH,* and *CD46*) were identified and correlated with the severity of phenotypes.

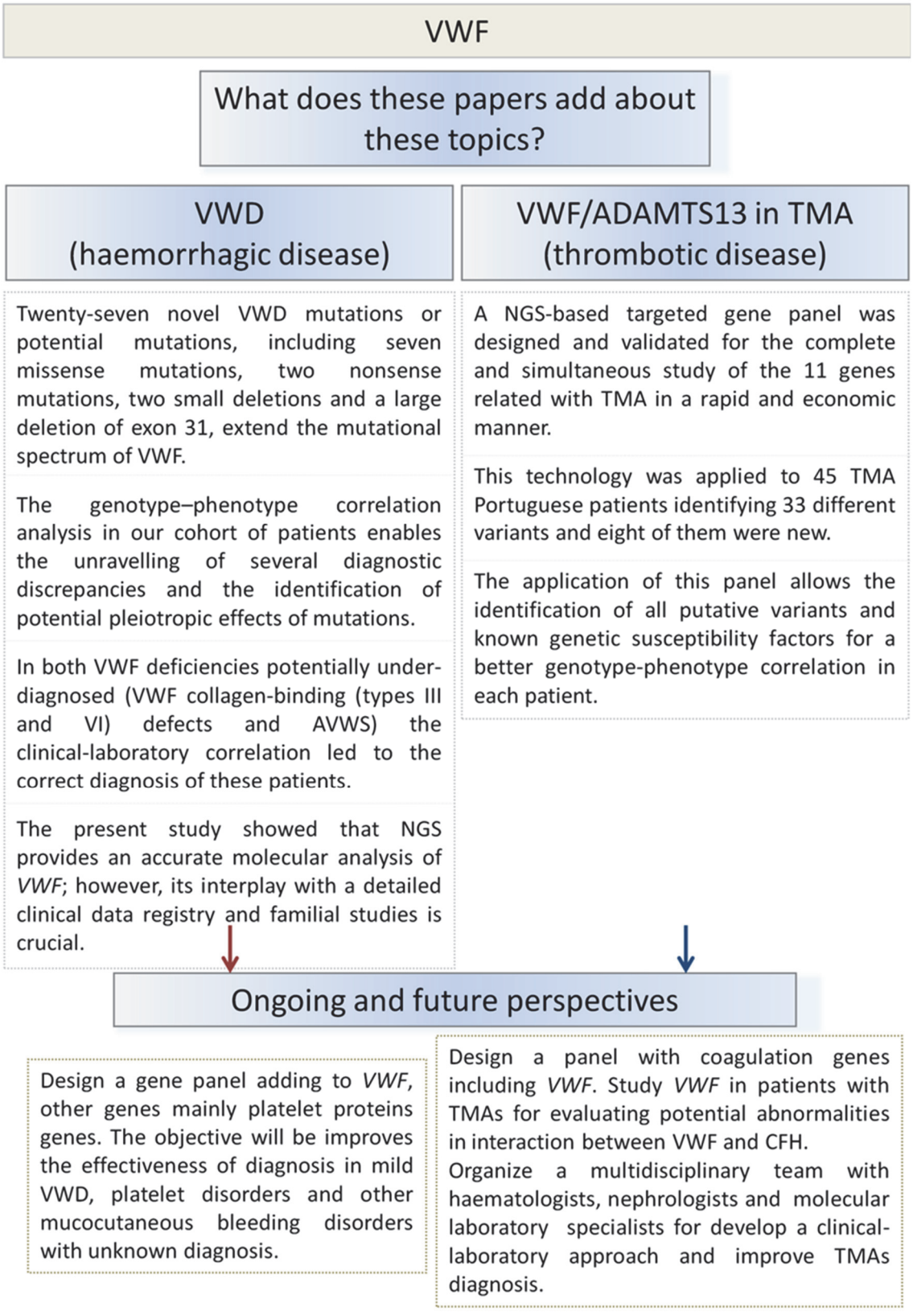
In most of these patients, there were associations with triggering/precipitating events (mainly infections), confirming that environmental factors are critical determinants of aHUS development. Nevertheless, this NGS study ended with 17 (50%) genetically unresolved aHUS patients, as described in other aHUS studies (Bresin *et al*, 2013; Córdoba *et al*, 2014). Therefore, the expectation that the whole-exome sequencing approach could identify other genes involved in aHUS is high; however, these studies still remain under investigation (Bu *et al*, 2015). Conversely, NGS-targeted gene panels being introduced into clinical practice provide substantial benefits for definitive diagnoses in haematological diseases, as demonstrated in several recent reports (Batlle *et al*, 2015; Del Orbe Barreto *et al*, 2016; Simeoni *et al*, 2016).

The knowledge of all putative variants for every patient permitted a broad overview of the pathogenicity and the combination of alleles that affected each patient. This accurate analysis was only possible because our custom NGS panel made it easier to simultaneously study ADAMTS13 and the complement genes. Moreover, this technology has changed the paradigm of routine molecular studies: in the face of multiple genetic changes found in every patient, the critical challenge became discriminating disease-associated variants from the broader background of variants

present in all patients' genomes. This analysis has led us to a well-established bioinformatics pipeline according to NGS guidelines (Matthijs *et al*, 2015; Richards *et al*, 2015); additionally, it provides evidence that a clinical-laboratory approach for each patient's genotypic data must be evaluated in consideration of their specific and differential clinical manifestations.

In conclusion, the study of these 45 TMA Portuguese patients contributes to the better understanding of the molecular genetics of ADAMTS13/complement gene-related phenotypes. Moreover, it provides evidence of the usefulness of the NGS panel as an excellent and advantageous technology that enables more rapid and cost-effective diagnosis of TMAs. These findings show that this is a valuable asset in clinical practice given that a correct diagnosis is essential for determining the most effective treatment for patients with this complex disease.

7.2. Concluding Remarks



CHAPTER

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SUPPLEMENTARY DATA

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- Suppl. Materials and Methods

Sanger direct sequencing VWF

Forty-seven primer pairs were used to amplify the 52 exons and the flanking splice regions of *VWF*. The primer sequences and polymerase chain reaction (PCR) conditions were previously described (Corrales *et al*, 2009). The following primer pair was used to study exon 2 of *GP1BA:* 5' CTGGAGAATCTCGACACCCTTC; 5' AGTGTGAAATTT GGGGTCCATCT. After amplification, the PCR products for all samples were sequenced using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and Big Dye Terminator v1.1 Cycle Sequencing Chemistry (Applied Biosystems), according to the protocols recommended by the manufacturer. Data from the Sequence Analysis Software was aligned against the native *VWF* sequence (GenBank no. NC_000012.10) using the SeqScape® v2.5 software (Applied Biosystems) for mismatch detection.

NGS VWF sequencing - depth of coverage and primers design

The minimum depth of coverage required for each nucleotide in the ROI to be identified/considered a candidate mutation was >=10X. However, the mean coverage for all the ROIs (although highly variable) was at least 500-fold coverage for each base in all patients. All designed primers were evaluated to corroborate the absence of SNPs in the primer binding sequences (particularly in the 3' region) that could result in preferential or single allele amplification. In addition, the primers were designed to be highly specific to avoid pseudogene (*VWFP1*) amplification. However, the optimal Fluidigm PCR conditions were not stringent enough, and approximately 4-5% of the reads obtained for each patient corresponded to pseudogene sequences. The specific alignment and segregation of reads corresponding to *VWF* and *VWFP1* was achieved by adjusting the parameters of the sequence analysis software (Batlle *et al*, 2015).

Sanger Sequencing of complement genes

One hundred and twenty-seven primer pairs were used to amplify all coding exons, promoter and the flanking splice regions of the *ADAMTS13, CFH, CFHR1, CFHR3, CFHR4, CFHR5, CFI, CFB, C3, CD46, THBD and DGKE*. The primer sequences and polymerase chain reaction (PCR) conditions were previously described (Levy *et al*, 2001; Schneppenheim *et al*, 2003; Maga *et al*, 2010). The exon 7 of *ADAMTS13* was amplified using GC-Rich PCR system (Roche Diagnostics, Rotkreuz, Switzerland), following the manufacturer's specifications. After amplification, the PCR products for all samples were sequenced using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and Big Dye Terminator v1.1 Cycle Sequencing Chemistry (Applied Biosystems), according to the protocols recommended by the manufacturer. Data from the Sequence Analysis Software was aligned against the native *ADAMTS13, CFH, CFI, CFB, C3, CD46, THBD* and *DGKE* sequence (GenBank no. NC_000009.12; NC_000001.11; NC_000004.12; NC_000006.12; NC_000019.10; NC_000001.11; NC_000020.11; NC_000017.11) using the SeqScape® v2.5 software (Applied Biosystems) for mismatch detection.

NGS gene panel for diagnostic of bleeding disorders developed by BST

A NGS custom panel to simultaneously analyze the 23 essential genes involved in inherited bleeding disorders (*VWF, F8, F9, F2, F5, F7, F10, F11, F12, F13A1, F13B, MCFD2, LMAN1, FGG, FGA, FGB, ITGB3, ITGA2B, GP1BB, GP9, GP1BA, ADAMTS13, MTHFR*) was designed and validated in the Banc de Sang i Teixits (BST) of Barcelona. The targeted exon enrichment GeneRead panel (QIAGEN) designed comprises a total of 1,285 amplicons (size average 169 bp) covering the 98.7% of the target genomic regions. Construction of libraries, including patient-specific indexation, was performed with NEBNext Ultra DNA Library Prep Kit. Between 24 and 48 libraries were sequenced together in every MiSeq (Illumina) run. Putative mutations were identified by GeneRead Variant Calling software and further validated by Sanger methodology, reaching 100% sensitivity.

Suppl. Results

Suppl. Table 1. Sixty- two different variants were identified in 60 families (92 individuals) with VWD. Distribution by the type of variant with assessment of the pathogenicity using different types of data: population, *in silico*, function and segregation.

Nucleotide Change	Amino Acid Change	Exon	MAF[a] (ExAC)	MAF[a] (EVS_EA)	rs ID	HGMD	VWF databases	*In silico* score[b] (Supp.Table 2)	VWD Subtype associated	Familial Segregation	Variants classification	Familial Studies Prop./relatives	Sanger	NGS
Missense variants														
c.440A>G	**p.Gln147Arg**[d]	5	ND	ND	NA	ND	ND	3	Type 1[d]	No	Pathogenic	1/0	•	•
c.1027G>T	p.Val343Leu	9	0.000008294	ND	NA	ND	ND	4	Co-inh./2M	No	Benign	1/0		•
c.1446C>G	p.Ile482Met	13	0.0001272	ND	rs569669757	ND	ND	4	Co-inh./2M	No	Benign	1/0		•
c.1892C>T	p.Ala631Val[d]	15	0.0002834	0.00029	rs199963222	ND	ND	3	Type 1[d]	Yes	Likely Pathogenic	1/2	•	•
c.2446C>T	p.Arg816Trp	19	0.00002489	ND	rs121964894	DM	Yes	5	Type 2N	No	Pathogenic	2/0	•	•
c.2561G>A	p.Arg854Gln	20	0.003081	0.005581	rs41276738	DM	Yes	3	Type 2N	Yes	Pathogenic	11/5	•	•
c.2637C>A	**p.Asp879Glu**	20	ND	ND	NA	ND	ND	5	Type 2N	Yes	Pathogenic	1/2	•	•
c.3388T>C	p.Cys1130Arg	26	ND	ND	rs267607323	DM	Yes	5	Type 2E	Yes	Pathogenic	1/2	•	•
c.3437A>G	p.Tyr1146Cys[d]	26	ND	ND	rs267607326	DM	Yes	5	Type 3[d]	Yes	Pathogenic	1/1	•	•
c.3485_3486 delinsTG	p.Pro1162 Leu	26	ND	ND	NA	ND	Yes	5	Type 3/ Type 2N	Yes	Uncertain significance	2/1	•	•
c.3590C>A	**p.Pro1197Gln**	27	ND	ND	NA	ND	ND	2	Co-inh./2B	No	Benign	1/0		•
c.3686T>C	**p.Val1229Ala**	28	ND	ND	NA	ND	ND	0	Co-inh./2N	No	Benign	0/1		•
c.3692A>C	p.Asn1231Thr	28	0.002190	0.000466	rs61749368	DM	Yes	2	Co-inh./2N	No	Benign	0/1		•
c.3814T>G	p.Cys1272Gly	28	ND	ND	rs61749372	DM	Yes	5	Type 2A	No	Pathogenic	1/0	•	•
c.3815G>T	p.Cys1272Phe	28	ND	ND	rs63524161	DM	Yes	5	Type 2A	No	Pathogenic	1/0	•	•
c.3916C>T	p.Arg1306Trp	28	ND	ND	rs61749384	DM	Yes	4	Type 2B	Yes	Pathogenic	4/4	•	•
c.3943C>T	p.Arg1315Cys	28	ND	ND	rs61749395	DM	Yes	4	Type 2M	No	Pathogenic	1/0	•	•
c.3944G>A	p.Arg1315His	28	0.00003310	0.000116	rs61749396	DM	Yes	4	Type 2M	Yes	Pathogenic	1/1	•	•
c.3946G>A	p.Val1316Met	28	ND	ND	rs61749397	DM	Yes	4	Type 2B	Yes	Pathogenic	1/1	•	•
c.4117G>T	**p.Asp1373Tyr**[d]	28	ND	ND	NA	ND	ND	4	Type 2M[d]	No	Pathogenic	1/0	•	•
c.4120C>T	p.Arg1374Cys	28	0.000008292	ND	rs61750071	DM	Yes	4	Type 2M	No	Pathogenic	2/0	•	•
c.4121G>A	p.Arg1374His	28	ND	ND	rs61750072	DM	Yes	4	Type 2M	No	Pathogenic	4/0	•	•
c.4195C>T	p.Arg1399Cys	28	0.00001660	ND	rs61750077	DM	Yes	4	Type 2M	Yes	Pathogenic	2/2	•	•
c.4257T>G	p.His1419Gln[d]	28	0.0001071	ND	rs569177726	ND	ND	2	Type 2M[d]	No	Benign	1/0	•	•
c.4517C>T	p.Ser1506Leu	28	ND	ND	rs61750100	DM	Yes	5	Type 2A	No	Pathogenic	2/0	•	•
c.4751A>G	p.Tyr1584Cys	28	0.002942	0.003023	rs1800386	DM	Yes	3	Type 1	No	Benign	0/1	•	•
c.4883T>C	p.Ile1628Thr	28	ND	ND	rs61750584	DM	Yes	3	Type 2A	Yes	Pathogenic	4/3	•	•
c.5140G>C	**p.Ala1714Pro**[d]	29	ND	ND	NA	ND	ND	4	Type 1[d]	Yes	Pathogenic	1/1	•	•
c.6890C>T	p.Pro2297Leu	39	0.00005100	ND	rs201372397	DM	Yes	4	Co-inh./2N	No	Likely Pathogenic	1/0		•
c.7400A>C	**p.Gln2467Pro**	43	ND	ND	NA	ND	ND	4	Type 1	No	Pathogenic	1/0	•	•
Nonsense variants														
c.3931C>T	p.Gln1311*	28	ND	ND	NA	DM	Yes	–	Type 3	Yes	Pathogenic	5/1	•	•
c.4666C>T	**p.Gln1556***	28	ND	ND	NA	ND	ND	–	Type 3	Yes	Pathogenic	4/3	•	•
c.7086C>A	**p.Cys2362***	42	ND	ND	NA	ND	ND	–	Type 3	No	Pathogenic	1/0	•	•

Small deletions and duplication														
c.100delT	**p.Arg34Aspfs*49**[d]	3	ND	ND	NA	ND	ND	–	Type 2N [d]	No	Pathogenic	1/0	•	•
c.5414_5415del	**p.Val1805Glyfs*8**	31	ND	ND	NA	ND	ND	–	Type 3	No	Pathogenic	1/0	•	•
c.6699_6702dup	p.Cys2235Argfs*8	38	ND	ND	NA	Yes	Yes	–	Type 3	Yes	Pathogenic	5/1	•	•
Large deletion														
c.5312-104_5455+642del (890bp)		31	ND	ND	NA	ND	ND	–	Type 1	Yes	Pathogenic	1/2		
Synonymous variants								(Supp.Table 3)						
c.1077C>T	p.(=)	9	0.001016	0.000814	rs71582884	ND	Yes	0	Co-inh./2N	No	Likely benign	0/1		•
c.3426T>C	p.(=)	26	ND	ND	NA	ND	Yes	0	Co-inh./3	Yes	Likely benign	2/1	•	•
c.7437G>A	p.(=)[d]	43	ND	ND	rs267607363	Yes	Yes	6 (DSS)	Type 1[d]	No	Pathogenic	1/0	•	•
c.7464C>T	p.(=)[d]	44	ND	ND	NA	ND	Yes	5 (DSS)	Type 2M[d]	No	Likely Pathogenic	1/0		•
c.8175C>T	p.(=)	51	0.000049	ND	NA	ND	ND	0	Co-inh./2M	Yes	Likely benign	1/1		•
Intronic variants		Intron												
c.55+8C>A		2	0.001773	0.000233	rs114713980	ND	Yes	0	Co-inh./2A	No	Likely benign	1/0		•
c.998-46C>T		9	ND	ND	NA	ND	ND	0	Co-inh./2M	No	Likely benign	1/0		•
c.1156+42C>T		10	ND	0.000233	rs376410074	ND	ND	0	Co-inh./2N	No	Likely benign	1/0		•
c.1293+109T>C		11	ND	ND	rs375092486	ND	ND	0	Co-inh./1	No	Likely benign	0/1		•
c.1533+1G>A[d]		13	ND	ND	NA	ND	ND	5 (DSS)	Type3[d]	Yes	Pathogenic	1/1	•	•
c.1534-38G>A		13	ND	ND	NA	ND	ND	0	Co-inh./2M	No	Likely benign	1/0		•
c.2821-57_-2821-56delTCinsA		21	ND	ND	NA	ND	ND	0	Co-inh./3	No	Likely benign	6/1		•
c.2821-102C>T		21	ND	ND	NA	ND	ND	0	Co-inh./2M	No	Likely benign	1/0		•
c.2968-105G>A		23	ND	ND	NA	ND	ND	0	Co-inh./2M	No	Likely benign	1/0		•
c.3380-92_3380-90delTAT		25	ND	ND	NA	ND	ND	0	Co-inh./3	No	Likely benign	0/1		•
c.5170+10C>T		29	0.00364	0.00465	rs61750601	ND	Yes	3 (DSS)	Co-inh./1, 2B	No	Uncertain significance	1/1	•	•
c.5620+33delA		32	ND	ND	NA	ND	ND	1 (DSS)	Co-inh./3	No	Likely benign	2/1		•
c.5621-139A>G		33	ND	ND	NA	ND	ND	0	Co-inh./3	No	Likely benign	0/1		•
c.6799-47G>A		38	ND	ND	NA	ND	ND	0	Co-inh./2M	No	Likely benign	1/0		•
c.7288-68G>A		42	ND	ND	NA	ND	ND	3 (DSS)	Co-inh./2A	No	Uncertain significance	1/0		•
c.7730-4C>G[d]		45	0.0001155	0.000116	rs71581030	ND	ND	1 (ASS)	Type 1[d]	Yes	Likely Pathogenic	1/3	•	•
c.7730-24A>G		45	ND	ND	NA	ND	ND	0	Co-inh./1	No	Likely benign	1/3		•
c.7730-223A>G		45	ND	ND	NA	ND	ND	1 (ASS)	Co-inh./1	No	Likely benign	1/0		•
c.7771-48G>A		46	ND	ND	NA	ND	ND	1 (ASS)	Co-inh./2B	No	Likely benign	1/1		•
c.7771-86G>A		46	ND	ND	NA	ND	ND	3 (ASS)	Co-inh./2M	No	Uncertain significance	1/0		•

[a]Minor allele frequency (MAF) values are from the Exome Aggregation Consortium (ExAC) and the European-American (EA) population from the Exome Variant Server (EVS). Novel variants (**in bold**) are those variants were not reported in the population databases (1000 Genomes, dbSNP database, ExAC, and EVS), Human Gene Mutation Database (HGMD) or VWD-specific databases (ISTH-SSC VWF, LOVD and VWFdb Hemobase). [b]The *in silico* score for missense variants was calculated using PROVEAN, SIFT, PolyPhen-2, MutationAssessor, and MutationTaster (Suppl. Table 2) and were considered deleterious when ≥ 3. [c]The *in silico* score for predicting potential splice site variants was calculated using HSF, MaxEntScan, NNSPLICE, NetGene2 and Splice View (Suppl. Table 3) and was considered deleterious for a score ≥ 3 (intronic variants) or score≥ 4 (exonic variants). [d]VWD subtype defined with the other variant in the compound heterozygous state. Co-inh./ designates the variants that were co-inherited with other variants and had an unknown functional effect on VWD subtype. Variants detected by Sanger and NGS sequencing are marked by a **dot.** Variants that initially were not screened using Sanger sequencing are marked by a grey cell. Prop. *propositus*; NA, not available; ND, no data; ASS, acceptor splice site; DSS, donor splice site.

Suppl. Table 2 - Summary of *in silico* analysis (PROVEAN, SIFT, Polyphen-2, Mutationassessor and Mutation Taster) for missense variants.

Nucleotide Change	Amino Acid Change	Exon	PROVEAN[a]		SIFT[b]		PolyPhen_2[c]		MutAss[d]		MutTaster[e]		
			Score	prediction	Score	prediction	Score	prediction	Score	prediction	Score	prediction	*In silico* score
c.440A>G	**p.Gln147Arg**	5	-1.227	Neutral	0.059	Tolerated	0.8	Possibly damaging	2.135	Medium	0.99	Disease causing	3
c.1027G>T	p.Val343Leu	9	-0.661	Neutral	0.04	Deleterious	0.992	Probably damaging	3.115	High	1	disease causing	4
c.1446C>G	p.Ile482Met	13	-2.107	Neutral	0.001	Deleterious	0.991	Probably damaging	2.99	Medium	1	Disease causing	4
c.1892C>T	p.Ala631Val	15	-1.689	Neutral	0.29	Tolerated	0.594	Possibly damaging	2.995	Medium	0.98	Disease causing	3
c.2446C>T	p.Arg816Trp	19	-4.378	Deleterious	0.001	Deleterious	1.0	Probably damaging	2.075	Medium	1	Disease causing	5
c.2451T>A	p.His817Gln	19	-2.144	Neutral	0.03	Deleterious	0.09	Benign	1.69	Low	1	Disease causing	2
c.2561G>A	p.Arg854Gln	20	-3.578	Deleterious	0.08	Tolerated	0.994	Probably damaging	1.765	Low	1	Disease causing	3
c.2637C>A	**p.Asp879Glu**	20	-3.841	Deleterious	0.0	Deleterious	0.982	Probably damaging	4.305	High	1	Disease causing	5
c.3388T>C	p.Cys1130Arg	26	-11.773	Deleterious	0.0	Deleterious	0.998	Probably damaging	3.825	High	1	Disease causing	5
c.3437A>G	p.Tyr1146Cys	26	-8.823	Deleterious	0.0	Deleterious	1.0	Probably damaging	4.18	High	1	Disease causing	5
c.3485_3486 delinsTG	p.Pro1162 Leu	26	-4.905	Deleterious	0.04	Deleterious	0.986	Probably damaging	2.5	Medium	1	Disease causing	5
c.3590C>A	**p.Pro1197Gln**	27	-1.535	Neutral	0.3	Tolerated	0.972	Probably damaging	1.75	Low	1	Disease causing	2
c.3686T>C	**p.Val1229Ala**	28	1.051	Neutral	0.34	Tolerated	0.0	Benign	0.0	Low	1	polymorphism	0
c.3692A>C	p.Asn1231Thr	28	-1.121	Neutral	0.39	Tolerated	0.651	Possibly damaging	1.73	Low	0.98	Disease causing	2
c.3814T>G	p.Cys1272Gly	28	-4.723	Deleterious	0.0	Deleterious	0.999	Probably damaging	3.32	Medium	1	Disease causing	5
c.3815G>T	p.Cys1272Phe	28	-3.564	Deleterious	0.0	Deleterious	0.998	Probably damaging	3.32	Medium	1	Disease causing	5
c.3916C>T	p.Arg1306Trp	28	-1.585	Neutral	0.0	Deleterious	0.981	Probably damaging	3.795	High	0.93	Disease causing	4
c.3943C>T	p.Arg1315Cys	28	-1.348	Neutral	0.0	Deleterious	1.0	Probably damaging	4.01	High	1	Disease causing	4
c.3944G>A	p.Arg1315His	28	-2.039	Neutral	0.0	Deleterious	0.799	Possibly damaging	3.66	High	1	Disease causing	4
c.3946G>A	p.Val1316Met	28	-1.065	Neutral	0.01	Deleterious	0.999	Probably damaging	3.955	High	1	Disease causing	4
c.4117G>T	**p.Asp1373Tyr**	28	-2.524	Deleterious	0.01	Deleterious	0.998	Probably damaging	3.455	High	0.98	Polymorphism	4
c.4120C>T	p.Arg1374Cys	28	-0.137	Neutral	0.0	Deleterious	0.999	Probably damaging	4.015	High	1	Disease causing	4
c.4121G>A	p.Arg1374His	28	-1.383	Neutral	0.0	Deleterious	0.998	Probably damaging	4.015	High	1	Disease causing	4
c.4195C>T	p.Arg1399Cys	28	-0.342	Neutral	0.01	Deleterious	0.998	Probably damaging	3.795	High	1	Disease causing	4
c.4257T>G	p.His1419Gln	28	-1.673	Neutral	0.09	Tolerated	0.819	Possibly damaging	2.955	Medium	1	Polymorphism	2
c.4517C>T	p.Ser1506Leu	28	-3.087	Deleterious	0.01	Deleterious	1.0	Probably damaging	4.4	High	1	Disease causing	5
c.4751A>G	p.Tyr1584Cys	28	-0.394	Neutral	0.02	Deleterious	0.981	Probably damaging	4.125	High	1	Polymorphism	3
c.4883T>C	p.Ile1628Thr	28	-0.879	Neutral	0.0	Deleterious	0.229	Benign	3.955	High	1	Disease causing	3
c.5140G>C	**p.Ala1714Pro**	29	-0.972	Neutral	0.03	Deleterious	0.974	Probably damaging	2.51	Medium	0.91	Disease causing	4
c.6890C>T	p.Pro2297Leu	39	-2.620	Deleterious	0.01	Deleterious	0.261	Benign	2.505	Medium	1	Disease causing	4
c.7400A>C	**p.Gln2467Pro**	43	-1.193	Neutral	0.02	Deleterious	0.898	Possibly damaging	2.015	Medium	1	Disease causing	4

[a]Provean uses the alignment and the measurement of the similarity between the variant sequence and the protein sequence homolog. The score threshold is set at -2.5 for binary classification (i.e., deleterious *vs* neutral). [b]SIFT is used to predict the effect of sequence changes on the protein function and is based on a homology search and the physical properties of amino acids – scores range from 0 to 1. An amino acid substitution is predicted to be damaging if the score is <=0.05 and is tolerated if the score is >0.05.

[c]PolyPhen is a tool that predicts the possible effect of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations. Scores between 0.909 and 1 denote a probably damaging variant, a score between 0.447 and 0.908 indicates a possibly damaging variant, and a score below 0.446 is a benign variant.

[d]MutationAssessor, The score predicts the functional impact of amino acid substitutions, with high scores denoting a more severe impact.

[e]MutationTaster uses a Bayes classifier to calculate the probability of whether the alteration in the sequence is a disease mutation or a harmless polymorphism. A probability close to 1 indicates a high certainty in the prediction. The *in silico* scores were considered deleterious when ≥ 3. Novel variants are marked in **bold**.

Suppl. Table 3. Summary of potential splice site variants and predicted impact on RNA processing. A higher score implies greater potential for splice site.

Nucleotide Change	HSF score native-mutated	MaxEntScan score native-mutated	NNSplice score native-mutated	NetGene2 score native-mutated	Splice View score native-mutated	MutPred Splice	*In silico* score	Comments
Intronic variants								
c.1533+1G>A	90.71 - 63.87 Broken DSS	10.29 - 2.1	0.98 - native DSS destroyed	1.00 - native DSS destroyed	90 - native DSS destroyed	NA	5	Heterozygous in *trans* with c.3437A>G, p.Tyr1146Cys
c.5170+10C>T	52.66 - 79.49 New DSS	No difference	0.84 - 0.97	No difference	Not predited – New DSS -78	NA	3	Heterozygous in *trans* with c.5140G>C, p.Ala1714Pro; Heterozygous in *trans* with c.3946G>A, p.Val1316Met
c.5620+33delA	68.43 - 9.69 Broken DSS	No difference	No difference	No difference	No difference	NA	1	Homozygous with c.3485_3486delinsTG
c.7288-68G>A	59.3 - 67.64 New DSS	2.89 - 6.3	No difference	No difference	Not predited – New DSS -75	NA	3	Heterozygous in *trans* with c.3815G>T, p.Cys1272Phe
c.7730-4C>G	36.76 - 65.71 New ASS	No difference	No difference	No difference	No difference	NA	1	Homozygous with c.1892C>T, p.Ala621Val
c.7730-223A>G	41.08 - 70.02 New ASS	No difference	No difference	No difference	No difference	NA	1	Heterozygous with c.6699_6702dupAGGC, p.Cys2235Argfs*8 and c.7437G>A, p. (=)
c.7771-48G>A	40.85 - 69.79 New ASS	No difference	No difference	No difference	No difference	NA	1	Heterozygous in *trans* with c.3916C>T, p. Arg1306Trp; Heterozygous in *trans* with c.3931C>T, p.Gln1311*
c.7771-86G>A	36.63 - 65.68 New ASS	3.82 - 8.73	Not predited - 0.98 New DSS	No difference	No difference	NA	3	Heterozygous with c.4121G>A, p.Arg1374His
Exonic variants								
c.2451T>A	40.74 - 69.68 New ASS	No difference	No difference	No difference	No difference	0.24 (SNV)	1	Heterozygous in *trans* with c.3485_3486delinsTG
c.2561G>A	50.04 - 78.99 New ASS	No difference	No difference	No difference	No difference	0.26 (SNV)	1	Homozygous
c.2637C>A	52.25 - 81.19 New ASS	-2.76 - 5.27	No difference	No difference	Not predited – New ASS -82	0.31 (SNV)	3	Heterozygous in *trans* with c.2561G>A p.Arg854Gln
c.3437A>G	41.44 - 68.27 New DSS	No difference	No difference	No difference	No difference	0.19 (SNV)	1	Heterozygous in *trans* with c.1533+1G>A
c.4257T>G	57.71 - 86.65 New ASS	-4.84 - 3.75	No difference	No difference	No difference	0.15 (SNV)	2	Heterozygous with c.1027G>T, p.Val343Leu; c.1446C>G, p.Ile482Met, c.4117G>T, p.Asp1373Tyr
c.7437G>A	92.11 - 81.53 Broken DSS	3.52 - 7.03	0.98-0.61	0.55 - 0.92	Not predited – New DSS -79	0.9 (SAV); Loss of natural 5' SS; (P < 0.000001)	6	Heterozygous in *trans* with c.6699_6702dupAGGC, p.Cys2235Argfs*8
c.7464C>T	No difference	2.72 - 10.47	Not predited - 0.99 New DSS	Not predited - DSS -0.63	Not predited – New DSS -94	0.97 (SAV) Cryptic 5' SS	5	Heterozygous in *trans* with c.3944G>A, p.Arg1315His

ASS: acceptor splice site; DSS: donor splice site; NA – not applicable; HSF score (0-100); NNSplice score (0-1); NetGene2 score (0-1); MaxEntScan score: maximum entropy score; Splice View score (0-100). MutPred Splice - Not predicted to disrupt splicing, Splice Neutral Variant (SNV), (general score <0.6); Confident calls of splicing variants - predicted Splice Affected Variant (SAV), (general score >=0.6). *In silico* scores were considered deleterious when ≥ 3 for intronic variants and when ≥ 4 for exonic variants. Novel variants are marked in **bold**.

Suppl. Table 4 - Summary of *in silico* analysis for missense variants using five pathogenicity prediction programmes (PROVEAN, SIFT, PolyPhen2, MutationAssessor and MutPred) to determine the degree of tolerance for each amino acid substitution on the basis of physio-chemical properties.

Gene	Nucleotide Change	Amino Acid Change	Exon	PROVEAN[a]		SIFT[b]		PolyPhen_2[c]		MutAss[d]		MutPred[e]		*In silico*
				score	prediction	score	prediction	score	prediction	score	prediction	score	prediction	score
ADAMTS13	c.1368G>T	p.Gln456His	12	-2.65	Deleterious	0.00	Damaging	0.00	Benign	1.88	Low	0.173	Neutral	2
	c.1370C>T	p.Pro457Leu	12	-7.29	Deleterious	0.05	Damaging	1.00	Probably damaging	2.66	Medium	0.715	High confidence	5
	c.1874G>A	p.Arg625His	16	-2.51	Deleterious	0.17	Tolerated	1.00	Probably damaging	2.38	Medium	0.558	Harmful mutation	4
	c.2074C>T	p.Arg692Cys	17	-3.58	Deleterious	0.07	Damaging	0.99	Probably damaging	2.35	Medium	0.862	High confidence	5
	c.2218G>A	p.Glu740Lys	18	-0.98	Neutral	0.26	Tolerated	0.22	Benign	0.55	Neutral	0.207	Neutral	0
	c.2260T>C	p.Cys754Arg	19	-8.51	Deleterious	0.00	Damaging	0.99	Probably damaging	4.61	High	0.900	High confidence	5
	c.2914C>T	p.Arg972Trp	23	-3.36	Deleterious	0.02	Damaging	0.99	Probably damaging	2.73	Medium	0.666	Harmful mutation	5
	c.3287G>A	p.Arg1096His	25	1.30	Neutral	0.407	Tolerated	0.001	Benign	-0.295	Neutral	0.458	Neutral	0
	c.3368G>A	p.Arg1123His	25	-3.48	Deleterious	0.0	Damaging	0.99	Probably damaging	2.67	Medium	0.649	Harmful mutation	5
CFH	c.240T>G	p.Cys80Trp	2	-6.74	Deleterious	0.001	Damaging	1.00	Probably damaging	2.8	Medium	0.837	High confidence	5
	c.335A>G	p.Tyr112Cys	3	-5.54	Deleterious	0.002	Damaging	1.00	Probably damaging	4.4	High	0.805	High confidence	5
	c.493G>T	p.Asp165Tyr	5	-4.41	Deleterious	0.005	Damaging	0.99	Probably damaging	2.015	Medium	0.552	Harmful mutation	5
	c.1864A>G	p.Ile622Val	12	0.69	Neutral	0.083	Tolerated	0.59	Benign	0.145	Neutral	0.501	Harmful mutation	1
	c.2669G>T	p.Ser890Ile	17	-0.71	Neutral	0.161	Tolerated	0.95	Probably damaging	0.54	Neutral	0.656	Harmful mutation	2
	c.2850G>T	p.Gln950His	18	-2.98	Deleterious	0.006	Damaging	0.80	Possibly damaging	1.06	Low	0.850	High confidence	4
	c.3019G>T	p.Val1007Leu	19	0.11	Neutral	0.649	Tolerated	0.00	Benign	0.3	Neutral	0.650	Harmful mutation	1
	c.3172T>C	p.Tyr1058His	20	0.13	Neutral	0.550	Tolerated	0.00	Benign	0.59	Neutral	0.544	Harmful mutation	1
	c.3178G>C	p.Val1060Leu	20	-0.09	Neutral	0.497	Tolerated	0.002	Benign	0.855	Low	0.516	Harmful mutation	1
	c.3226C>G	p.Gln1076Glu	20	1.34	Neutral	1.000	Tolerated	0.00	Benign	-1.18	Neutral	0.800	High confidence	1
	c.3562A>G	p.Lys1188Glu	22	-3.20	Deleterious	0.005	Damaging	0.999	Probably damaging	2.65	Medium	0.588	Harmful mutation	5
	c.3644G>T	p.Arg1215Leu	22	-5.98	Deleterious	0.004	Damaging	1.00	Probably damaging	3.38	Medium	0.776	High confidence	5
CD46 (MCP)	c.686G>A	p.Arg229Gln	6	0.69	Neutral	0.302	Tolerated	0.998	Probably damaging	-0.2	Neutral	0.551	Harmful mutation	2
	c.1148C>T	p.Thr383Ile	13	-0.44	Neutral	0.010	Damaging	0.001	Benign	0.345	Neutral	0.291	Neutral	1
C3	c.193A>C	p.Lys65Gln	2	-2.98	Deleterious	0.036	Damaging	1.00	Probably damaging	3.33	Medium	0.661	Harmful mutation	5
	c.1775G>A	p.Arg592Gln	14	-1.89	Neutral	0.050	Damaging	0.836	Possibly damaging	2.68	Medium	0.883	High confidence	4
	c.1407G>C	p.Glu469Asp	12	0.94	Neutral	1.000	Tolerated	0.001	Benign	0.045	Neutral	0.327	Neutral	0
CFI	c.452A>G	p.Asn151Ser	3	-4.50	Deleterious	0.000	Damaging	1.00	Probably damaging	2.07	Medium	0.814	High confidence	5
	c.1642G>C	p.Glu548Gln	13	-2.39	Neutral	0.052	Tolerated	0.99	Probably damaging	1.385	Low	0.658	Harmful mutation	2
CFB	c.1598A>G	p.Lys533Arg	12	-0.51	Neutral	0.400	Tolerated	0.009	Benign	0.81	Low	0.800	High confidence	1
CFHR5	c.329T>C	p.Val110Ala	3	-3.03	Deleterious	0.009	Damaging	0.887	Possibly damaging	2.62	Medium	0.686	Harmful mutation	5

[a]Provean uses the alignment and the measurement of the similarity between the variant sequence and the protein sequence homolog. The score threshold is set at -2.5 for binary classification (i.e., deleterious *vs* neutral). [b]SIFT is used to predict the effect of sequence changes on the protein function and is based on a homology search and the physical properties of amino acids – scores range from 0 to 1. An amino acid substitution is predicted to be damaging if the score is <=0.05 and is tolerated if the score is >0.05. [c]PolyPhen is a tool that predicts the possible effect of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations. Scores between 0.909 and 1 denote a probably damaging variant, a score between 0.447 and 0.908 indicates a possibly damaging variant, and a score below 0.446 is a benign variant. [d]MutationAssessor, the score predicts the functional impact of amino acid substitutions, with high scores denoting a more severe impact. [e]A missense mutation with a MutPred score of > 0.5 could be considered as 'harmful', while a MutPred score of > 0.75 should be considered a high confidence 'harmful' prediction. *In silico* scores of ≥ 3 were considered deleterious.

Suppl. Table 5. Thirty-three rare variants were identified in 28 individuals with TMA. Distribution by the type of variant with assessment of the pathogenicity using different types of data: *in silico*, population and phenotype.

Gene	Nucleotide Change	Amino Acid Change	Exon	Zygosity	Domain Region	*In silico* Score[a]	MAF[b] (ExAC_EA)	MAF[b] (EVS_EA)	rsID	HGMD, phenotype	FH aHUS, phenotype	Variants classification
ADAMTS13	**c.762_774del12pb**	**p.Pro256Serfs*12**	7	Htz	Metalloprotease	NA			ND	ND	—	Pathogenic
	c.1368G>T	p.Gln456His	12	Htz	Cystein–rich	2	0.012	0.0117	rs36220239	DM, TTP	—	Likely pathogenic
	c.1370C>T	p.Pro457Leu	12	Htz	Cystein–rich	5	0.6148	0.2467	rs36220240	DM, TTP	—	Pathogenic
	c.1874G>A	p.Arg625His	16	Htz	Spacer	4	0.01366	0.0581	rs36090624	ND	—	UVS
	c.2074C>T	p.Arg692Cys	17	Hmz	TSP1-2	5	0	ND	rs121908475	DM, TTP	—	Pathogenic
	c.2218G>A	p.Glu740Lys	18	Htz	TSP1-2	0	0.0061	0.0116	rs36221451	ND	—	Likely benign
	c.2260T>C	p.Cys754Arg	19	Hmz	TSP1-3	5	ND	ND	ND	DM, TTP	—	Pathogenic
	c.2914C>T	p.Arg972Trp	23	Htz	TSP1-6	5	0	ND	ND	ND	—	Pathogenic
	c.3287G>A	p.Arg1096His	25	Htz	TSP1-8	0	0.2379	0.3372	rs61751476	DM, ADAMTS13 deficiency	—	Likely benign
	c.3368G>A	p.Arg1123His	25	Hmz	TSP1-8	5	0.0015	ND	ND	DM, TTP	—	Pathogenic
CFH	**c.240T>G**	**p.Cys80Trp**	2	Htz	SCR 1	5	ND	ND	ND	ND	ND	Pathogenic
	c.335A>G	**p.Tyr112Cys**	3	Htz	SCR 2	5	ND	ND	ND	ND	ND	Pathogenic
	c.493G>T	**p.Asp165Tyr**	5	Htz	SCR 3	5	ND	ND	ND	ND	ND	Pathogenic
	c.1864A>G	**p.Ile622Val**	12	Htz	SCR 10	1	ND	ND	ND	ND	ND	Likely benign
	c.2669G>T	p.Ser890Ile	17	Htz	SCR 15	2	0.2742	0.2442	rs515299	DM? TTP, renal involvement	HUS	Likely pathogenic
	c.2850G>T	p.Gln950His	18	Htz	SCR 16	4	0.6084	0.616	rs149474608	DM, HUS	HUS	Pathogenic
	c.3019G>T	p.Val1007Leu	19	Htz	SCR 17	1	0.2757	0.2558	rs534399	DM?, HUS	HUS	Likely benign
	c.3172T>C	p.Tyr1058His	20	Htz	SCR 17	1	ND	ND	rs55679475	DM?, HUS	ND	Likely benign
	c.3178G>C	p.Val1060Leu	20	Htz	SCR 18	1	ND	ND	rs55771831	DM?, HUS	Non-DC	Likely benign
	c.3226C>G	p.Gln1076Glu	20	Htz	SCR 18	1	ND	ND	rs62625015	DM, Factor H deficiency	HUS	Likely pathogenic
	c.3562A>G	**p.Lys1188Glu**	22	Htz	SCR 20	5	ND	ND	ND	ND	ND	Pathogenic
	c.3644G>T	**p.Arg1215Leu**	22	Htz	SCR 20	5	ND	ND	ND	ND	ND	Pathogenic
CFHR5	c.329T>C	p.Val110Ala	3	Htz	SCR2	5	0.012	0.0116	rs140691305	ND	ND	Likely Pathogenic
MCP (CD46)	c.287-2A>G(splice acceptor)		Intron 2	Htz	SCR 1-2	NA	0.0064	ND	ND	DM, HUS	HUS	Pathogenic
	c.686G>A	p.Arg229Gln	6	Htz	SCR 4	2	0.0063	ND	ND	ND	ND	UVS
	c.800-821 del	**Thr267Ilefs*24**	6	Htz	SCR 4	NA	ND	ND	ND	ND	ND	Pathogenic
	c.1148C>T	p.Thr383Ile	13	Htz	CY	1	0.087	0.0581	rs146803767	DM, HUS	HUS	Benign
CFI	c.452A>G	p.Asn151Ser	3	Htz	SRCR	5	0.0015	ND	ND	DM, HUS	HUS	Pathogenic
	c.1642G>C	p.Glu548Gln	13	Htz	SP	2	0.02699	0.00	rs7437875	No phenotype recorded	Non-DC	Benign
C3	c.193A>C	p.Lys65Gln	2	Htz	MG1	5	0.0089	ND	rs539992721	DM, HUS	HUS	Pathogenic
	c.1407G>C	p.Glu469Asp	12	Htz	MG5	0	0.01648	0.0349	rs11569422	DM, HUS	HUS	Benign
	c.1775G>A	p.Arg592Gln	14	Htz	MG6β	4	ND	ND	rs121909583	DM, HUS	HUS	Pathogenic
CFB	c.1598A>G	p.Lys533Arg	12	Htz	SP	1	0.07887	0.0185	rs149101394	DM, HUS ?	-	Benign

Htz, Heterozygous; Hmz, Homozygous; TSP1, thrombospondin-like domain; SCR, short complement regulator; Cy, cytoplasmic region; MG, macroglobulin; SRCR, scavenger receptor cysteine-rich; SP, serine protease; [a]*In silico* scores of ≥ 3 for missense variants (Suppl. Table 1) were considered deleterious. [b]Minor allele frequency (MAF) values are from the Exome Aggregation Consortium - European (ExAC_EA) and the European-American (EA) population from the Exome Variant Server (EVS). NA, not available; ND, no data; DM, denotes a mutation reported to be disease-causing; DM?, denotes a mutation reported as likely disease-causing, but with questionable pathogenicity; TTP, thrombotic thrombocytopenic purpura; HUS, haemolytic uraemic syndrome; Non-DC, Non-Disease Causing; pdb, variants found only in the two population database; Novel variants (**in bold**) are those variants that were not reported in the population databases (1000 Genomes, dbSNP database, ExAC, and EVS), Human Gene Mutation Database (HGMD) or FH aHUS-specific databases.

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