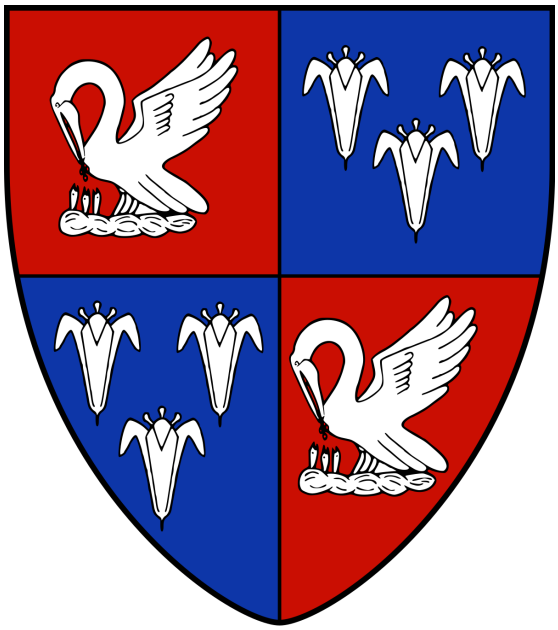
Pathogenicity assessment of genetic variants in von Willebrand disease using quantitative, qualitative, and functional approaches



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Declaration

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Acknowledgements section and specified in the text. No part of this thesis has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution. It does not exceed the prescribed word limit for the Clinical Medicine and Veterinary Medicine Degree Committee.

Name: Matthew Christopher Sims

Title: Pathogenicity assessment of genetic variants in von Willebrand disease using quantitative, qualitative, and functional approaches

Abstract

Von Willebrand disease (VWD) is the most common inherited bleeding disorder. It is defined by a deficiency or dysfunction of plasma von Willebrand factor (VWF), a glycoprotein with a multifaceted role in haemostasis. The majority of circulating VWF is synthesised and released by endothelial cells (ECs). VWD is caused by rare DNA sequence variants in the *VWF* gene. However, coupling genotype with phenotype is complicated by factors including incomplete penetrance and the trans-acting effect of the *ABO* histo-group. High throughput sequencing (HTS) is becoming the standard of care for the diagnosis of inherited bleeding disorders, including VWD. This raises several challenges. First, how should candidate *VWF* variants be searched for and their pathogenicity assessed? Second, if a pathogenic variant (PV) for VWD is identified how does this influence bleeding risk? Third, if the mechanism of the identified PV is unknown, how can its effect be elucidated? These questions are sequentially addressed in this thesis.

I curated 1,455 unique *VWF* variants into a single repository called VWDbase. Variants were only included if they had been previously linked to VWD. Two thirds of VWDbase variants had previously been deemed causal of VWD and were termed Putatively Aetiological VWD Variants (PAVVs). Of these, 194 PAVVs were identified in the whole exome sequencing data of 140,327 participants in UK Biobank (UKB). These data were used to accurately determine the minor allele frequency (MAF) of these PAVVs. The pathogenicity of each PAVV was then scrutinised using published data. Seventy three of 194 PAVVs were rejected as being pathogenic for VWD. In over half of cases this was because the PAVV occurred too frequently to be compatible with VWD prevalence. The PAVVs that were accepted as being pathogenic for VWD were identified in 401 UKB participants (the ‘genetically accepted VWD’[ga] group). Hospital inpatient data were analysed for UKB participants from 1997 to 2020. These were used to create the

ICD-bleeding assessment tool (ICD-BAT) to assess the presence or absence of bleeding episodes across 16 different domains and the time over which UKB participants lived without experiencing an episode (bleeding free survival). There was no difference in the ICD-BAT score or bleeding-free survival when the gaVWD group was compared to the rest of the UKB population. However, blood group O predicted for both an increased ICD-BAT score and a reduced risk of bleeding-free survival over the observation period.

VWDbase was then utilised to analyse 10 patients with VWD in whom no molecular diagnosis had previously been identified. The patient with the most severe (type 3) VWD phenotype was homozygous for a rare PAVV, c.8155+6T>A, situated in the donor splice site of the penultimate exon-intron junction. Analysis of platelet mRNA demonstrated that c.8155+6T>A results in a transcript with a frameshift and premature termination codon (PTC). Evaluation of patient-derived endothelial colony forming cells (ECFCs) revealed that c.8155+6T>A resulted in VWF that was mostly retained in a perinuclear position as opposed to being packed into Weibel-Palade bodies (WPBs). In order to overcome the finite supply of ECFCs and assess the effect of c.8155+6T>A in a different genetic context, a new cellular model of VWD was created. Human induced pluripotent stem cells (hiPSCs) were edited using CRISPR/Cas9 to contain a PTC in exon 50, positioned 10 nucleotides 5' of c.8155+6T>A. They were then differentiated to ECs and the findings in the patient ECFCs were replicated. The effect of c.8155+6T>A is likely to be due to the truncation of VWF prior to the C-terminal cysteine knot (CK), the domain which is crucial for VWF dimerisation and exit from the endoplasmic reticulum. In summary, this thesis highlights the utility of large reference populations and hiPSC-derived ECs (iECs) in the critical appraisal of PAVVs.

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Glossary

Frequently used terms are shortened to an abbreviation or acronym. The full names are provided in the table below, with definitions (in parentheses) for specialist terminology related to genetics and von Willebrand disease.

Abbreviation	Full name
AA(s)	Amino acid(s)
Acc	Acceleration ramp speed on centrifuge
ACMG/AMP	American College of Medical Genetics and Genomics/Association for Molecular Pathology
ADAMTS13	A disintegrin and metalloproteinase with thrombospondin type-1 repeats-13
AF	Alexa Fluor®
Ag	Antigen
BAT	Bleeding Assessment Tool
BP	Base pairs
BPD	Bleeding platelet and thrombotic disorders
(L)BV	(Likely) benign variant (based on ACMG/AMP classification system)
CADD	Combined Annotation-Dependent Depletion
Chr	Chromosome
CI	95% confidence interval
CK	C-terminal cysteine knot
ClinVar	National Center for Biotechnology Information Clinical Variant Database
CV	Coefficient of variance
D	Day
DB	Database
D-PBS	Dulbecco's phosphate buffered saline
DM	Disease-causing mutation (based on HGMD classification system)
DM?	Probable/possible disease-causing mutation (based on HGMD classification system)
DSS	Donor splice site

EAHAD-CFDB	European Association for Haemophilia and Allied Disorders Coagulation Factor Variant Database
EC	Endothelial cell
ECFC	Endothelial cell colony forming cell
ECIS	Electric cell-substrate impedance sensing
EHR	Electronic Health Record
ExAC	Exome Aggregation Consortium
F	Factor
FBS	Fetal Bovine Serum
FC	Flow cytometry
FVIII	Coagulation factor 8
GFP	Green fluorescent protein
gnomAD	Genome Aggregation Database
GRCh37/38	Genome Reference Consortium human genome build 37/38 (GRCh37/38)
HGMD	Human Genome Mutation Database
HGVS	Human Genome Variation Society
HMWM	High molecular weight multimer
HPC	High performance computer
HTS	High-Throughput Sequencing (term used in preference to, but taken to be synonymous with 'Next Generation Sequencing')
iEC	human iPSC-derived endothelial cell
(h)iPSC	(Human) induced pluripotent stem cell
Indel	Insertion deletion variant ≤ 50 bp in length
ICD-10	International Statistical Classification of Diseases and Related Health Problems 10th Revision
ISTH	International Society on Thrombosis and Haemostasis
LMWM	Low molecular weight multimer
(L)PV	(Likely) pathogenic variant
KO	Knockout
1000GP	One Thousand Genomes Project
MACS	Magnetic cell separation
MAF	Minor allele frequency
MDT	Multidisciplinary team
NBR	National Institute of Health Research BioResource
NCBI	National Center for Biotechnology Information
Nt	Nucleotide
1000GP	1000 Genomes Project
OR	Odds ratio
ORF	Open reading frame
PAM	Proto-spacer-adjacent motif
PAVV	Putatively Aetiological VWD Variant
PBMCs	Peripheral blood mononuclear cells
pLoF	Predicted loss-of-function
PRP	Platelet rich plasma
PTC	Premature termination codon

(L)PV	(Likely) pathogenic variant (based on ACMG/AMP classification system)
RT	Room temperature
SDM	Site-directed mutagenesis
SF + Y	Complete SF media containing Y-27632 at a final concentration of 10 μM
sgRNA	Single guide RNA
SNV	Single nucleotide variant
TSS	Transcription start site
UKB	UK Biobank
UKB BAC	UKB Bleeding Assessment Cohort
(UL) HMWM	(Ultra large) high molecular weight multimer
UTR	Untranslated region
VCF	Variant Call File
VEP	Variant Effect Predictor (tool from Ensembl)
VUS	Variant of uncertain significance (based on ACMG/AMP classification system)
VWF	Von Willebrand factor
VWF:Act	Plasma von Willebrand factor activity
VWF:Ag	Plasma von Willebrand factor antigen
VWF:CB	Plasma von Willebrand factor collagen binding
VWF DBs	Shorthand for describing databases which contain variants in *VWF* in the context of VWD (ClinVar, HGMD, EAHAD-CFDB)
WES	Whole exome sequencing
WGS	Whole genome sequencing
WHO	World Health Organisation
WPB	Weibel-Palade body
WT	Wild type

Nomenclature

The terminology used to describe sequence changes in the human genome has historically been ambiguously imprecise (Quintáns et al., 2014). The terms ‘mutation’, ‘polymorphism’, and ‘variant’ mean different things to different authors, particularly in terms of their minor allele frequency (MAF) (Karki et al., 2015) and causation of disease (Ogino et al., 2007). There is also a perception that the term ‘mutation’ is pejorative (Sheidley, 2016) and the cessation of its use is recommended by clinical genetics guidelines (Richards et al., 2015). Therefore, throughout this thesis the term ‘variant’ will be used, defined as a difference, at a specific DNA position (or positions), in the human

germline genome, as compared to a reference genome. In terms of other genetic nomenclature, introns have the format 'XX-YY', where XX represents the number of the adjacent exon in the 5' direction, and YY the adjacent exon in the 3' direction. Harmonisation of names of genes and their products improves accessibility and intelligibility across the biomedical community (Fujiyoshi et al., 2021). Therefore, throughout this thesis I have used the approved gene and protein names from UniProt (Apweiler et al., 2004) outlining the full name in the first instance and then using the short form thereafter. To differentiate between gene and protein names, the former are italicised. When variants are described with reference to the protein sequence, names are provided using the Human Gene Variation Society terminology (den Dunnen et al., 2016), normally prefixed 'p.' only on first usage. In the context of protein position, amino acids (AAs) are referred to by their three letter abbreviation rather than single letters to improve readability. DNA, RNA, and protein sequence positions, for instance 'exon 50' or 'C-terminal cysteine knot', refer to *VWF* or VWF unless stated otherwise. Types 1, 2, and 3 von Willebrand disease (VWD) are abbreviated as VWD1, VWD2, and VWD3, respectively. Likewise subtypes 2A, 2B, 2M, and 2N are referred to as VWD2A, VWD2B, VWD2M, and VWD2N. For consistency, plasma VWF levels (abbreviated as just 'VWF level' hereafter) are provided in IU/ml including conversions from IU/dL to these units from published articles. Non-integers are rounded to 1 decimal place unless otherwise specified.

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1. Introduction

Haemostasis is the term used to describe the physiological cessation of bleeding at the site of vascular injury (Gale, 2011; Monroe, 2017). Von Willebrand factor (VWF) is a multi-domain secreted glycoprotein that plays a number of roles in this process, particularly as an important bridge between the disrupted vessel wall and platelets, but also by forming a complex with coagulation factor VIII (FVIII). Deficiency and/or domain-specific dysfunction of VWF results in von Willebrand disease, the most common inherited bleeding disorder. This thesis is focussed on rare DNA sequence variants in the *VWF* gene and whether they cause VWD (i.e. their pathogenicity), a problem I tackle using a combination of approaches including population genomics, electronic health records, and in vitro endothelial cells (ECs). Therefore in the first section I outline the basic biochemistry and functions of VWF and in the second section describe the clinical and laboratory manifestations of VWD, discuss its epidemiology, and the complex relationship between genotype and phenotype. I finish by describing the tools - large scale population datasets and cell models - which I used to generate my results.

1.1. Von Willebrand factor

1.1.1.The gene

Von Willebrand factor (VWF) was localised to the short arm of chromosome 12 nearly 40 years ago (Ginsburg et al., 1985; Verweij et al., 1985) (Figure 1.1A, page 17). It is 178kb long and contains 52 exons, of which exons 2 - 52 are protein coding. There is a non-transcribed pseudogene, on chromosome 22, which contains *VWF* exons 23-34 with 97% sequence homology (Mancuso et al., 1991).

1.1.2. Expression in endothelial cells, megakaryocytes, and platelets

The open reading frame (ORF) of *VWF* is 8439 nucleotides in length (Verweij et al., 1986). Transcription of this mRNA is confined to ECs and megakaryocytes (MKs) (Jaffe et al., 1973; Nachman et al., 1977). VWF is found in four main pools: blood plasma, sub-

Figure 1.1

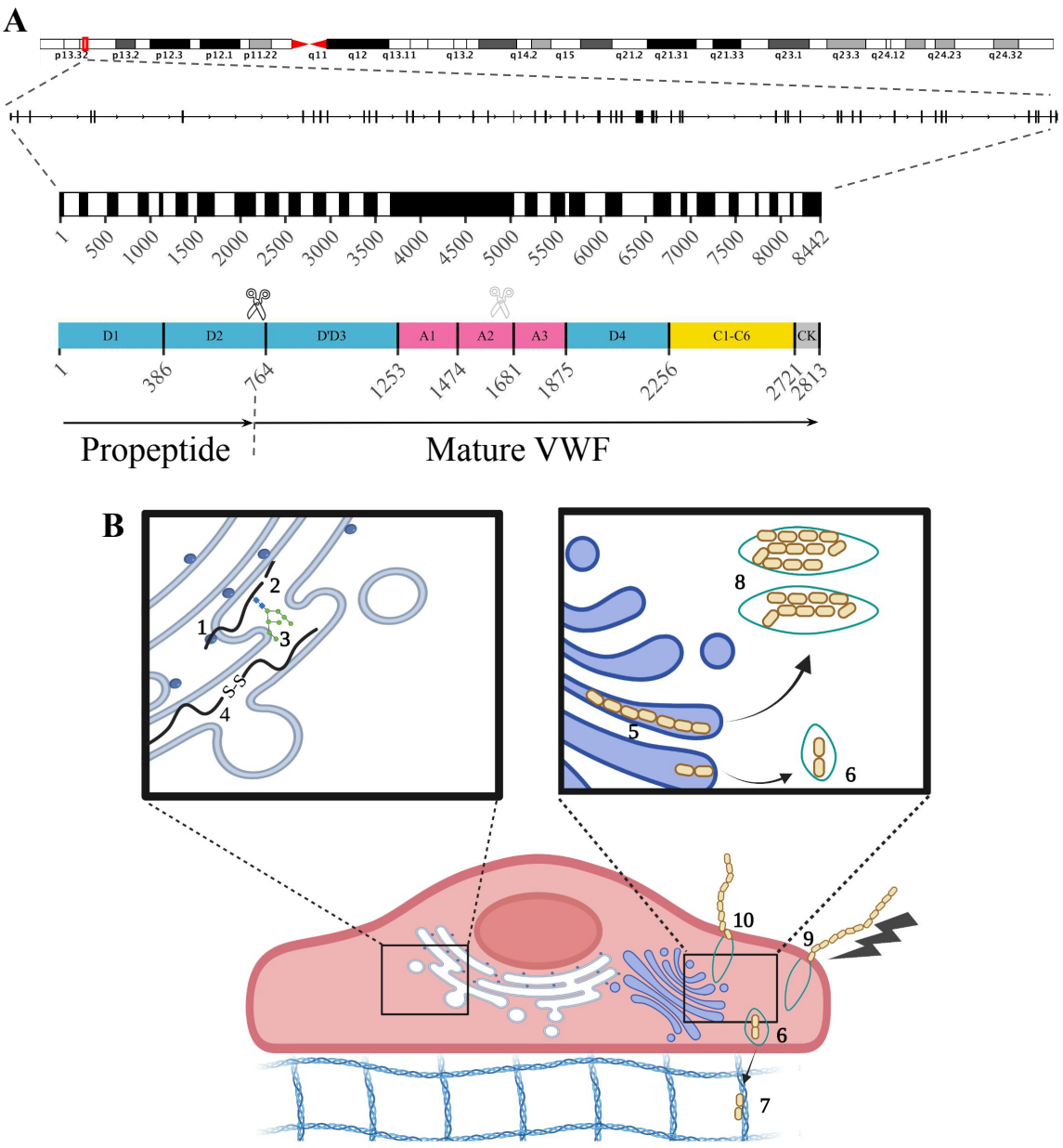


Figure 1.1 | Biosynthesis of VWF. (A) *VWF* is on chromosome 12p13.31. The three figures below the chromosome show, from top to bottom, the *VWF* gene, ORF, and protein sequence from 5' (left) to 3' (right). The position of the furin and ADAMTS13 cleavage sites are marked by a pair of black and grey scissors, respectively. (B) Cartoon showing the biosynthetic pathway of VWF in an EC sitting on an extracellular matrix, with the rough ER shown as hollow tubules dotted by ribosomes and the Golgi in purple. Key steps are numbered. 1: the VWF polypeptide chain entering the ER through the translocon. 2: the SP is cleaved off. 3: N-linked glycosylation. 4: C-terminal dimerisation. 5: VWF quanta (indicated by the brown lozenge shapes) coalesce in the Golgi. 6: LMWM VWF in anterograde vesicle for constitutive release. 7: VWF bound to collagen in the subendothelial matrix. 8: HMWM in WPBs. Regulated release of VWF from WPB in response to agonist (9) or basal release without an agonist (10). N.B. (B) was created with BioRender.

endothelial matrix, storage organelles of ECs (Weibel-Palade bodies [WPB] [Wagner et al., 1982]), and platelets and their precursor, the MK (stored in alpha granules [Zucker et al., 1979]). There are several key differences between EC- and platelet-derived VWF (EC-VWF and plt-VWF, respectively) which are functionally and diagnostically relevant to VWD.

First, although the same VWF polypeptide is synthesised in both ECs and MKs, this is then subjected to different post-translational modifications (PTMs) in each of the cell types (Ward et al. 2019). The most important of these differences is that the asparagine-linked glycan side chains of EC-VWF are capped with A or B glycoantigens whereas these are absent from plt-VWF (Brown et al., 2002; Matsui et al., 1999). This A and/or B capping is determined by an individual's *ABO* histogroup genotype (Matsui et al., 1992; Yamamoto et al., 1990) (Subsection 1.1.8, pages 25 - 26), which is the strongest determinant of plasma VWF levels in the general population (McCallum et al., 1983; Mohanty et al., 1984; Orstavik et al., 1985; Stormorken & Erikssen, 1977) (Subsection 1.2.4, page 51).

Second, ECs and platelets do not contribute equally to plasma VWF (McGrath et al., 2010). The majority of this is derived from ECs, with estimates of their contribution ranging from 85 - 90% depending on the model system used (Dhanesha et al, 2016; Nichols et al., 1995); furthermore, this still may be an underestimate of the proportion of plasma VWF derived from ECs. This is because plt-VWF in plasma may be an artefact of the in vitro activation of platelets during (Burns et al., 1984) or subsequent to (Kennedy et al., 1997) venesection, with the resultant release of plt-VWF into the blood tube prior to plasma quantitation (Kanaji et al., 2012). Several pro-haemostatic proteins, such as coagulation factor V and fibrinogen are actively endocytosed from plasma into alpha granules at either the MK and/or platelet stage of maturation (Bouchard et al., 2005; Handagama et al., 1989; Harrison et al., 1989; Suehiro et al., 2005). No such mechanism exists for VWF, meaning that EC- and plt-VWF are physically discrete pools.

Third, although stimulated release of VWF from platelets results in its high local concentration at sites of platelet aggregation (Fernandez et al., 1982) (Subsection 1.1.9,

page 28), this locally-restricted pool alone is, by itself, insufficient to maintain haemostasis (Blair & Flaumenhaft, 2009). This is in contrast to EC-VWF. For example, mice deficient in plt-VWF but replete in EC-VWF had no difference in the tail bleeding time (BT) when compared to wild type (WT) controls, but in contrast the BT was significantly prolonged in EC-VWF deficient, but plt-VWF replete, mice (Dhanesha et al., 2016), with similar observations made in pigs (Nichols et al., 1995).

Fourth, there is a difference in the extent to which plt- and EC-VWF bind to other haemostatic ligands. As outlined in Subsection 1.1.9 (page 28), the first platelet receptor to which VWF attaches to when it is in its active state (Figure 1.1B, page 17) is glycoprotein Ib alpha chain (GP-Ibα). National Institute of Health researchers demonstrated that plasma VWF binds 4-5 times more avidly to GP-Ibα as compared with plt-VWF (Williams et al., 1994). In plasma, VWF and FVIII are tightly complexed, which protects the latter from proteolytic degradation (Foster et al., 1987; Koedam et al., 1988) (Subsection 1.1.9, pages 26 - 28). Murine models have shown that plt-VWF can bind FVIII if the latter is ectopically expressed in MKs (Yarovoi et al., 2003). However, mice which expressed plt-VWF only were found to have a plasma FVIII concentration (FVIII:C) which was 20% of that measured in WT controls (Kanaji et al., 2012). In contrast, the mice which expressed EC-VWF only had FVIII:C at 93% of WT levels. Although the physiological relevance of this to humans has not been determined, these findings suggest that under basal conditions EC-VWF is more important than plt-VWF in determining plasma FVIII levels. This is also the case in response to agonists (a process called regulated secretion [Subsections 1.1.6 and 1.1.9, pages 24 & 28]). Both VWF and FVIII are co-released from ECs in response to histamine (Turner and Moake, 2015). The synthetic agonist, DDAVP, also triggers VWF release from ECs (Hashemi et al. 1990). Although not directly proven, the co-release of both VWF and FVIII from ECs (from specific vascular beds) is thought to be the explanation for the rapid rise in plasma concentrations of both proteins in response to DDAVP administration (Haberichter et al., 2006), meaning that it is therapeutically useful for mild forms of both VWD and haemophilia A, conditions in which there is reduced, but not absent plasma VWF and FVIII:C, respectively (Mannucci et al., 1977) (Subsection 1.2.1, page 33). In contrast, although the effects of DDAVP on

platelet activation are contested (Persyn et al., 2022), it has not been shown to stimulate release of plt-VWF (Balduini et al., 1999) and cannot do so for FVIII as it is not contained within platelets (Shi and Montgomery, 2010).

Fifth, there are distinctions between plt- and EC-VWF in how they are multimerised, stored, and released. Multimerisation refers to the end-to-end binding of VWF dimers into higher order chains that can include > 40 individual monomer units (Stockschlaeder et al., 2014) (Subsection 1.1.6, pages 23-24). A range of research groups have consistently shown that platelets contain a greater proportion of higher molecular weight VWF multimers as compared with ECs (Gralnick et al., 1985; Rodeghiero et al., 1990; Ruggeri and Zimmerman, 1980). Postulated mechanisms to explain this include the more extensive interdimer disulphide bond formation of VWF in MKs (Sporn and Marder, 1986; Williams et al., 1994) and less extensive proteolysis by ADAMTS13 (Dent et al., 1991) (Subsection 1.1.7, pages 24-25). Although there are ultrastructural similarities between alpha granules and WPBs (Cramer et al., 1985) there are several differences between the two organelles. The synthesis of WPBs is VWF-dependent, the characteristic rod shape (Weibel and Palade, 1964) being driven by concertinaed tubules of VWF (Metcalf et al., 2008; Wagner et al., 1982). In the absence of VWF expression, rod-shaped WPBs are not formed at all (Schillemans et al., 2019), as supported by the WPB-membrane protein, P-selectin, routing to alternative, lysosomal vesicles (Denis et al., 2001) in VWF knockout (KO) mice. In contrast, alpha granules form normally in the absence of VWF (Nurden et al., 2009; Swinkels et al., 2021). Furthermore, alpha granules only exocytose their cargo, including VWF, in response to agonist stimulation (Flaumenhaft et al., 2005; Heijnen & Van Der Sluijs, 2015) with no reports of basal, continuous, secretion. This is in contrast to WPBs where both mechanisms are active, with basal secretion the predominant source of plasma VWF under resting conditions (Giblin et al., 2008; Lopes da Silva & Cutler, 2016) (Subsection 1.1.6, page 24).

In summary, ECs are the predominant source of plasma VWF (VWF antigen [VWF:Ag] (Subsection 1.2.2, pages 35 - 36)). Plasma VWF is the pool critical for haemostasis, mediated by its high platelet-binding activity (via GP-Ibα [quantified in clinical

laboratories as VWF:Act - Subsection 1.2.2, pages 36 - 37]) and role as the protective chaperone of circulating FVIII. As detailed in Subsection 1.2.2 (pages 39 - 40), VWD is defined by reduction in plasma VWF:Ag and/or VWF:Act. Furthermore it is EC-VWF which is therapeutically useful in treating VWD as evidenced by the use of DDAVP.

Therefore, ECs are the source of VWF which is most clinically relevant to VWD. Furthermore the differences in post-translational biochemistry, intracellular handling, and exocytosis of EC- as compared to plt-VWF means that it cannot be assumed that potentially causal VWD variants in *VWF* will have equal effects on both EC- and plt-VWF. For both of these reasons, I have focussed the remainder of this thesis on EC-VWF. In particular, Chapter 5 is focussed on the development of a new approach to modelling VWD in ECs. However, I acknowledge that the specific contribution of plt-VWF to the pathogenesis of VWD, and how this may be affected by variants in *VWF*, are topics that are understudied and require further exploration (Mannucci, 1995; Fogarty et al., 2020), but are outside the scope of this thesis.

The endothelium comprises the thin layer of cells that provides the interface between the blood or lymph running through the vessels it lines with the parenchymal cells of the adjacent organ (Aird, 2012; Karkkainen et al., 2002). The embryonic origin of vascular ECs is the middle germ cell layer, the mesoderm, with which they share a common precursor with haematopoietic cells (Choi et al., 1998). Three of the proteins commonly used to define EC identity also reveal insights into the roles of the endothelium. Cadherin-5 (CDH5), better known as vascular endothelial (VE) cadherin, forms tight junctions between ECs (Aird, 2007). Tight junctions are one of the components that influence the differential permeability of the endothelium, in a tissue-dependent context (Corada et al., 1999) and the response to physiological stimuli. Platelet endothelial cell adhesion molecule (PECAM1) is one of the surface ligands that plays a role in transmigration of leucocytes from the blood into underlying tissues (Springer, 1994). And finally, VWF participates both directly and indirectly in haemostasis as outlined in Subsection 1.1.9 (pages 26-29).

1.1.3. Initial steps in VWF synthesis

Full length, pre-pro-VWF comprises 2813 amino acids (AAs), and residue numbering and variants that affect the protein sequence of VWF are reported using this full sequence (although this has not always been the case [Goodeve, 2010]). The first 22 AAs of pre-pro-VWF comprise the signal peptide (SP). Its role in trafficking of VWF has not been specifically studied. However, because SPs are ubiquitous on proteins destined for cellular secretion via the Sec61 secretory pathway, it has been widely inferred (Denis, 2002; Luken, 2008; Ruggeri, 1997) that this directs the nascent VWF polypeptide-ribosome complex to the Sec61 translocon, on the surface of the endoplasmic reticulum (ER). It is through the translocon that the translated polypeptide chain then emerges directly into the ER lumen (Lumangtad & Bell, 2020), and the SP is then cleaved (Sadler, 1998) (Figure 1.1B, page 17). This leaves pro-VWF, so named because the propeptide remains attached. The propeptide comprises the N-terminal 741 AAs (Figure 1.1A). About 8% of AAs in pro-VWF are cysteine residues, which have an -SH (thiol) group. Covalent disulfide bonds are formed between thiol groups on other cysteine residues in the same polypeptide chain, important for correct protein folding and stability that facilitates the progression of VWF through its biosynthetic pathway (Mamathambika & Bardwell, 2008; Marti et al., 1987).

The domain structure of the VWF polypeptide is shown in Figure 1.1A (page 17). A, C, and D domains are not exact repeats of each other but share sequence homology (Springer, 2014). Detailed descriptions of the domains most relevant to VWD are provided later in the introduction, however I provide a brief overview here. The D1 and D2 domains in the propeptide facilitate multimer assembly in the Golgi. A1 contains the binding site for the platelet receptor, platelet glycoprotein Ib alpha chain (GP-Ibα). Collagen binding is also mediated by A1 but also A3. Sandwiched between A1 and A3, the A2 domain contains the proteolytic cleavage site for the enzyme named ‘a disintegrin and metalloproteinase with thrombospondin type-1 repeats-13’ (ADAMTS13). C1-C6 form the stalk of the ‘bouquet’ formed by VWF dimers and contribute to the great length of VWF once it is multimerised (Springer, 2014). The C-terminal cysteine knot (CK) contains key cysteine residues that are needed for VWF dimerisation.

1.1.4. N-glycosylation

Pro-VWF moves through sequential stages of post-translational modification that start within the ER. The first step in this process is called N-glycosylation. A 14-sugar oligosaccharide, $Glc_3Man_9GlcNAc_2$ is co-translationally (Preston et al., 2013) added to the asparagine (N) residues (Figure 1.1B, page 17) which are positioned at the start of NXS or NXT triplet sequences, where X corresponds to any amino acid except proline, S is serine, and T is threonine (Brehm, 2017). The N-linked oligosaccharides undergo further modifications in the ER as VWF progresses through its biosynthetic pathway. Once N-glycosylated, pro-VWF then folds, a process involving enzymes including protein disulfide-isomerase A3 (PDIA3) (Allen et al., 2001). Bound PDIA3 catalyses intramonomer disulfide bridging (Ellgaard & Frickel, 2003) between the majority of thiol residues of pro-VWF with the exception of some cysteines in the N- and C- termini reserved for intermolecular bonds.

1.1.5. C-terminal dimerisation

Still in the ER, the folded pro-VWF monomer undergoes dimerisation (Figure 1.1B, page 17). This occurs 'tail-to-tail' (Sadler, 1998) secondary to disulfide bonding between three cysteine residues (Cys2771-Cys2773', Cys2771'-Cys2273, Cys2811-Cys2811' where ' denotes the second monomer in the dimeric pair (Katsumi et al., 2000; Zhou & Springer, 2014). These residues are all in the CK domain containing 93 AAs at the C-terminus of pro-VWF (McDonald & Hendrickson, 1993). This domain has sequence and functional homology shared with other extracellular proteins, including mucins (Zhou & Springer, 2014). Protein disulfide-isomerase (PDI) binds to CK in its N-terminal portion prior to C2771 (Lippok et al., 2016) and catalyses the disulfide bonding between the cysteine residues mentioned above.

1.1.6. Multimerisation and exocytosis of WPBs

The pro-VWF dimers are then allowed to move to the cis-Golgi along anterograde pathway (Nightingale & Cutler, 2013). Additional disulfide bonds form between the N-terminal D'D3 domains of mature VWF (Vischer & Wagner, 1994) and Furin cleaves the propeptide from mature VWF at AA residue 763 (Figure 1.1A, page 17), but the

propeptide remains noncovalently associated until VWF exits the cell, providing an essential stabilising function (Wise et al., 1988) in the next step of VWF processing, the sequential concatemerisation and compaction of VWF into tightly packed tubules that double back on themselves multiple times (Springer, 2014). These higher-order concatemers beyond dimers are referred to as multimers. They vary in size between 0.5 and 20 MDa (Sadler, 1998), with each nascent unit of VWF called a quantum (Ferraro et al., 2014) (Figure 1.1B, page 17).

Having progressed through the Golgi, VWF is at a waypoint: some buds off into anterograde carriers containing low molecular weight multimers (LMWMs) (Lopes da Silva & Cutler, 2016) (Figure 1.1B, page 17). This is directly to the basolateral membrane where it is incorporated into the subendothelial matrix. Higher molecular weight multimer (HMWM) VWF instead merges as it traverses the Golgi and ultimately buds off into membrane-bound WPBs (Schillemans et al., 2019; Zenner et al., 2007). VWF in WPB is then either released from the cell in a basal or regulated manner, depending on whether it is in the absence of an agonist (such as thrombin) or triggered by one. The unloading of VWF from WPBs at the apical membrane into the vascular lumen is called exocytosis and is orchestrated by at least 30 different proteins (McCormack et al., 2017) including Guanosine-5'-triphosphate (GTP)-ases (Nightingale et al., 2009) and soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes (Fu et al., 2005). The VWF released through the basal pathway contains both LMWMs and HMWMs whereas the regulated release results in the latter only, which have greater haemostatic capability (as discussed in Subsection 1.1.9).

1.1.7. ADAMTS13 regulates multimer size

Plasma VWF is cleaved by ADAMTS13, a protease that cleaves the bond between the Tyr1605 and Met1606 residues in the A2 domain (Figure 1.1A, page 17) resulting in digestion of HMWMs into smaller fragments (Fujikawa et al., 2001; Furlan et al., 1996; Gerritsen et al., 2001; Tsai, 1996; Zheng et al., 2001). ADAMTS13 performs an essential function as exemplified by thrombotic thrombocytopenic purpura (TTP), a condition in which there is severe deficiency of this factor. In TTP, ultra large HMWMs occlude the

arterioles of the brain, heart, and kidney (Sadler, 2017) causing almost universal death if untreated (Sarode et al., 2014). Proteolytic fragmentation of VWF by ADAMTS13 is not thought to hasten its clearance from the circulation (Badirou et al., 2010).

1.1.8. The ABO histo-group is a determinant of plasma VWF clearance

The *ABO* gene encodes a glycosyltransferase enzyme (Yamamoto et al., 1990). There are three main alleles: *A, B,* and *O* (Bernstein, 1924). The first two result in functional transferases, while the latter is silent. *A* and *B* are co-dominant and *O* is recessive (Blancher, 2013). Therefore, phenotypically, the human population can be divided into four categories - O, A, B, and AB - that occur in the UK blood donor population at a frequency of 49%, 38%, 10%, and 3% respectively (NHS Blood and Transplant, 2018). Although ABO was first discovered in the context of red blood cell transfusion (Landsteiner, 1901), its role extends beyond erythrocytes to all tissues that contain epithelial and endothelial cells (Holborow et al., 1960), hence the preference for the term ABO histo-group as opposed to ABO blood group (Clausen & Hakomori, 1989). This is because the ABH antigen - the saccharide substrate modified by the histo-blood group ABO system transferase - is found attached to a wide range of glycoproteins and glycolipids (Szulman, 1962). This includes ~ 16% of the N-glycan termini of VWF (Matsui et al., 1992) (Subsection 1.1.4, page 23). Both A and B transferases add a monosaccharide to ABH: A adds N-acetylgalactosamine (Hearn et al., 1968) and B adds galactose (Kobata et al., 1968). There is indirect evidence that, for VWF, this transferase step occurs within ECs as opposed to post-exocytosis (Matsui et al., 1999). As discussed later in Subsection 1.2.4 (pages 51-52), *ABO* is an important determinant of plasma VWF levels: *OO* individuals (unmodified VWF) have the lowest plasma VWF:Ag (Shima et al., 1995) which has implications for VWD diagnosis. Furthermore, the ABH backbone itself is important: individuals with the Bombay phenotype who do not synthesise ABH at all were found to have a median VWF:Ag that was lower than those who were O group (O'Donnell et al., 2005).

So what is behind the ABO effect? The predominant theory is that it pertains to the rate of clearance of plasma VWF. O and non-O group volunteers were injected with DDAVP, an

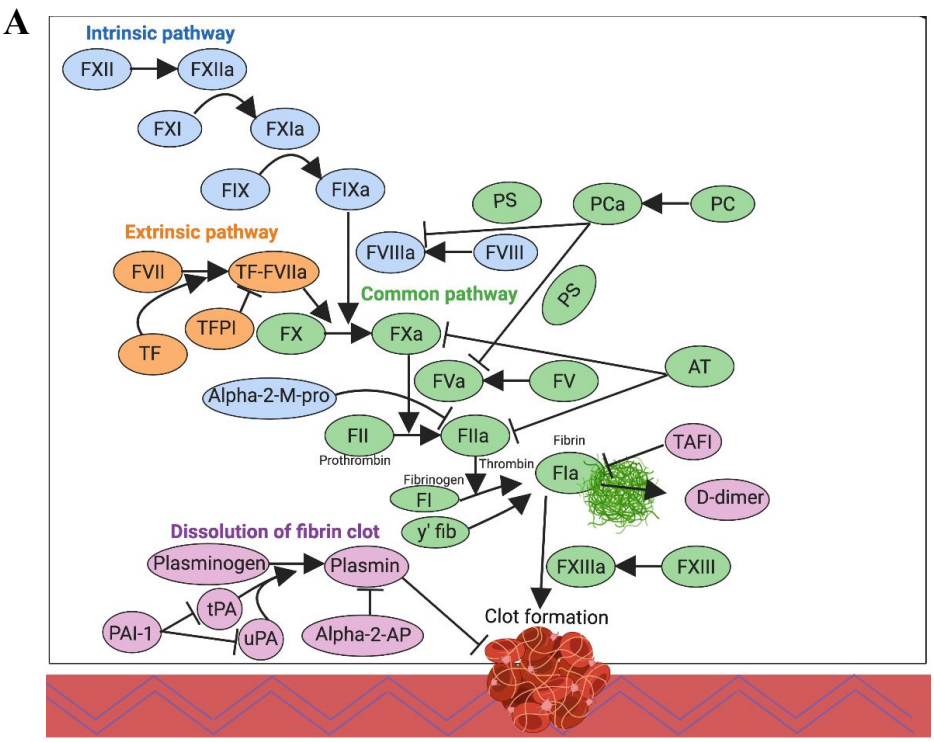
agonist that stimulates the release of VWF from WPBs (Gallinaro et al., 2008). The half-life of VWF was 10 hours in the O group compared to 25 hours in non-O individuals. The prevailing explanation for this is that type O VWF is more avidly taken up by receptors on cells of the liver (Ward et al., 2020). As discussed in Subsection 1.2.4 (pages 52-53), this is supported by the fact that several protein quantitative trait loci for plasma VWF levels encode for these receptors (Smith et al., 2010). However it is likely that ABO is also having a 'supply-side' effect. Thirty five individuals had their post-mortem pulmonary arterioles stained for VWF. The lowest intensity of staining was in O group patients (Murray et al., 2020), suggesting an effect of the ABO glycosyltransferase on intracellular handling of VWF.

1.1.9. VWF is a multi-functional actor in haemostasis

Haemostasis is canonically classified into primary and secondary phases (Clemetson, 2012; Gale, 2011). The former refers to the creation of a platelet plug on a damaged vessel wall. The latter is the series of enzymatic steps called the coagulation cascade (Figure 1.2A, page 27), which generates insoluble, cross-linked fibrin (from soluble fibrinogen) that enmeshes the platelet plug in order to form a stable blood clot. In reality, the two pathways are interlinked. For example, platelet polyphosphate released by degranulating platelets triggers secondary haemostasis through activation of factor (F) XII (Verhoef et al., 2017). When considering VWF's role in haemostasis, the primary versus secondary distinction is helpful. The purview of VWF with respect to the latter is limited so this is discussed first.

Activated FVIII and FIX together form the tenase complex that are critical in delivery of the 'thrombin burst' that stabilises the initial fibrin clot (van Geffen & van Heerde, 2012). However, free FVIII has a half life of only 2 hours (Pipe et al., 2016). VWF binds non-covalently to FVIII via the N-terminal portion of its D'D3 domain (Foster et al., 1987). This slows the rate of inactivation of FVIII by the protease, activated protein C (Koedam et al., 1988). Furthermore FVIII in complex with VWF is less readily taken up by the ubiquitous endocytic receptor, LRP1 (Lenting et al., 1999). Therefore VWF contributes indirectly to secondary haemostasis by helping to maintain physiological levels

Figure 1.2



B

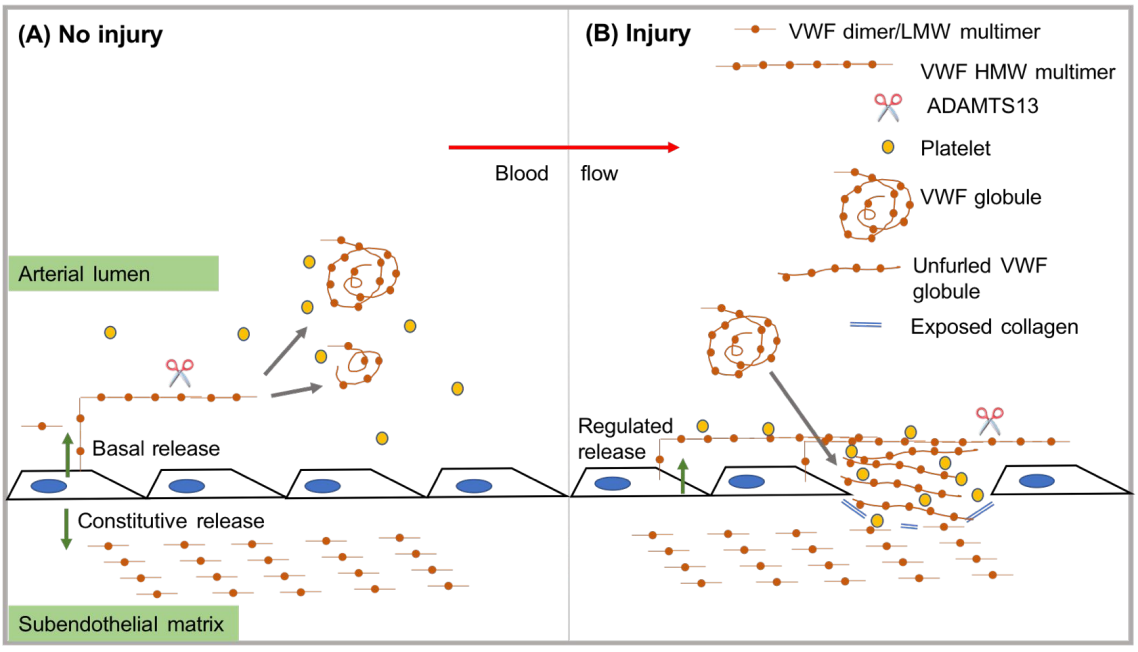


Figure 1.2 | Haemostasis. (A) The coagulation cascade. Adapted from Harshfield et al., 2020. The cofactor function of FVIIIa is shown. (B) A cartoon representing the different states of VWF (coiled and inactive on the left and stretched out and activated on the right) due to vessel wall injury and increased shear stress.

of FVIII. This function of VWF occurs in its quiescent, globular form (Bonazza et al., 2015 (Figure 1.2B, page 27).

However, in order to expose its multiple adhesive binding domains, VWF needs to be unfurled. This is limited to vessels with high shear stress, i.e. arteries and arterioles (Sakariassen et al., 1979, Ni et al., 2000). When the endothelium is disrupted by injury (Figure 1.2B, page 27), endogenous agonists stimulate the release of VWF via the regulated pathway, predominantly as HMWMs. These coalesce into strings up to 3mm long (Dong et al., 2002) that orient to the direction of flow. Some of these are capable of binding platelets, as the A1 domain becomes exposed and can bind GP-Ibα (Fujimura et al., 1986; Hoylaerts, 1997). The strings can associate laterally with each other to form bundles and are transiently anchored to the EC surface by P-selectin and the integrin $\alpha_V\beta_3$ (Huang et al., 2009; Padilla et al., 2004). These strings are then cleaved within minutes by ADAMTS13 (Dong et al., 2002; Padilla et al., 2004). In addition, vessel injury itself exposes subendothelial collagen. VWF binds to this through its A1 and A3 domains (Cruz et al. 1995; Flood et al. 2015; Pareti et al., 1987). This promotes the unfurling of the globular structure of VWF, thereby exposing the A1 domain, which binds GP-Ibα that arrests platelets on the VWF surface (De Ceunynck et al., 2013). This results in the aggregation and activation of platelets and facilitates the triggering of secondary haemostasis through delivery of FVIII to the platelet plug (Peyvandi et al., 2011). As a result of their activation, platelets release the cargo contained within alpha granules, which includes VWF, into the adjacent interstitial space (McGrath et al., 2013). This amplifies further platelet adhesion and activation (Williams et al.,1994).

Plasma VWF has two further roles in primary haemostasis: binding to GPIIb-IIIa and self-association. Platelet activation stimulates ‘inside-out’ signalling that induces conformational change to the integrin GPIIb-IIIa receptor, which is then permissive to ligand-binding (Shattil et al., 1985). Although in competition with fibrinogen, the principal GPIIb-IIIa ligand (Marguerie et al., 1984), VWF does bind to this receptor through its Arg-Gly-Asp integrin binding motif in the VWC4 domain (Haverstick et al., 1985; Plow et al., 1985; Ruggeri et al., 1983; Sadler et al., 1985) (Figure 1.1, page 17) which facilitates

platelet spreading (Weiss et al., 1993). Self-association is the process by which VWF, arrested at the site of vascular injury, binds to other circulating VWF (Savage et al., 2002). Initially, self-association was thought to be mediated by multiple domains, more recent work suggests that the A2 domain is the main regulator of this process (Zhang et al., 2019).

Given VWF's multifaceted roles in haemostasis it is not surprising that alterations to its behaviour, function, and/or quantity can result in thrombosis, or at the opposite end of the spectrum, bleeding. As plasma VWF level rises with the acute phase response (Pottinger et al., 1989), it had been initially difficult to resolve the cause-versus-effect conundrum posed by data showing that patients with myocardial infarction, stroke, and deep vein thrombosis have higher VWF levels (Bombeli et al., 2002; Chion et al., 2007; Folsom et al., 1999). Recent studies using Mendelian Randomisation, a statistical approach to disentangle cause and effect, have suggested a causal relationship between VWF and cardio- and cerebro-vascular disease (Harshfield et al., 2020; Zhao & Schooling, 2018). My thesis is focussed on the quantitative deficiency and/or qualitative dysfunction in VWF that is linked to bleeding in the inherited disorder, von Willebrand disease (VWD).

1.2. Von Willebrand disease

1.2.1. Bleeding assessment and treatment

In 1926, Erik von Willebrand, a Finnish clinician scientist travelled to the Scandinavian Åland archipelago. His trip was prompted by a clinical observation. A five year old girl, Hjördis, had been brought to von Willebrand's clinic in Helsinki by her parents. She had uncontrollable epistaxis and had been rendered semi-conscious following difficult-to-control haemorrhage after a cut lip (Berntop, 2007). Hjördis and her parents were Åland islanders living on Föglö (Nilsson, 1999). So von Willebrand followed them there to investigate further; he painstakingly documented the bleeding history of Hjördis' extended pedigree, including 58 individuals some of which are shown in Figure 1.3A (page 30). This led him to three main conclusions which still apply today. First, the bleeding disorder he had discovered affected both males and females. Second, the bleeding

Figure 1.3

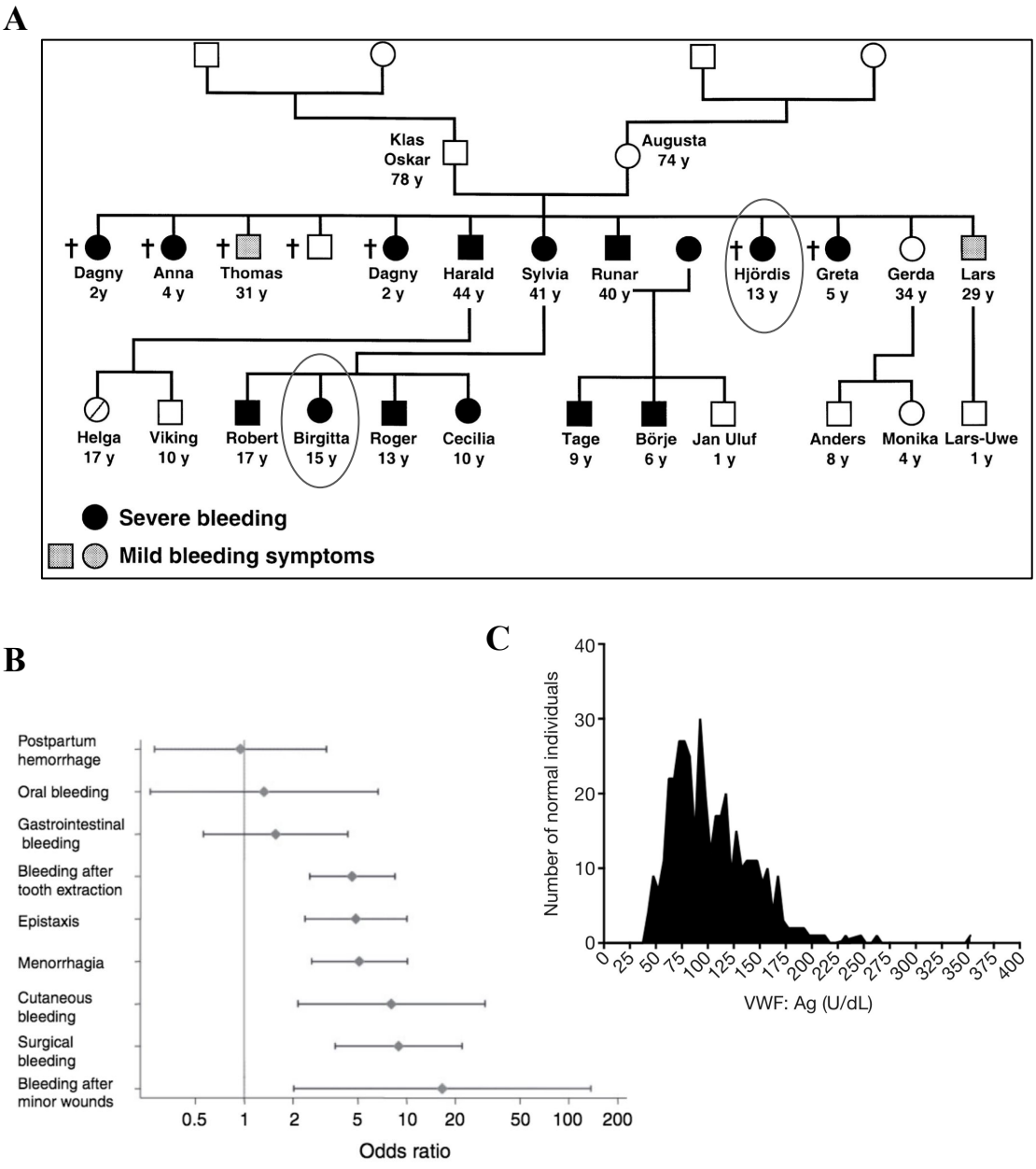


Figure 1.3 | Bleeding and VWF levels with respect to VWD. (A) Family tree of the Föglö family reproduced from Nilsson, 1999. Circled is Hjördis, the propositus in whom von Willebrand first described pseudohaemophilia, now known as VWD. Birgitta, also circled, was the first patient treated with a plasma concentrate. B) The association between specific bleeding symptoms and the likelihood of being diagnosed with type 1 VWD, reproduced from Tosetto et al., 2006. Diamonds indicate OR estimate and bars 95% confidence interval. (C) Histogram of the VWF:Ag results of "nearly 400 individuals", reproduced from Favaloro and Lippi, 2017.

symptoms of affected individuals were predominantly from the nose, mouth, and in affected women during menstruation. This pattern of bleeding became known as ‘mucocutaneous’ (Duemling & Norman, 1961) and supports the notion that VWF has a more important role in primary as compared to secondary haemostasis (Subsection 1.1.9, pages 26 - 29). Moreover, haemarthrosis and haematomas were rare (Nilsson, 1999). Third, following on from the first two points, it was clear that the affliction affecting the Föglö pedigree was distinct from haemophilia, an entity which by von Willebrand’s time had already been firmly identified as a rare bleeding diathesis affecting boys in which severe joint bleeding was characteristic (Ingram, 1976). Henceforth von Willebrand named the disorder affecting Hjördis and her kindred as ‘pseudohemophilia’ (von Willebrand, 1926).

Bleeding attributed to VWD has been reported in the majority of organs, including the digestive (Makris, 2006), integumentary (Sidonio Jr et al., 2012), lymphatic (Jones et al., 2021), muscular (Sood et al., 2013), nervous (Lak et al., 2020), reproductive (Ragni et al., 1999), respiratory (Nagarajan et al., 2021), and skeletal (van Galen et al., 2015) systems. What has become apparent in the century since von Willebrand first described the disorder is that there is heterogeneity in the bleeding phenotype of patients with VWD (Levy & Ginsburg, 2001), even within specific subtypes (Ziv & Ragni, 2004), and between family members with the same VWD variant (Ginsburg, 2005).

Therefore, in an attempt to objectively assess bleeding symptoms, an international consortium devised a semi-quantitative, ‘Bleeding Assessment Tool’ (BAT) score and applied this to individuals with type 1 VWD (VWD1). This condition is defined by a reduction, but not complete absence, of VWF (Rodeghiero et al., 2005) (Table 1.2, page 40). An ordinal score comprising the levels - zero, one, two, and three - was applied to 10 different bleeding symptoms, with higher scores reflecting a greater severity of bleeding. Over the subsequent five years, further refinements were made to the original scoring system, including condensation of the number of questions asked (Bowman et al., 2008), and inclusion of the negative integer, -1, to represent events where there was adequate haemostasis when the individual was challenged with surgery or another procedure

(Tosetto et al., 2006). In an effort to standardise the approach to bleeding assessment and widen the applicability to bleeding disorders beyond VWD, a consensus, 'International Society on Thrombosis and Haemostasis (ISTH)-BAT' was created (Rodeghiero et al., 2010), a copy of which is in Appendix 7.1 (pages 241-243).

Based on the data collected in studies using BATs, the following conclusions can be drawn regarding the profile of bleeding symptoms in VWD:

1. Bleeding levels are variable. They are most severe in type 3 VWD (VWD3), in which individuals have absent, or minimally detectable VWF levels (Table 1.2, page 40). Bleeding is least severe in VWD1, with type 2 VWD (VWD2) occupying the middle ground (Bowman et al., 2008) (VWD2 is a qualitative dysfunction of VWF [Table 1.2, page 40]).
2. Procedural and mucocutaneous bleeding symptoms increase the likelihood of being diagnosed with VWD1 (Tosetto et al., 2006) (Figure 1.3B, page 30).
3. There is uncertainty about the positive predictive value of muscle haematomas and central nervous system (CNS) bleeding for VWD because of the rarity of these events in both VWD and control populations (Spradbrow et al., 2020).
4. The presence of three different haemorrhagic symptoms occurs in 50% of individuals with VWD1 but less than 1% of the general population (Rodeghiero et al., 2005; Tosetto et al., 2008).
5. Gastrointestinal bleeding is over-represented in VWD2 - specifically related to those subtypes in which there is a deficiency of HMWMs (Fressinaud & Meyer, 1993) (Table 1.2, page 40).

BATs have been particularly useful in reducing the need for laboratory testing in those with a low pretest probability of VWD (Fasulo et al., 2018). However, they have limitations including recall bias, a lack of adjustment for age-related accumulation of bleeding events, and the small selective nature of the control populations used. This is further outlined in the background to Chapter 4 (Section 4.1, pages 138-139).

Medications are given to individuals for the specific treatment of VWD in three contexts. First, acute treatment to arrest haemorrhage. Second, prior to a haemostatic challenge with common examples including childbirth, surgery, or a dental procedure. Third, long-term prophylaxis to reduce "anticipated bleeding" (Miesbach & Berntorp, 2021). The medications used include tranexamic acid (TXA), desmopressin (DDAVP), and concentrates containing VWF.

TXA retards fibrinolysis (Hoylaerts et al., 1981), the pathway which dissolves a fibrin clot (Figure 1.2A, page 27). It is widely used for the treatment of VWD (Lavin & O'Donnell, 2016), but recommendations regarding its use are based principally on expert opinion (Pasi et al., 2004), and evidence translated from TXA's use in haemophilia (e.g. Forbes et al., 1972), with no well-designed clinical trials on the use of TXA in VWD itself (van Galen et al., 2019).

DDAVP mediates release of VWF via interaction with vasopressin receptor 2 on the surface of some vascular EC subtypes including the lung microvasculature (Kaufmann & Vischer, 2003). Exposure of ECs to DDAVP leads to rapid induction of WPB exocytosis (Kaufmann et al. 2000) via the regulated pathway of release (Figure 1.1B, page 17), thus causing VWF levels to rise. The most common use of DDAVP is in patients with mildly reduced VWF levels (VWD1) (Table 1.2, page 40) prior to an operation or procedure. Depending on whether an intranasal, subcutaneous, or intravenous route is used, DDAVP is typically administered 30 - 120 minutes before an intervention (Mannucci et al., 1997).

Concentrates for intravenous administration containing VWF (with or without FVIII) are reserved for situations including procedures, surgery or acute bleeding, in individuals in whom DDAVP will be or has been demonstrated to be ineffective, is contraindicated, or where it is deemed that it will provide insufficient cover (Windyga et al., 2016b). Until the mid 2010s, VWF concentrates were plasma derived (pd). Recombinant (r)VWF (vonicog alfa) has been available for use in the UK since 2020 (NHS England, 2020). However, whethether pd- or rVWF is chosen, the principles of use remain the same. Concentrates are typically administered within an hour prior to surgery, or at the time of a bleed, and

subsequent doses are given daily for two to three days afterwards, until haemostasis is secure (Castaman, 2020). In rare circumstances pdVWF concentrates may also be used for prophylaxis to reduce the risk of future bleeds and prevent complications such as joint arthropathy secondary to recurrent haemarthroses. No published data is currently available for the use of rVWF as prophylaxis so it is not currently licensed for this purpose. In resource-limited settings, VWF concentrates may not be available, meaning that cryoprecipitate may be the only alternative (Dorgalaleh et al., 2018; Mannucci, 2019; Nascimento et al., 2014; Sahoo et al., 2020). Cryoprecipitate is obtained from slowly thawing fresh frozen plasma at 1-6ºC, which results in the precipitation of cold-insoluble (cryoproteins) rich in VWF and FVIII which explains why it was the mainstay of VWD treatment until the advent of concentrate therapy (Bennett & Dormandy, 1966; Yang et al., 2012).

1.2.2. Laboratory diagnosis: easy as 1,2,3?

It took 30 years before it was confirmed that VWD resulted from the deficiency of a plasma clotting factor in 1956. Fittingly, it is also an Åland island story. Hjördis' niece, Birgitta (Figure 1.3, page 30), was also suspected of having VWD because of uncontrolled uterine bleeding. By that time an early assay for FVIII was available, at the time referred to as antihemophilic globulin (AHG). Birgitta had an AHG result of 1-5% (reference range of the normal group was 60-160%). When Birgitta was infused with a precipitant of normal plasma, fraction I-0 (Blombäck & Blombäck, 1956), known to contain AHG, this corrected her AHG plasma level to 55% and controlled her bleeding. However, more remarkably the infusion normalised Birgitta's bleeding time (BT), which had been > 60 minutes prior to its administration. The BT is a measurement of the time taken for a small skin cut to stop bleeding when blotted with absorbent paper every 30 seconds, and is a method for measuring primary haemostasis. The BT was known to be prolonged in individuals with thrombocytopenia but normal in haemophilia (Duke, 1910). Hence, the observation in Birgitta was crucial: the BT correction couldn't have been from AHG because of the known fact that patients with haemophilia have a normal BT. From this, two lessons were learnt which have relevance for the modern laboratory diagnosis of VWD. First, that there is a plasma factor, separate to FVIII, that is missing in VWD, this would

later be discovered and named as VWF. Secondly, FVIII is also often low in patients with (more severe forms of) VWD because of the tight complex which they form in plasma (Subsection 1.1.9, pages 26-28).

There are seven key assays used for the diagnosis and subtyping of VWD. These are summarised in Table 1.1 (page 36). In the initial work up of a patient with a suspected diagnosis of VWD, the assays detailed in the top four rows are routinely carried out (Favaloro, 2010a) and if these are normal, the additional assays in the bottom three rows do not need to be performed. All these assays are carried out by hospital haemostasis laboratories on blood collected into citrate tubes. Based on the techniques used in the respondents to the UK National External Quality Assessment Scheme for Blood Coagulation (UK NEQAS, 2017) (for the top four rows) and literature review (for the three bottom rows), the method(s) for conducting the assays are now discussed in the same order as that presented in the table.

FVIII:C can be either measured based on the activated partial thromboplastin time (APTT) 1-stage method or the chromogenic assay. The APTT involves measuring the time recalcified plasma takes to clot in the presence of a 'contact' activator of the intrinsic pathway of coagulation (Figure 1.2A, page 27) and is sensitive to the concentration of FVIII in the sample. Therefore, FVIII:C can be (indirectly) quantified from the proportional shortening of the APTT when the test plasma is added to plasma which is deficient in FVIII. In the first step of the chromogenic assay, FXa is generated in the test plasma through the addition of excess phospholipid, calcium, FIXa, and FX (Moser & Funk, 2014). In the second step, a source of fibrinogen and prothrombin are added to the test plasma and a chromogenic substrate is added to the test plasma which hydrolyses in the presence of FXa (Rosen, 1984). This can then be quantified by a spectrophotometer and is proportional to the FVIII:C (Adcock et al., 2018).

An immunoturbidometric assay for the measurement of VWF:Ag (Veyradier et al., 1999; Sukhu et al. 2000) is the common method used (Chandler et al., 2011) mainly driven by

Assay [Number of centres in NEQAS]	Method(s)	Median coefficient of variance (CV, %))
FVIII activity (FVIII:C) [347]	APTT 1-stage; chromogenic substrate	10.8; 10.0
VWF antigen (VWF:Ag) [216]	Immunoturbidometry	15.8
VWF platelet-binding activity (VWF:Act) [152]	Immunoturbidometry; ristocetin cofactor activity; chemiluminescence	41.9*; 71.6; 5.0
VWF collagen-binding activity (VWF:CB) [34]	Technoclone ELISA	42.0
VWF multimer analysis	Gel electrophoresis; densitometry	NA
Ristocetin-induced platelet agglutination (RIPA)	Platelet agglutination using low and standard dose ristocetin	NA
VWF:FVIII binding activity (VWF:FVIIIB)	ELISA	NA

Table 1.1 | Summary of the seven laboratory assays used in hospital laboratories to measure the function and quantity of plasma VWF and FVIII. The top 4 assays are listed in descending order of the number of centres participating in the NEQAS scheme for the given assay, which is detailed in square brackets. For these 4 assays, the methods for performing the assays are in descending order of the frequency of use and for which there were sufficient number of centres for CVs to be reported, as provided in the third column. The assays in the three bottom rows are normally carried out manually, often using in-house methods and are not part of the NEQAS scheme. NA = not available; *Mean of three different analysers.

the ease of automation compared with earlier techniques (enzyme linked immunosorbent assay [ELISA]). The immunoturbidometric method involves the addition of a reagent containing latex particles - the surface of which is coated with a polyclonal antibody to VWF - to the test plasma (Villa et al., 2001). The subsequent agglutination of the latex beads impedes the passage of light shone through the test plasma, which is quantified photometrically and is proportional to VWF:Ag (Smith, 2017).

The measurement of the ability of VWF to bind to platelet GP-Ibα is called VWF:Act (Vicente et al., 1990). The original method was called ristocetin cofactor activity (VWF:RCo) and was still in use by 18 laboratories reporting to NEQAS, despite being superseded by the techniques below in many laboratories. Under physiological conditions, the A1 domain-GP-Ibα interaction only occurs under high shear stress because otherwise the A1 domain is not exposed in VWF's globular conformation (Subsection 1.1.9, page 28). Cationic ristocetin unfolds the tertiary structure of VWF and hence permits A1

domain-GP-Ibα binding under the static conditions used for laboratory analysis. Therefore, the degree of ristocetin induced agglutination is a reflection of the platelet binding capacity of the VWF in a given sample. This can be detected by measuring the optical density of a sample using platelet aggregometry (Born and Cross, 1963). The most widely used immunoturbidometric method (HemosIL^{TM} VWF:RCo) employs the same principle: magnetic beads are coated with the GP-Ibα fragments, which in the presence of ristocetin and VWF in the plasma sample cause agglutination and an absorbance change that can be detected on the automated analyser, which is a measure of VWF:Act (Stufano et al., 2014). The least commonly used system for ascertaining VWF platelet binding is based on chemiluminescence. This assay is similar to the HemosIL^{TM} VWF:RCo in that recombinant GP-Ibα is attached to microparticles in the presence of ristocetin. However, rather than optical density being quantified, isoluminol-VWF antibody is instead added at the end, which in the presence of hydrogen peroxide leads to luminescence that is measured by the analyser (Tous et al., 2009).

In addition to platelet binding at the site of vascular injury the main other role of VWF is to bind subendothelial collagen (Subsection 1.1.9, page 28), known as VWF:CB. The predominant method in use is the Technozym^{TM} VWF:CBA ELISA. The 96-well plates contain a fixed amount of covalently-bound pepsin-digested type III collagen derived from human placenta (Siekmann et al., 1998). Test plasma(s) and reference samples are added to the plate, incubated, and then washed. After which a polyclonal VWF antibody conjugated to horseradish peroxidase is added to each of the wells of the plate and incubated. Finally a chromogenic substrate is then added and the absorbance measured on a plate reader is used to determine the VWF:CB of the test sample.

The conventional method for assessing the sizes and relative distribution of plasma multimers is the electrophoresis of unreduced VWF on low agarose percentage gels (Counts et al., 1978; Fass et al., 1978; Ruggeri and Zimmerman, 1981). In addition, the action of ADAMTS13 can be evaluated - as LMWMs in plasma are proteolysed into fragments that appear as lower intensity ‘satellite’ bands flanking a central, higher intensity, band on the gel (Dent et al., 1991; Ledford-Kraemer, 2010). In the past five

years, there have been efforts to standardise and semi-automate multimer analysis to make it more suitable for hospital laboratories, for example the HYDRAGEL VW™ system in which all test equipment is supplied (Favaloro and Oliver, 2017). Furthermore this system includes a method to measure the optical density of each of the bands, therefore making the approach semi-quantitative and facilitating the development of reference ranges for low, intermediate, and high molecular weight multimers in normal plasma (Vangenechten and Gadisseur, 2020).

To assess whether there is enhanced binding between VWF and platelet GP-Ibα, low dose ristocetin induced platelet agglutination (RIPA) is performed. The basic principles of obtaining platelet rich plasma, provoking agglutination through the addition of ristocetin at 1.0mg/ml, and measurement of the resultant change in turbidity using an aggregometer are the same as when the approach was initially developed (Howard and Firkin, 1971; Howard et al., 1973). That a subset of VWD patients had abnormal agglutination at low doses of ristocetin (0.2 - 0.9 mg/ml) was first demonstrated by the Italian Working Group (Mannucci, 1977; Ruggeri et al., 1980). A laboratory standard of defining the low dose of ristocetin as 0.5 - 0.7 mg/ml has been proposed by the ISTH (Catteneo et al., 2013) with 0.5mg/ml the most commonly used dose (Frontroth et al., 2010; Kruse-Jarres & Johnsen, 2018; Goodeve, 2010).

The non-covalent association of VWF with FVIII prolongs the half-life of the latter (Pipe et al., 2016) and underpins VWF's role in secondary haemostasis (Miesbach & Berntorp, 2017) (Subsection 1.1.9, pages 26 - 28). The most common method for assaying the FVIII binding activity of VWF (VWF:FVIIIB) is by ELISA (Mohammed & Favaloro, 2017), first developed by Italian investigators (Casonato et al. 1998), with further optimisation (Caron et al. 2002; Veyradier et al., 2011; Zhukov et al., 2009). In brief, the main steps are as follows. First, 96-well plates are coated with polyclonal VWF antibodies. Then, between wash steps, test and reference plasma samples are added to the wells and any VWF contained within the plasma will be immobilised onto the surface of the plate. Endogenous FVIII is subsequently removed through addition of calcium chloride and washes. Subsequently, recombinant FVIII is added to the samples followed by a secondary

antibody that is conjugated to horseradish peroxidase (HRP). Finally a chromogenic HRP substrate is added and the colour intensity is proportional to the bound FVIII. By comparison with the calibration curves of the reference samples and normalisation against the measured VWF:Ag concentration, the relative VWF:FVIIIB is determined.

The low concentrations of VWF and FVIII in the blood means that absolute quantification of both antigen and activity levels has - to date - not been adopted in clinical haematology laboratories but instead relative quantification against a reference standard is used (Raut & Hubbard, 2010). The mean VWF:Ag, VWF:Act, VWF:CB, and FVIII:C plasma concentrations in the population are denominated as 1.00 international units per ml (IU/ml) (Raut & Hubbard, 2010), where IU represents the amount of factor that is being assayed by the given technique in 1ml of plasma pooled from a range of donors (Hubbard & Heath, 2004). The typical clinical laboratory reference range for these assays is set between 0.50 - 2.00 IU/ml, reflecting the wide range of variation of plasma VWF levels in the general population (O'Donnell & Laffan, 2001). The values are not normally distributed (Favaloro et al., 2005; Favaloro & Lippi, 2017; Sagheer et al., 2016) (Figure 1.3C, page 30). International (World Health Organisation [WHO]) standards are available for calibration of FVIII:C, VWF:Ag, VWF:Act, and VWF:CB. Multimer, RIPA, and FVIII:B analysis do not have WHO standardisation

The definition and classification of VWD is controversial and has altered over time (Federici et al., 2014; Laffan et al., 2004; Laffan et al., 2014; Ruggeri & Zimmerman, 1987; Sadler, 1994; Sadler et al., 2006). Recent guidelines have been developed by international haemostasis experts drawn from four patient and professional groups (James et al., 2021). Their position is that a diagnosis of VWD requires:

1) A low VWF level (either VWF:Ag and/or VWF:Act);
2) The presence of an abnormal bleeding history in individuals in whom there is only a modest reduction in VWF level (typically 0.30 - 0.50 IU/ml) but is not essential where the level is below 0.30 IU/ml.

Further information about how the subtypes are defined is outlined in Table 1.2 (page 40).

Based on the latest figures from UK haemophilia centres (United Kingdom Haemophilia Centre Doctors' Organisation 2021), there are 11,152 patients registered with a diagnosis of VWD, of which 7,071 are female and 4,081 are male. The subtype most frequently diagnosed is VWD1 (4,878/11,152). There were 1,701 with VWD2 and 179 with VWD3, therefore comprising 15% and 2% of the total VWD population, respectively. Some patients fell outside of the above classification either because their subtype was unreported or they were given a diagnosis of 'Low VWF' (discussed in Subsection 1.2.4, pages 55 & 57).

<table>
<tr><th>Type</th><th>VWF pathology</th><th colspan="2">Laboratory definition[1,2,3,4]</th></tr>
<tr><td>1</td><td>↓ secretion and/or
↑ clearance</td><td colspan="2">VWF:Ag and/or VWF:Act < 0.30 IU/ml or
< 0.50 IU/ml and bleeding symptoms</td></tr>
<tr><td>2A</td><td>↓ binding to platelet GP-Ibα and ↓ HMWMs</td><td rowspan="3">Meets type 1 criteria and VWF:Act/ VWF:Ag < 0.70 +</td><td>↓ or absent HMWMs on gel electrophoresis</td></tr>
<tr><td>2B</td><td>↑ binding to platelet GP-Ibα</td><td>Agglutination of platelets with low dose ristocetin</td></tr>
<tr><td>2M</td><td>↓ binding to platelet GP-Ibα or collagen</td><td>Preserved HMWMs on gel electrophoresis</td></tr>
<tr><td>2N</td><td>↓ binding to FVIII</td><td colspan="2">FVIII:C/VWF:Ag < 0.5 & ↓ FVIII:B</td></tr>
<tr><td>3</td><td>Absent or near-absent secretion</td><td colspan="2">VWF:Ag < 0.05 IU/ml</td></tr>
</table>

Table 1.2 | Classification system of von Willebrand disease. [1]IU/ml apply to all values provided. [2]The definitions for VWD1, VWD2A, 2B, and 2M[2]; VWD2N[3]; and VWD3[4] are based on James et al., 2021, Mazurier et al., 2001, and Baronciani et al., 2021, respectively.

In VWD1 there is a concordant reduction in both VWF:Ag and VWF:Act - whereas in VWD2A, 2B, and 2M - this ratio is discordant, with VWF:Act being disproportionately reduced compared to VWF:Ag (Federici, 1998; Holmberg & Nilsson, 1972; Ruggeri et al., 1980), with the lowered ratio taken as a proxy for abnormal VWF structure and function (Tosetto & Castaman, 2015; Meyer et al., 2011). In the diagnosis of type 2N, the functional abnormality is the interaction between VWF and FVIII, reducing the proteolytic protection offered to FVIII:C, reducing its level to at least 50% of VWF:Ag (Mazurier et al., 2001; Tosetto & Castaman, 2015). Although some authors argue that the hallmark of VWD3 is a

complete deficiency of VWF:Ag (Leebeek & de Wee, 2010; Mannucci, 2001), more recent literature acknowledge that VWD3 also includes individuals in whom there is near-absence of plasma VWF (Favaloro, 2020; Fogarty et al., 2021) up to 0.05 IU/ml (Baronciani et al., 2021).

The mechanisms leading to reduced VWF levels in the plasma in VWD1 are diverse and - broadly - can be split into two groups. The most common reason is impaired, but not complete absence of the production of VWF which can occur at any point in the biosynthetic journey of this protein in ECs (Lillicrap, 2013) right from the failure of the VWF promoter to initiate transcription of VWF (Othman et al., 2010) through to a problem with exocytosis of WPBs (Wang et al., 2013b). A minority of VWD1 results from enhanced clearance from plasma, called VWD1C (Haberichter et al., 2006).

VWD3 is also a quantitative defect, and most commonly results from the complete absence of VWF being produced in ECs because of biallelic predicted loss-of-function variants (pLoFs) (Baronciani et al., 2003; Shahbazi et al., 2009; Sutherland et al., 2009) (Subsection 1.2.4, page 53), but also can arise due to alleles that result in VWF being translated but result in a severely reduced synthesis, trafficking, or release (Eikenboom, 2001; Lillicrap, 2013).

Both VWD2A and most cases of VWD2M are characterised by reduced or absent binding of VWF to GP-Ibα. However, there are differences in the underlying reasons why this occurs. HMWMs are more effective at binding GP-Ibα (Moake et al., 1986; Federici et al., 1989). Therefore in VWD2A, VWF:Act is reduced because HMWMs are depleted or absent from plasma, whereas low and intermediate molecular weight forms are unaffected. The relative HMWM plasma deficit may be due to defective multimerisation, enhanced degradation, or problems in storage granule formation in ECs (Jacobi et al., 2012). An alternative pathomechanism of VWD2A is the hyper-susceptibility of VWF to ADAMTS13 proteolysis, meaning that HMWMs of VWF are quickly depleted from the circulation (Hassenpflug et al., 2006). In VWD2M, HMWMs are intact but there is a specific loss of function defect; most commonly this is an inability to bind GP-Ibα

(Doruelo et al., 2013) but in some cases there is impaired capacity to bind collagen, therefore causing VWF:CB/VWF:Ag to be reduced (< 0.6) but with a normal VWF:Act/VWF:Ag (Favaloro et al., 2021).

Paradoxically, individuals with VWF that binds too readily to GP-Ibα also have a form of VWD, called VWD2B (Kruse-Jarres & Johnsen, 2018). The heightened VWF-GP1bα interaction results in depletion of HMWMs from the circulation, explaining the reduced VWF:Act/VWF:Ag ratio. There is a reduction in the shear threshold at which VWF binds GP-Ibα and aggregates platelets, which in turn makes the multimers holding the platelets together more vulnerable to ADAMTS13 proteolysis (Yago et al., 2008). Some individuals with VWD2B also are thrombocytopenic, due to macrophage uptake of VWF-platelet complexes and a shortened half life of platelets (Casari et al., 2013). One in seven apparent cases of VWD2B (Hamilton et al., 2011), are actually a phenocopy called platelet-type VWD, PT-VWD (Miller & Castella, 1982; Takahashi, 1980; Weiss et al., 1982). PT-VWD and VWD2B can be phenotypically distinguished using a low-dose RIPA that is performed with patient platelets and normal plasma and compared to a low-dose RIPA performed with patient plasma and normal platelets - a modification known as a mixing RIPA assay (Othman et al., 2016).

VWD2N is characterised by impaired binding of VWF to FVIII (Nishino et al., 1989; Mazurier et al., 1990). The low FVIII:C means that VWD2N is an important mimic of haemophilia A, HA (Schneppenheim et al., 1996). Although VWD2N can often be distinguished clinically from HA because of an autosomal inheritance pattern (Mazurier, 1992) pedigree information is not always readily available. Although there is not an international standard for FVIII:B, VWD2N should be suspected if the VWF:Ag/FVIII:B ratio is reduced (Casonato et al., 2018; Veyradier et al., 2011).

There are a number of limitations - and challenges in the usage - of assays used to subtype VWD. First, the wide coefficient of variance around the mean values (Table 1.1, page 36) increases the chances of both false positive and false negative results for individuals with only modest reductions in VWF levels. Therefore contemporary guidelines recommend

repeat testing for borderline cases (James et al., 2021), which is an inconvenience given the 100s of kilometres some patients have to travel (McClellan et al., 2006)). Second, VWF and FVIII:C levels can be falsely normal (even when the individual has VWD), which is why it is recommended that VWD should be diagnosed when an individual is at their 'baseline' status of health (James et al., 2021). This is plausible for pregnancy and infection, which cause two-to-five-fold increases in VWF:Ag (Goshua et al., 2020; Pottinger et al., 1989; Sie et al., 2003), but which are transient. What is more challenging is for individuals with cardiovascular disease and/or obesity - who may be at their baseline - but whose FVIII:C and VWF level may be increased because of these conditions (Lip & Blann, 1997; Mertens & Gaal, 2002). Given that there is some evidence that age-normalisation of VWF levels in VWD1 patients over the age of 65 does not reduce their bleeding phenotype (Sanders et al., 2014) there remains a challenge about how such patients should be diagnosed and monitored using existing assays. Third, existing assays miss some aspects of VWF's diverse functions that may result in a clinically meaningful phenotype, such as capacity to bind GPIIb-IIIa (Bolton-Maggs et al., 2012). Fourth, abnormal results can occur as a result of the way the assays are performed but without in vivo relevance. As outlined earlier (pages 36-37), ristocetin circumvents the problem of the A1 domain-GP-Ibα interaction normally occurring under shear stress. However, a family has been identified who have a reduced VWF:Act but have no other clinical features suggestive of VWD. The reduced VWF:Act co-segregated with the p.Pro1467Ser variant in the family pedigree. This variant is situated in the distal portion of the A1 domain known to bind to ristocetin, but does not seem, in vivo, to have any physiological relevance. This is consistent with this being a spuriously low result (Flood et al., 2009).

1.2.3. How common is VWD (really)?

To-date, there have been two approaches used to try and estimate the prevalence of VWD: population- and referral-based (Sadler et al., 2000). These are addressed, in turn, below.

In 1987, a team of Italian researchers published the first study that determined the population prevalence of VWD (Rodeghiero et al., 1987). Questionnaires regarding bleeding symptoms were completed by the parents of 1,218 school-attending adolescents

equally split by sex. The investigators took into consideration a combination of factors. These included a personal history of bleeding, whether the VWF:Act level was below the 2.5 percentile of a control group, and the co-segregation of bleeding symptoms with reduced VWF:Act in family members. Based on these variables they estimated that 0.82% of children in their cohort had a diagnosis of VWD. The approach of the Italian group has become a template for the majority of other population-based estimates of VWD prevalence, which have focussed on children, adolescents, and young adults in education (Abu-Douleh et al., 2018; Şap et al., 2013; Werner et al., 1993; Yilmaz et al., 2005).

As detailed in Table 1.3 (pages 45-46), twelve studies have attempted to estimate VWD prevalence in the general population. The mean of the estimates of prevalence based on the values calculated by the authors is 0.82%, however this varies between 0.01 and 1.60%, likely due to inter-study differences in the definition of VWD. As shown in the last two columns on the right side of the table, the majority of studies provided sufficient information to define study participants as having VWD against the latest international criteria (James et al., 2021), thus allowing a more meaningful comparison. The mean estimates for prevalence based on these figures are 0.51 and 0.22%, respectively, depending whether VWD was defined as VWF:Ag or VWF:Act < 0.50 IU/ml in the presence of bleeding or, more strictly, by VWF:Ag or VWF:Act < 0.30 IU/ml irrespective of bleeding. This demonstrates that the authors' definitions for VWD were more lenient than the criteria that is now advised (James et al., 2021).

Referral-based prevalence is based on individuals with a known diagnosis of VWD. One approach is to determine a numerator VWD population based on the referrals made to tertiary (haemophilia) centres and then divide this over the known catchment population (Rodeghiero & Castaman, 2001). Another way of calculating this is based on regional or national registries of VWD patients, the first of which was created in the UK in the 1960s (Aznar et al., 2009). Practical help in setting up national registries was provided by the World Federation of Haemophilia (WFH) at the start of the 21st century (Evatt, 2005). I have used this data - as reported to the 2020 Annual Global Survey (World Federation of

Hemophilia, 2021) and combined with published information to determine an estimated referral prevalence for 120 of the world's countries (Appendix 7.2; Figure 1.4, page 47),

Year	First author (country)	No.[1]	Setting	Age range in years	VWD prevalence (%, 2 d.p.)		
					Author determined	VWF:Act or VWF:Ag < 0.30 IU/ml	VWF:Act or VWF:Ag < 0.50 IU/ml and bleeding
1987	Rodeghiero (Italy)	1,218	School	11-14	0.82	0.08	0.24
1987	Miller (USA)[2]	NA	Blood donors	NA	1.6	NA	NA
1991	Meriane (Turkey)[2]	NA	Adult students	NA	1.23	NA	NA
1993	Werner (USA)	600	School	2-18	1.33	0.33	1.33
1999	Biron (France)	832	Hospital (pre-op)	1-82	0.60	0.36	0.96
2004	Koscielny (Germany)	5649	Hospital (pre-op)	17-87	0.96	NA[3]	NA[3]
2005	Yilmaz (Turkey)	3485	School	6-13	0.44	NA[4]	NA[4]
2010	Bowman (Canada)	4592	Hospital out-patients	≤ 18	0.11	0.07	0.11
2010	Bowman (Canada)	10,258	Primary care	NA[5]	0.09	NA[3]	0.09
2013	Şap (Turkey)	4592	School	14-19	1.04	0.35	0.60
2018	Abu-Douleh (Saudi Arabia)	2000	Uni[6]	17-22	1.60	0.10	0.25
2018	Badagabettu (India)	25,625	Home[7]	<13 to > 40 years	0.01	NA[3]	NA[3]
				Mean	0.82	0.22	0.51

Table 1.3 | Population-based estimates of the prevalence of VWD. [1]Number of participants. [2]Data only published in an abstract which could not be located; therefore data extracted from Table 2, Rodeghiero & Castaman, 2001. [3]No individual participant details were included so this calculation could not be made. [4]Only the abstract in English was obtained and no individual participant details were included in this so these calculations could not be made. [5]The authors (Bowman et al., 2010a) note that the majority of participants were adults but do not specify an age range for the entire cohort. [6]University. [7]Rural healthcare professionals screened individuals in their homes with a standardised questionnaire regarding bleeding symptoms.

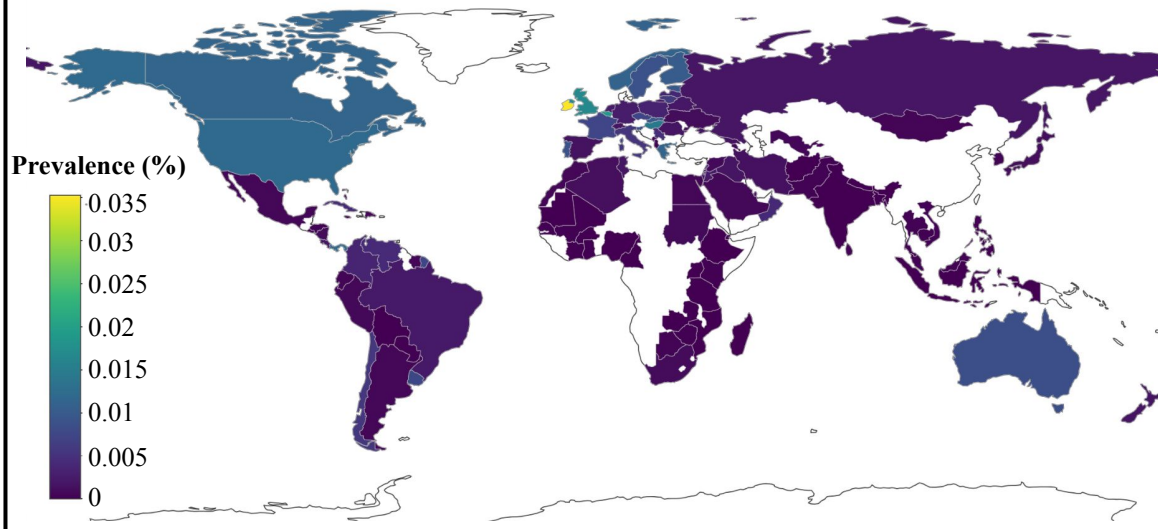
excluding publications in which no denominator population was provided (Khan et al., 2014; Kumar et al., 2010; Manisha et al., 2002; Trasi et al., 2005; Zhang et al., 2003). The referral-based prevalence varies from 0% to 0.035%, with a median of 0.0009%, corresponding to ~ 1/111,000. Three conclusions can be drawn. First, the highest referral-based prevalence is reported in western Europe and North America. Second, the lowest prevalence is in Africa, South America and Asia. Third, the countries for which data are lacking are principally in southeast Europe, central and east Asia, and central Africa.

One potential explanation for the apparent regional disparity in referral-based prevalence is differing prevalence of VWD across ethnic groups due to founder effects, which have been reported in both Hungarians (Mohl et al., 2008) and Finns (O'Brien et al., 1988), who have the fourth and tenth highest referral prevalence respectively (Figure 1.4B, page 47). However, I think this is unlikely as in the latest USA registry dataset (Soucie et al., 2021), individuals of black, asian, and hispanic ancestry were all represented. Although the authors found that their proportional representation in the US VWD population was up to two fold lower than in the general population, this is not sufficient to explain a difference as marked as the ~10,000 fold higher number of cases in Ireland compared to Bangladesh.

So it is likely that there are other factors at play. Indeed the mean GDP per capita of the five countries with the highest prevalence (Ireland, Belgium, United Kingdom, Hungary, and Panama) is $40,008, whereas the per capita income of the five countries with the lowest prevalence (Burkina Faso, Ethiopia, Bangladesh, Afghanistan, and Nigeria) is $1,274 (World Bank, 2020). Therefore it would seem likely - at least in part - that referral-based prevalence is linked to the higher wealth of a country and the capacity of its healthcare system to be able to carry out diagnostic investigations to identify individuals

Figure 1.4

A



B

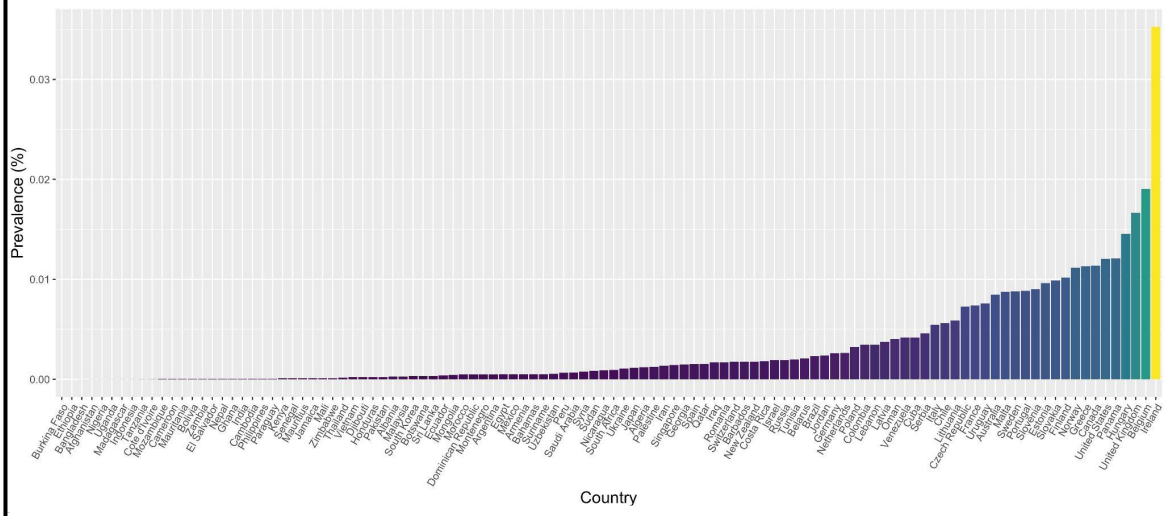


Figure 1.4 | Estimation of referral-based prevalence of VWD. Prevalence was determined for 110 countries using a combination of published data using referral or national registration statistics and the most recent World Federation of Haemophilia annual global survey, as listed in full in Appendix 7.2. The only exclusions were data from countries that no longer exist (the former Yugoslavia) and those estimating grouped prevalence across more than one nation (i.e. the Nordic countries in Berntorp and Önundarson, 2005). Where there was more than one estimate for a country, the value displayed is the mean. This data is represented as (A) a world heatmap and (B) bar graph, ordered left to right in order of increasing prevalence. The legend from (A) also applied to (B).

with VWD and set up and maintain a registry (Federici, 2016; Mahony et al., 2017; Nair et al., 2011). In addition, there are a number of other variables that influence the referral-based prevalence. First, there is a lack of uniformity between countries - and over time - about the definition of VWD (Kalot et al., 2020). Second, there may be underreporting of patients to national registries (Bloom & Giddins, 1991), particularly if care is not universally provided in haemophilia centres (Seaman et al., 2021; Soucie et al., 2021). Third, the quality of the registry data itself varies (Keipert et al., 2015), with potential problems including duplicate entries and failures to remove individuals from the registry who are deceased.

In summary, estimates of population-based prevalence of VWD are much higher than referral-based estimates, with less variation between studies probably due to the controlled and time-limited settings in which these were executed. Even if the strictest interpretation of the latest international guidelines is applied, the mean population prevalence of 0.22% (~ 1 in 500) - is six-times higher than the most liberal referral-based estimate from Ireland (Figure 1.4, page 47), 0.035%, or ~ 1 in 2,900. This would suggest that ~ 83% of the population with VWD are not known to a haemophilia centre, with this proportion being much closer to 100% in poorer countries. Partly this can be attributed to logistical factors, i.e. the lack of a patient registry in some countries or geographical variability in haemophilia centre provision. However, it is also probable that the majority of individuals with VWD are simply not seen in haemophilia centres. This is likely to be for manifold reasons which could include absence of haemostatic challenges, mild or absent haemorrhagic symptoms meaning that no medical attention is sought (Trasi et al., 2005), or that the diagnosis is missed because of presentation with bleeding to non-haematologists (Sidonio Jr et al., 2020). This “never-seen” group of individuals with VWD are understudied and require further attention as discussed in Chapter 4.

1.2.4. The challenge of linking *VWF* genotype to VWD phenotype

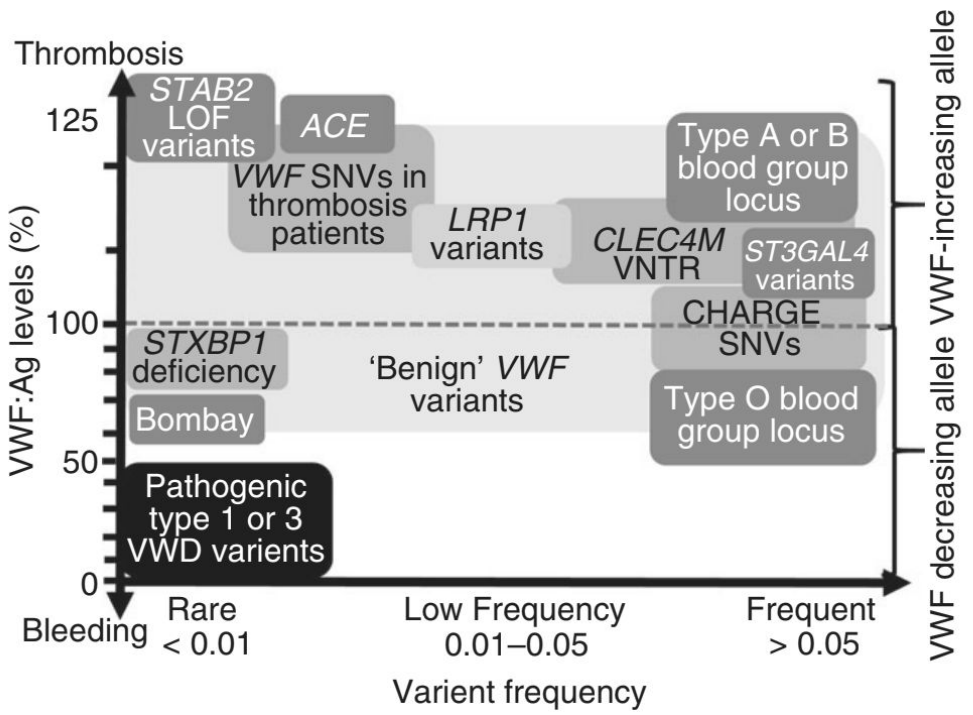
The first scientific report confirming the long held suspicion that VWD can result from rare DNA variants in the *VWF* gene was published over 30 years ago, in which deletions encompassing the whole of *VWF* were identified in two patients with VWD3

(Shelton-Inloes et al., 1987). Since then, clinically accredited sequencing of the *VWF* gene has become widespread in the diagnostic laboratories of developed nations and, as outlined later in this chapter, at least 750 potentially causal VWD variants have now been reported (De Jong and Eikenboom, 2017). Therefore, given the limitations of laboratory assays for the diagnosis of VWD (Subsection 1.2.2, pages 42-43) and the frequency of the condition in the population (Subsection 1.2.3, pages 43-48), there is increasing interest in not only using genetic analysis of *VWF* to confirm a phenotypic diagnosis, but also utilising it, upfront, in the diagnostic workup of an individual suspected of having the condition (James et al., 2021; Smock & Moser, 2021). This has a number of potential advantages. For example, it could reduce the need to provide hyper-specialised, but infrequently performed coagulation assays (i.e., the bottom three rows of Table 1.1, page 36) where there is paucity of provision (Castaman et al., 2018). Moreover, given the increasing availability and use of multi-gene, high-throughput sequencing (HTS), panel tests for the diagnosis of inherited bleeding, platelet and thrombotic disorders (Bastida et al., 2018; Downes et al., 2019; Simeoni et al., 2016), which includes VWD, this could potentially simplify the diagnostic evaluation of patients with these conditions. Despite these advantages, there are challenges in realising the wider rollout of genetic testing for VWD. These relate to the need to consider the results generated in the light of trans-acting determinants of VWF levels, the different modes of inheritance and incomplete penetrance of the phenotype of VWD, and heterogeneity in the published reports of genetic variants which makes it difficult to ascertain their contribution to VWD. In this subsection I cover each of these points and finish by discussing why the pathogenicity appraisal of potentially causal VWD variants needs to be revisited.

A useful conceptual framework for thinking about genetic variants and their influence on plasma VWF levels has been put forward by Swystun and Lillicrap (2018) (Figure 1.5A, page 50). The focus of my thesis are those variants that sit in the bottom left hand corner of this figure, i.e. rare variants in *VWF,* and their pathogenicity for VWD. However, as highlighted in the figure, there are a number of loci outside of *VWF* that affect VWF:Ag. The majority of these are variants with common minor allele frequencies (MAFs) (> 0.01 [1%]).

Figure 1.5

A



B

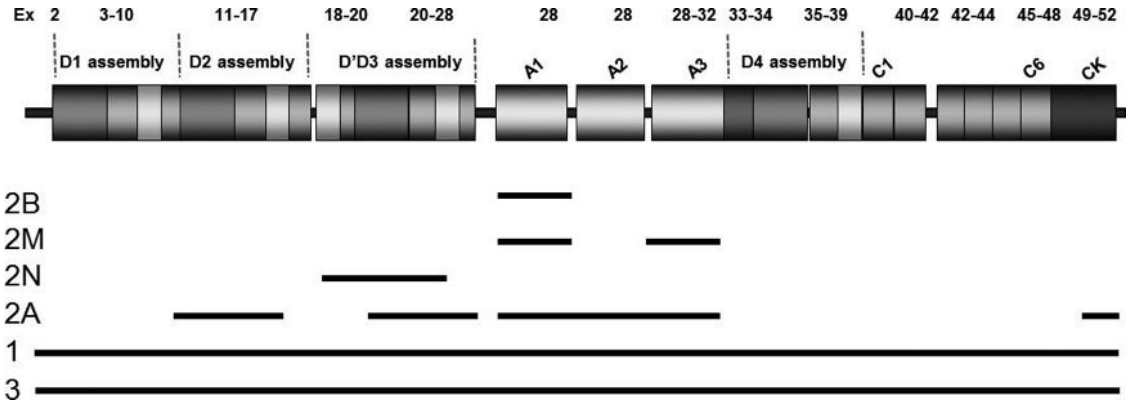


Figure 1.5 | The genetic basis of VWD. (A) An illustrative plot, reproduced from Swystun & Lillicrap, 2018, demonstrating the groups of genetic variants that influence VWF level (on the y-axis) and their relative frequency (on the x-axis). (B) Exons and domains of VWF with the positions in which variants (normally) occur in each subtype marked by a horizontal black line below. Reproduced from Berntorp et al., 2018.

The most important trans-acting locus affecting VWF:Ag levels is *ABO*. Starting in the late 1970s, four studies enrolled several hundreds of participants between them and demonstrated an association between ABO (based on red cell typing) and VWF:Ag (Stormorken & Erikssen, 1977; McCallum et al., 1983; Mohanty et al., 1984; Orstavik et al., 1985). Three out of the four studies showed that the average VWF:Ag was lowest in group O individuals. This observation was subsequently confirmed and expanded upon in a study with 10 times as many participants (Gill et al., 1987). The authors showed that amongst ~ 1,100 US blood donors, the subgroups - AB, B, A, and O - had mean plasma VWF:Ag values of 1.23, 1.17, 1.06, 0.75 IU/ml respectively, with all pairwise comparisons reaching statistical significance except B compared with AB. Similar studies have consistently replicated the observation that group O individuals have ~ 20-35% lower plasma VWF:Ag than non group O individuals and that this difference holds up across a variety of ancestry groups and settings (Caekebeke−Peerlinck et al., 1989; Miller et al., 2003; Schleef et al., 2005; Souto et al., 2000). As discussed, the mechanism is thought to be secondary to reduced VWF production and/or increased clearance in group O individuals (Subsection 1.1.8, pages 25-26).

Until 2010, *ABO* was the only trans-acting locus that had been consistently shown to affect VWF levels. The advent of genome wide association studies (GWAS) in the last 15 years has changed this. The GWAS method relies on single nucleotide variant (SNV) genotyping array technology. SNV arrays were introduced at the beginning of the twenty first century and facilitated the first systematic cataloguing of MAFs for common variants (International HapMap Consortium, 2005). Shortly thereafter it was appreciated that this technology could be used to agnostically assess the effect of genome-wide SNVs against a trait of interest (Klein et al., 2005; LaFramboise, 2009; Ozaki et al., 2002). The first GWAS studying plasma VWF:Ag was from the Cohorts for Heart and Aging Research in Genome Epidemiology (CHARGE) consortium (Smith et al., 2010). Since then, five further GWAS' have been published (Desch et al., 2013; Huffman et al., 2015; Sabater-Lleal et al., 2019; Tang et al., 2015; Williams et al., 2017), which have identified 18 known protein quantitative trait loci (pQTL) for VWF:Ag (Table 1.4, page 52) with *ABO* consistently having the greatest effect.

Gene(s) (Chr)	Lead SNV	MAF	Effect size (VWF:Ag IU/ml)
ABO (9q34)	rs687289	0.63	0.20
ST3GAL4 (11q24)	rs35458154	0.01	0.06
OR13C5/NIPSNAP3A/NIPSNAP3B (9q31)	rs6479259	0.10	−0.06
VWF (12p13)	rs2238109	0.40	0.05
STAB2 (12q23)	rs4981022	0.69	0.04
SLC39A8 (4q24)	rs6855246	0.13	−0.03
STXBP5 (6q24)	rs9390460	0.51	−0.03
SCARA5 (8p21)	rs4276643	0.64	−0.03
STX2 (12q24.3)	rs4759787	0.49	0.02
CLEC4M (19p13.2)	rs2277998	0.23	−0.02
TC2N (14q32)	rs4904820	0.53	0.02
RPL3/TAB1/SYNGR1/PDGFB (22q13)	rs5750823	0.71	−0.02
FCHO2/TMEM171/TNPO1 (5q13)	rs548630	0.46	−0.02
C2CD4B (15q22)	rs6494314	0.80	−0.02
DAB2IP (9q33	rs10985344	0.20	0.02
PDHB/PXK/KCTD6 (3p14)	rs55954186	0.25	0.02
GIMAP7/GIMAP4 (7q36)	rs7788962	0.71	−0.01
HLA-C (6p21) *(?)*[15]	rs9271597	0.39	−0.01

Table 1.4 | Protein quantitative trait loci for VWF:Ag. The information is based on that published by Sabater-Lleal et al., 2019. The table is ordered, top-to-bottom, in descending order of effect size (irrespective of the direction of the effect). The left hand column lists the genes at the loci for which a significant association with VWF:Ag was found. The lead SNV is given using dbSNP refSNP (rs)ID (Barnes, 2002) and reflects the SNV at the locus with the lowest *P* value. Both MAF and effect size are given to 2 decimal places.

For some of the more recently discovered loci (Sabater-Lleal et al., 2019), a candidate gene could not be definitively allocated. Therefore there are 28 genes potentially linked to variants that affect VWF:Ag levels. Not all of the loci are trans-acting: there were also common *VWF* variants significantly associated with VWF:Ag levels. In terms of the 27

trans-acting genes, the functional evidence that has been gathered so far suggests that there are three main mechanisms through which these pQTLs exert their effects:

1) Altered post translational modification. ST3GAL4, for example, adds sialic acid to the terminal branches of glycosylated VWF and, in mice, has been shown to affect its clearance (Ellies et al., 2002).
2) WPB trafficking and release. STXBP5, for example, is part of the SNARE machinery controlling WPB exocytosis (Zhu et al., 2014).
3) Clearance receptor function. SCARA5, for example, is a putative splenic receptor for VWF. In a double VWF/SCARA5 mouse KO model, human VWF was less rapidly cleared compared to the VWF KO (Swystun et al., 2019).

As illustrated by Figure 1.5A (page 50), rare variants in *VWF* can have much greater effects on VWF:Ag than those described above and is why they have been conventionally linked to VWD. The challenge, however, is figuring out which ones these are. Using the type 1, 2, 3 phenotype definition (Table 1.2, page 40) also helps in understanding the genetic basis of VWD and how this relates to the VWF protein (Figure 1.5B, page 50).

VWD3 is due to homozygosity or compound heterozygosity for deletions, frameshift, nonsense, or splice site variants (Baronciani et al., 2003). In the majority of cases, this results in the absence of VWF translation (Castaman et al., 2010; Corrales et al., 2011; Eikenboom et al., 1992; Platè et al., 2010; Shahbazi et al., 2012; Xie et al., 2007). A less well reported mechanism is where a nonsense or frameshift variant results in a premature termination codon (PTC) that does not affect mRNA stability (Eikenboom et al., 1992; Platè et al., 2010) and the truncated VWF is translated, but is intracellularly retained and the "mutant protein is only detectable at trace levels in homozygous patient plasma" (Mohlke et al., 1996).

Subtypes of VWD2 are caused by missense variants which change the protein sequence but not its length (Tosetto & Castaman, 2015). These localise to a specific domain, as shown in Figure 1.5B (page 50). For example, both VWD2B and 2M can result from A1 domain variants. In VWD2B the reported variants localise to a region of the A1 domain

which comes into proximity to the N-cap of the GP-Ibα receptor (de Jong & Eikenboom, 2017) and result in a gain-of-function interaction with it (Subsection 1.2.2, page 42). In contrast, VWD2M variants that occur in a range of positions throughout the A1 domain have been reported to result in a loss-of-function interaction with GP-Ibα through mechanisms including disordered conformational binding under shear stress (Tischer et al., 2014). Apart from VWD2N, which is normally recessive (Mazurier et al., 2001), the remaining subtypes of VWD2 are autosomal dominant (Tosetto & Castaman, 2015).

VWD1 shares some similarities with VWD2 in that the variants that do cause it are generally missense and autosomal dominant (Goodeve et al., 2007; James et al., 2007). However, unlike VWD2, the variants occur throughout the gene, including the promoter (Flood et al., 2019). An issue which is debated is whether the pLoFs that cause VWD3 in homozygosity or compound heterozygosity result in a VWD1 phenotype when only on one allele, with such individuals often being referred to VWD3 obligate carriers (Ewenstein, 1997). Some research groups suggest that pLoFs function as codominant alleles with a resultant increased risk of reduced VWF levels (and a consequent VWD diagnosis) and a bleeding phenotype (Bowman et al., 2013; Castaman et al., 2006). On the other hand, different groups emphasise the variable expressivity of phenotype in pLoF heterozygotes (Christopherson et al., 2022).

Indeed, ‘incomplete penetrance’ of phenotype is a major challenge, more broadly, for understanding potentially causal VWD variants. When considering phenotype in VWD this can mean bleeding (Subsection 1.2.1, pages 29-34) or laboratory phenotype (Subsection 1.2.2, pages 34-43; Table 1.2, page 40). A difference in bleeding tendency between family members with the same potentially causal VWD variant is reported to mainly occur in VWD1 (Miller et al., 1979; Levy & Ginsburg, 2001), but this may be a reflection of its higher prevalence. Potential explanations for varying bleeding expressivity include undiagnosed compound heterozygosity for a second VWD variant (Levy & Ginsburg, 2001) and discrepancies in the number and nature of haemostatic challenges experienced (most notable for females compared to males). Furthermore the same variant can present with a laboratory phenotype of VWD2 in one individual from a pedigree but

appear as VWD1 in another, blurring the distinction between types 1 and 2 (Chen et al., 2015).

Given the heterogeneity in the types of variants that cause VWD, its multimodal method of inheritance, and varying definitions of phenotype, how can one decide whether a given VWD variant is pathogenic, i.e. having the variant causes the disease? General guidelines designed for the clinical genetics community at large can help inform the answer to this question. The most influential are those published by the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) (Richards et al., 2015). With respect to a disease of interest the ACMG/AMP propose categorisation of variants into five tiers: pathogenic; likely pathogenic, uncertain significance, likely benign, and benign. When adjudging a variant against the criteria the guidelines recommend a multi-layered approach considering a range of evidence. This includes the type of variant being considered (e.g. pLoF versus missense) alongside a body of clinical, laboratory, and epidemiological data. The latter includes MAF with a variant being classified as benign if it has a MAF "greater than expected for [the] disorder".

Large sequencing studies of patients with VWD took off in 2007 with the publication of both the MCMDM1-VWD (Goodeve et al., 2007) and Canadian cohort (James et al., 2007) projects which focussed on the genetic aetiology of VWD1. With the ACMG/AMP guidelines in mind, Table 1.5 (pages 56 - 57) summarises these two studies and others published between then and 2021 that enrolled only VWD patients.

Three conclusions can be drawn from the data presented in Table 1.5.

First, for studies including VWD1 patients, a wide definition of VWD was used, either a VWF level < 0.50 IU/ml or a historic diagnosis of VWD. This means that individuals with VWF:Ag and/or VWF:Act between 0.30 and 0.49 IU/ml had been included. It is a moot point about whether individuals with VWF levels in this range have VWD (James et al., 2021) or 'Low VWF' (Laffan et al., 2014) that reflects a potential risk factor for bleeding (Sadler, 2009). Notwithstanding semantics, the Low VWF group is perhaps better thought

First author surname, date	Participant inclusion Criteria	VWF levels[1]	Pathogenicity assertion made	MAF threshold for variant inclusion
Goodeve, 2007	Historic VWD1	Y	N	< 0.01
James, 2007	Index family member VWF:Ag & VWF:Act 0.05 - 0.50 IU/ml	Some	Y	None
Johansson, 2011	Historic VWD1	N	N	None
Yadegari, 2012	Based on ISTH 2006 guidelines[2]	Y	N	None
Bowman, 2013	VWF:Act < 0.05 and FVIII:C < 0.10 IU/ml	Y	N	None
Kasatkar, 2014	VWF:Ag ≤ 0.05 IU/ml; FVIII:C < 0.10 IU/ml	Y	Y	None
Batlle, 2016	Multiple[3]	N	N	None
Flood, 2016	Historic VWD1	N	N	< 0.01
Veyradier, 2016	VWF levels < 0.30 IU/ml[4]	Some	N	None
Borras, 2017	Multiple[3]	Y	Y	<0.01
Lavin, 2017	VWF levels 0.30 - 0.49 IU/ml	Some	Y	None
Liang, 2017	Based on ISTH 2006 guidelines[2]	Y	Y	None
Manderstedt, 2018	Historic VWD1	N	Y	< 0.005
Elayaperumal, 2018	Historic VWD3	Y	N	< 0.02
Vangenechten, 2019	Multiple[5]	Y	Y	None
Rassoulzadegan, 2020	VWF:Act/Ag < 0.60	Some	Y	None
Baronciani, 2021	VWF levels < 0.05, bleeding and recessive inheritance	N	Y	< 0.01

Table 1.5 | VWD patient sequencing studies since 2007. Only those with > 50 VWD patients are included. The table is ordered from top to bottom in chronological order by year. [1]Refers to whether the individual VWF levels of participants were provided in the publication. [2]Sadler et al., 2006: did not specify thresholds for diagnosis. [3]One or more of: VWF:Ag, VWF:RCo and/or VWF:CB ≤ 0.30 IU/ml; abnormal multimers; reduced FVIII:C and VWF:FVIIIB; positive RIPA. [4]Whether VWF:Act,VWF:Ag, VWF:CB levels were used was not specified. [5]VWD1: < 0.35 IU/ml; VWD2: decreased RIPA and/or VWF:Act/VWF:Ag < 0.60, unexplained thrombocytopenia and/or suspected VWD and/or positive RIPA, VWF:CB/VWF:Ag < 0.60, FVIII:C/VWF:Ag < 0.50; VWD3: VWF:Ag and VWF:Act < 0.05 IU/ml.

of as the lower end of a quantitative trait as opposed to a Mendelian disorder (Collins et al., 2008). In support of this is the fact that one tail of the VWF:Ag distribution in O group blood donors (0.36 - 1.57 IU/ml) overlaps with the range used to define Low VWF (O'Donnell et al., 2002). Moreover, there is a significant enrichment of individuals with a red cell serotype of O in the Low VWF group compared to the background population (89% vs 55% in Lavin et al. 2017).

Second, only 6 out of 17 studies used a MAF threshold in which to include variants for further analysis. Where a threshold was applied it was most commonly set at 0.01, i.e. 1% of alleles. As discussed above, given that the majority of VWD can be considered autosomal dominant, a MAF of 0.01 corresponds to 2% of individuals, more than double the estimates of VWD prevalence based on the available published data (Table 1.2, page 40).

Third, although the majority of publications did make some assertion about pathogenicity, generally the focus was on the aggregated output of in silico algorithms (as discussed in Subsection 3.5, pages 135-137), as opposed coming to an overall decision about the reported variant's causal relationship with VWD. This is understandable given that 9 out of 17 studies presented in Table 1.5 (pages 56-57) were published in 2016 or beforehand, and the ACMG/AMP guidelines were published in 2015. However, the most recent study included in the table (Baronciani et al., 2021) did include overall assessments of variant pathogenicity using an online tool (Patel et al., 2017), suggesting that this approach may start to be adopted for new reports of potentially causal VWD variants.

In summary, due to the use of liberal phenotypic and genotypic definitions of VWD, the

authors of large sequencing studies of individuals with VWD have adopted an approach that is likely to have been sensitive for the detection of potentially causal VWD variants but not specific. These studies have been useful in highlighting the challenge of coupling phenotype to genotype, especially in VWD1 (Lillicrap, 2009), as well as the breadth of sequence diversity of the *VWF* gene (Babushok & Cuker, 2012). However, there is increasing appreciation that, genome-wide, the majority of rare variants are expected to be function neutral (Dudley et al., 2012) and that the (understandably) small size of control populations of early sequencing studies often resulted in the overattribution of pathogenicity to rare variants across a variety of disease groups (de Andrade et al., 2017; Lanktree et al., 2018; Walsh et al., 2017). Thus, there is a strong argument for the systematic reappraisal of previously reported VWD variants, which uses a data-led approach to the definition of VWD frequency (Subsection 1.2.3, pages 43-48), is informed by recent VWD (James et al. 2021) and ACMG/AMP guidelines (Richards et al., 2016), and harnesses the genotype and phenotype data generated on large populations.

1.2.5. Big data

Recent government-backed, collaborative research endeavours have collated high throughput sequencing (HTS) data on hundreds of thousands of individuals (Agrawal & Prabakaran, 2020). This provides, for the first time, datasets of sufficient size, to calculate, more accurately, the MAFs of rare variants potentially causal of a disease phenotype (Whiffin et al., 2017). The increasing ethnic diversity of the individuals included in these projects has highlighted the marked variation in the MAFs of such variants between different ancestry groups (Manrai et al., 2016). In populations where these genotype data have been combined with relevant medical information, this facilitates an examination of disease outcomes with potentially less bias than case series, which were, until recently, the main way of studying the clinical impacts of rare diseases (Bolignano & Pisano, 2016). In this subsection I first provide an overview of HTS, as I use the data generated by this approach throughout the thesis. I then describe the collections of individuals - UK Biobank (UKB) and the Genome aggregation database (gnomAD) - whose HTS data I use in my analysis. I finish by providing a background to the three databases that house the *VWF* variants which I interrogate in Chapter 3.

The HTS methods employed by Illumina™ are by far the most commonly used at the time of writing (Reuter et al., 2015) and are what I described here. This high throughput (or next generation) sequencing technology employs a principle similar to Sanger sequencing but scales this up (Kircher & Kelso, 2010). That is, the 'read' of the sequence is determined by the detection of the signal generated by Watson-Crick base pairing of a fluorescent deoxy-nucleotide matching its complementary nucleotide on the template sequence (Heather & Chain, 2016). What distinguishes HTS from Sanger sequencing is the parallel nature of this process such that multiple reads of the same region of the template can be generated at the same time by clonal amplification on a flow cell (Voelkerding et al., 2010). The read length is typically between 100-150 bases, a restriction imposed by the limitations of the reversible cyclic termination of the fluorescent deoxy-nucleotide (Chen et al., 2013). Hence this technology is also referred to as short read sequencing (Kumar et al., 2019). Read depth relates to the number of times a targeted nucleotide is seen in the data (Goldman & Domschke, 2014) and is lower for whole genome sequencing (WGS) than whole exome sequencing (WES) (Alfares et al., 2018). The short reads are mapped to a reference (see below) in the analysis step (Li et al., 2008) to generate the sequence of the diploid template. Variant calling is the detection of differences between what has been read from the template and the 'reference'. For the purposes of this thesis this means the Genome Reference Consortium human genome reference builds 37 and 38 (GRCh37 and 38 respectively [Church et al., 2011 and [Schneider et al., 2017]). Both of these represent updates to the original Build 35 assembly released nearly 20 years ago (International Human Genome Sequencing Consortium, 2004) which was constructed from bacterial artificial chromosome (BAC) clones containing overlapping regions (contigs) of the genome sequence (Zhang & Wu, 2001). Limitations of variant calling include the challenge of mapping reads to repetitive sequences of the genome (Li et al., 2008) and the reductionism that inevitably results from the idea of a single reference (derived from a handful of individuals [Snyder et al., 2015]), which is incompatible with the representation of allelic diversity across different ancestries (Shumate et al., 2020).

There are a number of large international population sequencing projects that are ongoing, which include the 100,000 Genomes Project (100,000 Genomes Project Pilot Investigators, 2021), Million Veteran Program (Gaziano et al., 2016), UKB, and gnomAD. I chose to use the latter two for assessment of *VWF* variant pathogenicity for two main reasons: accessibility of the data (Subsection 2.1.4, pages 73-75) and because the data did not specifically include individuals from projects that included rare inherited bleeding disorders, which would have carried the risk of enriching for the alleles of potentially causal VWD variants causing their MAF to appear higher than that expected in the background population.

UKB is a longitudinal, multi-centre follow-up study of ~ 500,000 prospectively enrolled individuals in Great Britain (Ollier et al., 2005). Participants were invited into the project by means of a mail invitation (Sudlow et al., 2015). Between 2006 and 2010 participants attended 22 recruitment centres and were aged between 38 and 73 years (Chudasama et al., 2020). At the initial study visit, participants completed a self-reported questionnaire which included details about their past medical history. Anonymised information from their primary and secondary electronic health record (EHR) is also linked to each participant (Eastwood et al., 2016). In addition blood samples were taken from which DNA and plasma were extracted. The DNA has been genotyped using a whole genome array containing markers for both SNVs and indels (Bycroft et al., 2018) and analysed by WES (Van Hout et al., 2020). Some of the limitations of the resource include its overrepresentation of individuals who are healthier and socioeconomically more advantaged than the general UK population (Batty et al., 2020), which has the potential to confound genotype-phenotype associations (Haworth et al., 2019). In contrast to UKB, which contains both individual level genotype and phenotype data, gnomAD only contains aggregated genomic data for ~ 195,000 individuals with removal of first- and second-degree relatives (Gudmundsson et al., 2022). Arguably one its strengths - the compilation of individuals from disparate epidemiological studies - is also its weakness, as the different sequencing methodologies used can introduce erroneous rare variant calls (Atkinson et al., 2022) despite attempts to control for this (Karczewski et al., 2020).

Descriptions of the five genotypically determined ancestry populations in both UKB and gnomAD is covered extensively in Chapter 3 (Subsection 3.4.1, pages 112-113; Subsection 3.4.5, pages 127-133). As detailed in Table 3.5 (page 128), there were sufficient numbers of individuals in each of five genetically determined ancestral super-populations to determine MAFs for *VWF* variants within each of these groups. Whilst this demonstrates that there is a move away from the European ancestry centricity for large population datasets there is still much progress to be made (Petrovski & Goldstein, 2016).

As the starting point for collation of *VWF* variants previously linked to a phenotype of VWD I turned to three databases (DBs). These are the National Center for Biotechnology Information (NCBI) Clinical Variant Database (ClinVar), European Association for Haemophilia and Allied Disorders Coagulation Factor Variant Database (EAHAD-CFDB), and the Human Gene Mutation Database (HGMD). The *VWF* variants in these DBs and pathogenicity classification is described in Subsection 3.3.3 (pages 102-107). However, I provide a brief overview here of the history, accessibility, and deposition strategy of each DB. ClinVar launched in 2012 and is run by the National Institutes of Health (Landrum et al., 2014; Landrum et al., 2018). Variants are deposited by academic or clinical genetics laboratories, with the latter contributing the majority of entries (Landrum & Kattman, 2018). Therefore, the key advantage of ClinVar is that it does not require variants to be published in order for them to be shared, which is done through an openly accessible website (Baker, 2012). Likewise, this is also the case for EAHAD-CFDB, hosted by the Leiden Open Variation Database (LOVD) (Fokkema et al., 2011). Submitters also in this case are researchers or clinical genetics laboratories, but those participating from the latter group seem to be confined to the Netherlands. Advantages over ClinVar include the inclusion of VWD specific fields such VWF and FVIII:C levels. HGMD is the oldest of the three DBs, launching initially as a book (Cooper & Krawczak, 1993) and then going online three years later (Krawczak & Cooper, 1997). This is the most comprehensive curated repository of published *VWF* variants linked to VWD, but has the disadvantage that the up-to-date version of the portal is only available via a commercial licence with the freely available version lagging three years behind in terms of content (Stenson et al., 2014).

1.2.6. Model systems and CRISPR/Cas9 gene editing

Functional genomic evaluation of potentially causal VWD variants complements the clinical and epidemiological assessment described previously. In this subsection I outline the advances that have been made using animal models of VWD. I then provide an overview of in vitro methods using human cells, highlight the recent use of CRISPR/Cas9 to knockout (KO) *VWF*, and flag up why there is a need for new EC approaches for modelling VWD.

Porcine VWF shares ~ 85% sequence homology with its human counterpart (Lozier & Nichols, 2013). Bone marrow transplant experiments in pigs with VWD3 have revealed two important insights (Denis & Wagner, 1999). First, VWF is not endocytosed into platelets (Roussi et al., 1995). Second, that the majority of plasma VWF derives from the endothelium (Bowie et al., 1986; Nichols et al., 1995). Experiments in mice have been helpful in developing our understanding of VWF clearance mechanisms (Subsection 1.2.4, page 53). However, there are a number of ways in which animal models differ with respect to VWF physiology which means caution is required when translating these findings to humans. For example, in VWD3 pigs the FVIII level is not reduced to the same extent as in humans (Denis & Wagner, 1999), which may reflect the higher basal levels in the pig (Bowie et al., 1973).

For variants likely to affect the intracellular handling of VWF, in vitro cell models have been used. Heterologous systems refer to the transfection of a plasmid containing the cDNA of the ORF of VWF into cell lines which do not endogenously express VWF but have high transfection efficiencies. HEK293T are the most commonly used because when they ectopically express VWF, this forms storage organelles that resemble WPBs (Michaux et al., 2003). They have proven useful for studying VWD variants affecting trafficking and multimerisation (Wang et al., 2011). However, as an overexpression system, they are less useful for modelling pLoF variants or for variants that result in reduced but not absent translation (Rodenburg, 2018). In such scenarios studying the variant in ECs is the preferred option as the variant will be under the control of *VWF*'s

endogenous promoter. There are a couple of EC models which have proven useful in VWD to-date.

Human umbilical vein endothelial cells (HUVECs) are fetal endothelial cells (Oettel et al., 2016) that line the cord providing the connection between mother and fetus during pregnancy. As they reliably produce elongated WPBs (Zenner et al., 2007) these are frequently used to study the storage and release of VWF in many different contexts (McCormack et al., 2017). HUVECs also successfully recapitulate VWD phenotypes such as increased platelet binding in VWD2B (De Groot et al., 1989) and reduced mRNA expression and basal secretion of VWF in VWD1 (Ewenstein et al., 1990). The downside is that there is only a narrow window of opportunity in which to collect HUVECs in the context of individuals with VWD and this is often before diagnosis. Furthermore HUVECs' refractoriness to transfection is a major roadblock in the use of genome editing tools such as CRISPR/Cas9 (Abrahimi et al., 2015). Therefore HUVECs have limited scope in modelling VWD.

CRISPR/Cas9 has recently been used to manipulate *VWF* in endothelial cell colony forming cells (ECFCs). I describe my CRISPR/Cas9 experiments in Chapter 5 (Subsection 5.5, pages 220-232). Therefore I provide only a brief summary of it here before moving to ECFCs. Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas is the prokaryotic, highly flexible, acquired immune system evolved to fight other bacteria and viruses (Horvath & Barrangou, 2010). In this system the invading DNA sequence (the spacer) with a defined short nucleotide sequence (the proto-spacer-adjacent motif [PAM])) is incorporated into the host genome (Makarova et al., 2011). Then in response to subsequent attack from the same pathogen this immune system is activated with the spacer and PAM directing the site at which the Cas endonuclease cleaves the invading DNA. In 2011, two groups simultaneously demonstrated that components of *Streptococcus pyogenes* CRISPR/Cas9 system could be delivered into human cells. They showed the system could be guided to a targeted portion of the human genome using a 20 bp (Mali et al., 2013) or 30 bp (Cong et al., 2013) spacer with a complementary sequence. At this position a double stranded break is created. Repair by non homologous end joining

(NHEJ) results in indels which can frameshift the sequence and introduce PTCs, or homology directed repair (HDR) using a guide sequence can direct a specific sequence change (Ran et al., 2013).

ECFCs are also known by other names including blood outgrowth endothelial cells. They can be derived by culturing peripheral blood mononuclear cells (PBMCs) obtained either from cord (Ingram et al., 2004) or peripheral blood (PB) (Lin et al., 2000). Cord blood (CB) ECFCs produce more colonies than those derived from PB. Therefore they provided a sufficient number of starting cells for a recent CRISPR/Cas9 KO of VWF which ablated both its expression and WPB production (Schillemans et al., 2019). PB-derived ECFCs are a much more accessible substrate than CB and have been used recently to highlight the heterogeneity of EC phenotypes in VWD1 (Starke et al., 2013). However, in up to 10% cases ECFCs cannot be generated (Ormiston et al., 2015). Furthermore ECFCs limited proliferative capacity places a ceiling on clonal expansion post CRISPR/Cas9 and the subsequent availability of sufficient cells for downstream assays (Schillemans et al., 2019). Therefore, as tackled in Chapter 5, there is a need to develop an alternative EC model of VWD which is less dependent on VWD donor individuals, can generate sufficient numbers of cells for genome editing, and has the functionality to explore the cellular consequences of variants in *VWF*.

1.3. Summary

Of the rare inherited bleeding disorders, VWD is the most common (Favaloro, 2011). However, until recently, the lack of a consensus of how to define VWD has hampered accurate estimations of its prevalence (Connell et al., 2021). In Subsection 1.2.3 (pages 44 - 48), I applied the most recent, internationally-agreed, definition of VWD (James et al., 2021) to historic epidemiological studies of VWD (Table 1.3, pages 45-46). This highlighted that, across a number of different countries, community VWD prevalence is fairly consistent and much higher than that based on the number of patients referred to, and seen at, haemophilia centres. Therefore, it follows that the study of VWD should not be limited to those patients seen in these specialised clinics, but should be also explored in the

general population (Sidonio Jr et al., 2020). As highlighted in Subsection 1.2.5, there are cohorts of hundreds of thousands of genetically diverse individuals who have undergone high throughput sequencing (HTS) of their whole exomes and genomes. This revolution coupled with the recent ACMG/AMP guidelines that aid variant interpretation (Richards et al., 2016), means that, there is now an unprecedented opportunity to accurately determine the MAF of potentially causal VWD variants and explore potential disparities between different ancestral populations. This is essential given that previous sequencing studies linking genotype to phenotype in VWD have used widely varying definitions of VWD, MAF thresholds above the prevalence for the disorder, and only had small, European-centric, reference populations available for comparison (Subsection 1.2.4, pages 55-58; Table 1.5, pages 56-57).

In addition, the coupling of genotype data to the electronic health record (EHR) in UK Biobank (UKB) participants opens up the opportunity to study the effect of *VWF* variants on bleeding phenotype. This is relevant given that the systematic appraisal of referred patients with VWD using bleeding assessment tools (BATs) has indicated that these individuals have a higher haemorrhagic burden than those without VWD (Rodeghiero et al., 2005; Tosetto et al., 2008) (Subsection 1.2.1, page 32), including heterozygotes of pLoF variants (Bowman et al., 2013; Castaman et al., 2006) (Subsection 1.2.4, page 54). However, BATs have limitations including recall bias and the selective nature and small sample size of control individuals used as comparators. Although population-level HTS data can provide relevant information about the MAF and predicted consequence of a *VWF* variant, it remains important to experimentally validate those deemed to be potentially pathogenic for VWD (Swystun & James, 2017). This is because VWD can potentially arise secondary to disruption of its complex biosynthesis in ECs, function after release into the bloodstream, or clearance from the circulation (Subsections 1.1.2 - 1.1.8). In vitro models to evaluate the former have been limited to heterologous cell lines transfected with a vector carrying *VWF*, such as HEK293Ts, which cannot model pLoF variants, and primary endothelial cells which have finite replication capacity and are challenging to genetically manipulate. Therefore, given the hundreds of variants reported to cause VWD (De Jong and Eikenboom, 2017) (Subsection 1.2.4, page 49), there remains an urgent need

to develop alternative cell models in order to mechanistically understand their effects as this could open the way to alternative therapeutic approaches beyond traditional prohaemostatic agents (Subsection 1.2.1, pages 33-34), such as small-interfering RNAs recently employed to correct a multimerisation defect due to a variant causal of a VWD2A phenotype (De Jong et al., 2020).

1.3.1. Hypotheses

1) Exploration of potentially causal VWD variants in UKB and gnomAD will show:
    a) There are carriers of variants previously deemed causal of VWD in the general population;
    b) That following determination of the MAF, and review of the published literature, a substantial proportion of these variants will be shown not to be credible pathogenic variants for VWD;
    c) Those variants which are deemed pathogenic for VWD will result in a bleeding phenotype in the UKB participants who carry them.

2) An alternative in vitro, endothelial cell approach can be developed that:
    a) Models important aspects of VWF biology relevant to VWD;
    b) Is amenable to genetic editing for the purposes of evaluating the pathomechanism of potentially causal VWD variants.

1.3.2. Aims

1) Build a combined resource of *VWF* variants reported to be associated with a phenotype of VWD from three major variant databases (DBs): ClinVar, EAHAD-CFDB, HGMD. This resource will be known as VWDbase. Restrict the variants included in VWDbase to those amenable to detection by HTS (SNVs and insertion deletions (indels) $\leq$ 50 bp in length).

2) Based on the assessment of VWDbase variants by their source DB(s), narrow the scope of VWDbase to a set of variants with an increased prior probability of being

causal of VWD. This set will be known as putatively aetiological VWD variants (PAVVs).

3) Apply the whole exome sequencing (WES) data of 140,327 participants in UKB and 125,748 participants in gnomAD to evaluate PAVVs for the purpose of:
    a) Accurately determining their overall MAF;
    b) Generating an aggregated MAF in each of five major, genetically-determined ancestral populations (African/African-American [AFR], Admixed American/Latino [AMR], East Asian [EAS], European [EUR], South Asian [SAS]).

4) Systematically appraise PAVVs which are present in the WES data of at least one UKB participant based on the outcome of 3) and the published literature, focussing on whether the MAF of the variant is consistent with the prevalence of VWD and if the previously reported clinical and laboratory data support pathogenicity. This approach will be used to create a shortlist of PAVVs that can be 'accepted' as pathogenic for VWD.

5) Use the EHR of UKB participants to devise an approach for ascertaining the burden of bleeding symptoms experienced by these participants since this data first became available. Focus on UKB participants carrying PAVVs, specifically those accepted as pathogenic, to explore whether they experience an increased risk of bleeding as compared to participants without PAVVs. Stratify this analysis according to whether the variant is a predicted loss of function (pLoF) or protein-altering, because of the ongoing debate as to the expressivity of haemorrhagic symptoms in pLoF heterozygotes (Subsection 1.2.4, page 54).

6) Utilise VWDbase, particularly the PAVV list to analyse the whole genome sequencing data of patients with molecularly unexplained VWD for the purpose of identifying an aetiological variant suspected to affect the synthesis of EC-VWF.

7) Model the *VWF* variant identified in 6) using a new in vitro approach. This will be using endothelial cells derived from human induced pluripotent stem cells (iECs), as introduced in the background to Chapter 5 (pages 196 - 198). Specifically test whether the iECs:
    a) Have properties consistent with endothelium including expression of relevant surface immunotype markers; capacity to synthesise VWF transcripts and protein; release VWF in response to agonist stimulation.
    b) Can be made to model the variant identified in 6) by genetically editing them using CRISPR/Cas9 and comparing the resultant clones to wild type iECs.

2. Materials and methods

2.1. Bioinformatics and clinical studies

2.1.1. Data storage, format, and analysis

All clinical and sequencing data were stored and analysis performed using the password-protected Cambridge University HPC. No individual identifiers such as name or date of birth were stored. The majority of analyses and all statistical tests were performed in R version 3.6.3 using the interactive platform RStudio (Build 351). The packages used for analysis that were installed in addition to base R are listed in Table 7.3 (page 249-250). Unix shell scripts or commands were used for some preliminary analysis, principally to filter large sequencing and variant call files for the relevant data, which was then small enough to be loaded into R. Nucleotide and amino acid reference sequences were read into R in FASTA format (Pearson & Lipman, 1988). Variant data from reference databases, UKB, and the NIHR BioResource was stored in Variant Call File (VCF) format (Danecek et al., 2011). Filtering of VCFs was carried out using BCFtools (Li et al., 2009) v1.9 from the command line in order to create .txt files containing only the region of interest prior to being read into R.

2.1.2. Statistical tests

For comparison of categorical variables, 2 x 2 contingency tables were created, and a two-sided Fisher's Exact Test was used to determine odds ratios (ORs). Unless stated otherwise, ORs were calculated using this method. To compare central tendency between two populations, both were analysed using the Shapiro-Wilk Normality Test (Royston, 1995) to assess whether they were normally distributed. If they were then then means were compared using a two-sided t-test. However, if one or both populations were not normally distributed then medians were computed and the populations compared using a two-sided Wilcoxon Rank Sum Test (hereafter Wilcoxon Test). All other, less commonly performed statistical tests are specified in the text.

2.1.3. VWDbase

VWDbase was built to contain unique SNVs and indels in *VWF* that had a prior association with VWD. Indels were defined as insertion-deletion variants ≤ 50 bp. The final filtering step when extracting data from each of the *VWF* DBs was to filter for SNVs and indels.

VWF variants in ClinVar were downloaded from the FTP site (https://ftp.ncbi.nlm.nih.gov/pub/clinvar/) on 20 March 2021 in VCF format with GRCh38 positions. Only variants in the *VWF* scaffold region, i.e. between and including chromosome (chr) 12 positions 5948877 and 6126975, were retained. The variant table was supplemented with further information accessible in a different place on the ClinVar website https://www.ncbi.nlm.nih.gov/clinvar/?term=VWF%5Bgene%5D, accessed on 20 March 2021. A HGMD Professional account was created and used to access version 2019.4 on 20 December 2019. HGMD listed summaries of variants under different categories. The .html file of each summary page for *VWF* was downloaded individually and then aggregated using the 'rvest' R package. Variants tagged as 'complex' or 'regulatory', with a cDNA name indicating a sequence change longer than 50 bp (e.g. c.5312-104_5455+642), and those with a phenotype label not pertaining to VWD were removed. LOVD v.3.0 Build 22 of EAHAD-CFDB was downloaded from https://databases.lovd.nl/shared/variants/VWF/unique on 03 January 2020. All SNVs and indels were retained except for four putative gene conversion events with the VWF pseudogene, c.3686_3692con, c.3789_3797con, c.3789_3835con, c.3797_3835con (James et al., 2007) as the converted sequence could not be established from the deposition in EAHAD-CFDB or the original publication.

The *VWF* scaffold was used to facilitate merging of the three *VWF* DBs and is defined in Subsection 3.3.1 (pages 101-102). The first step was to define the correct transcript. Although there are nine predicted transcripts of VWF (Ensembl, 2021), only ENST00000261405.10 corresponds to the full-length 2813 amino acid polypeptide chain of VWF and so this one was used. The sequence of nucleotides comprising the

ENST00000261405 transcript in GRCh38 coordinates were downloaded from Ensembl on 25 June 2020. A total of 175,082 nucleotide positions were identified. The transcription start site (TSS) of *VWF* is known to be located at the sixth nucleotide of exon 1 (Bonthron & Orkin, 1988). The GRCh38 positions of the TSS and transcript end site were downloaded using an online query of Ensembl BioMart (https://www.ensembl.org/biomart/martview) on 30 December 2020 and the start position of the scaffold was defined as the nucleotide position 2305 nucleotides upstream of this. The end of the scaffold corresponded to the last nucleotide of the transcript because no variants were identified in the curated repositories that were 3' of this position. The start position of the transcript, 12-6125670, has a higher number than the end position, 12-5948877, because *VWF* is assigned to the reverse strand of chromosome 12. To append nucleotides to each scaffold position, the GRCh38 FASTA sequence was downloaded from Ensembl on 19 February 2022. In addition, the start and end coordinates of the scaffold in GRCh37 coordinates, 12-6236141 and 12-6058043 respectively, were determined using the Ensembl Assembly Convertor (http://www.ensembl.org/Homo_sapiens/Tools/AssemblyConverter), which uses CrossMap (Zhao et al., 2014) for the coordinate liftover, and the FASTA sequence of the nucleotides between these coordinates was downloaded. Subsequently, the GRCh37 positions and nucleotides were annotated onto corresponding GRCh38 positions and nucleotides. To ensure concordance between GRCh37 and GRCh38 across the entire scaffold, the two downloaded nucleotide sequences were compared and found to be identical.

After creation of the scaffold, the variants extracted from each of the three *VWF* DBs were then mapped to it. Variants downloaded from HGMD and EAHAD-CFDB could not be mapped by GRCh38 position to the scaffold as this was not consistently provided in these DBs. Therefore nucleotides, and where applicable, amino acids were annotated in accordance with the international standards of the Human Genome Variation Society (HGVS) nomenclature v20.05 (den Dunnen et al., 2016; HGVS, 2020) and 'root' terms defined. For HGVSc, variants have the structure 'c.{transcript position}{transcript reference nucleotide}>{transcript alternate nucleotide}'. Therefore, the 'root' for the

scaffold contained all terms prior to '>'. For HGVSp, variants have the structure p.{reference amino acid}{residue number}{alternate amino acid}, so for the purposes of the scaffold the first two terms were determined, as exemplified below.

All 8442 nucleotides involved in the coding sequence of *VWF* (from the first nucleotide of the translation initiation codon to, and including, the last nucleotide of the translation stop codon) were named c.1 through to c.8442 and suffixed with the nucleotide of the corresponding cDNA. However, given that genomic coordinates from GRCh37 and GRCh38 are from the strand arbitrarily defined as the forward strand, and *VWF* is transcribed from the reverse strand, the nucleotide of the cDNA is complementary to the genomic position. For example, the first nucleotide of the coding sequence of *VWF* is located on chromosome 12 at position 6123196 of GRCh38 and the forward strand nucleotide is T, so this corresponds to c.1A. Positions in the 5' and 3' regions of introns were annotated with respect to their position from the last nucleotide of the preceding exon (5') or the first nucleotide of the subsequent exon (3'). For example, the last nucleotide of exon 5 is c.532, so the 16th nucleotide of the intron between exon 5 and 6 (intron 5-6), is named c.532+16A, and the first nucleotide of exon 6 is c.533, so the 10th nucleotide from the 3' end of intron 5-6 is c.533-10T. Positions 5' of the translation initiation codon, and 3' of the translation stop codon to the last A of the poly-A (3'UTR), were prefixed with "-" and "*" respectively and numbered, for example the 10th nucleotide upstream of c.1A was labelled as c.-10G (as there is no c.0) and downstream of c.8442A was labelled as c.*10A.

To annotate in protein coding information, the FASTA amino acid sequence of the isoform of VWF corresponding to the ENST00000261405.10 transcript was identified as P04275-1 (Ensembl, 2021) and then downloaded (UniProt, 2011), such that all coding triplets were annotated with the corresponding amino acid residue, and then formatted using the HGVS nomenclature, e.g. the proline residue at sequence position 1480 was notated as p.Pro1480.

After collation of reported VWD variants from all three repositories, the names used for indel variants were standardised in accordance with HGVS nomenclature (den Dunnen et al., 2016). The variants were systematically tidied: unnecessary trailing letters from

deletions were removed and incorrect annotations were amended. For further simplification ≤50 bp variants labelled as conversions with suffix '-con' were instead labelled as delins variants (Hong, 2020). This had the effect of removing redundancy (a known problem with indel entries in variant databases [Assmus et al., 2013]). In order to obtain standardised, left-aligned positions for each of the indels, these were inputted, on bulk, into Ensembl Variant Recoder (https://www.ensembl.org/Homo_sapiens/Tools/VR), the VCF read back into R and the POS, REF, and ALT sequences re-annotated into VWD base.

2.1.4. UKB and gnomAD

To obtain access to phenotype and genotype data I became an approved collaborator of the approved UKB project, 13745, the Principal Investigator of which is Dr Astle (Acknowledgements, page 7). Dr Stefanucci (Acknowledgements, page 7) downloaded the WES VCF data, selected only genetically unrelated participants, and annotated in gnomAD v2.1 allele frequencies (Karczewski et al., 2020). He then filtered these data to retain individuals with Putatively Aetiological VWD Variants (PAVVs), and determined the genetically determined ancestry super-population group of each individual (Table 3.3, page 113). This was based on the 1000 Genomes Project definition (International HapMap Consortium, 2005) using the Somalier tool (Pedersen et al., 2020). Somalier uses a predetermined set of common variants which are polymorphic in different ancestry groups. It uses these to perform a principal component analysis and subsequently determine the ancestry from genotyping data.

The exome VCF data used was the data-field 23151 release (https://biobank.ndph.ox.ac.uk/ukb/field.cgi?id=23151) which included 200,000 participants. The protocols are described in the paper that accompanied the initial tranche of exomes being released (Van Hout et al., 2020). In brief, genomic DNA from each participant was analysed by Regeneron Genetics using an exome capture technique. The sequencing was performed on an Illumina NovaSeq 6000 using 75 bp paired-end reads with ~ 95% of the targeted regions covered at 20x read depth.

The effect of the 22 UKB accepted pLoFs were assessed using published data regarding bleeding and VWF levels (Figure 3.7, pages 120-121). Only individuals in whom the pLoFs were in hetero- or homozygosity in the absence of other *VWF* variants potentially causal of VWD were included. Hence, if the publication used a limited sequencing strategy they may have missed a relevant variant on the other *VWF* allele. Individuals were adjudicated to have a bleeding propensity if the bleeding score was ≥ 5 (consistent with Tosetto et al., 2007), if the bleeding was described as moderate or severe (Baronciani et al. 2000; Casaña et al., 2000) or pronounced (Zhang et al., 1992), or if ≥ 2 different bleeding symptoms were listed. Individuals were categorised as having no bleeding propensity if there was a documented absence of bleeding, or the symptoms were described as mild (Casaña et al., 2000) or vague (Zhang et al., 1992) or if only one bleeding symptom was listed (Lethagen et al., 2002). NA was used if the bleeding history was not provided or there were confounders (i.e. in Ahmad et al., 2010 the individuals with VWD were also carriers of or have Glanzmann Thrombasthenia).

The 194 UKB PAVVs and candidate variants from the NBR-VWD analysis were annotated with v1.6 Combined Annotation-Dependent Depletion (CADD) scores (Rentzch et al., 2021) by uploading a VCF of these variants onto the CADD server (https://cadd.gs.washington.edu/score) on 14 March 2022.

The hospital episode statistics (HES) data were those under the field 'Category 2002' (UKB, 2020a) and were made available to the UKB from the NHS. Drs Sun and Stefanucci (Acknowledgements, page 7) downloaded these data from UKB in March 2021 and assigned the date of the first bleeding event under each ICD-BAT category using the CALIBER algorithm. These data included age, sex, UKB assessment centre, and date of recruitment. I carried out the remainder of the analysis as described in Section 4.3 (pages 141-166).

Each recruitment centre was open for a median duration of operation of 348 days between 2006 and 2010. Age of participants on 01 April 1997 (the point from which English HES data was available) was estimated by calculating the difference between the mid date on

which an assessment centre was operational and 01 April 1997 and then subtracting this from the participant's assessment age.

2.1.5. NIHR BioResource Rare Diseases

Access to the NIHR BioResource Rare Diseases (NBR-RD) phenotype and sequencing data was granted following approved study (NBR33) and genotype access applications in 2018. The phenotype data was made available to me by Dr Ernest Turro and represented the collective work of the members of the NBR Bleeding, Thrombotic and Platelet Disorders (NBR-BPD) domain (Turro et al., 2020; Westbury et al., 2015); additional clinical data was then retrieved using the Gene Docs portal (Acknowledgements, page 8). The NBR-RD whole genome sequencing (WGS) pipeline is outlined in the consortium paper (Turro et al., 2020). In brief, samples were sequenced with short reads at a mean coverage of 35x on an Illumina HiSeq 2500 or HiSeq X instrument. The read length of NBR 1-5, 7, 8, 10 was 150 bp. For NBR 6 and 9 it was 125 bp. For my reanalysis of the data of participants NBR 1-10, the annotation with gnomAD v3.0 MAFs was using the VCF downloaded 25 June 2020 from gnomad FTP site https://gnomad.broadinstitute.org/downloads and included all variants that passed the quality control (QC) filter. The position of the c.8155+6T>A variant in NBR 1 was confirmed by visualisation of the BAM file in the Integrative Genomics Viewer 2.3 (Robinson et al., 2017). Regions of homozygosity were searched for in NBR 1's WGS data using PLINK (Purcell et al., 2007) and this analysis was performed by Dr Sanchis-Juan (Acknowledgements, page 8).

2.1.6. Clinical evaluation of NBR 1 and control participants

For both the studies outlined below, participants provided written consent. Both studies were approved by the East of England Research Ethics Committee (REC). NBR 1 consented into the "Genetic analysis of inherited platelet and bleeding disorders (BPD)" (REC 10/H0304/66) study, also known as BRIDGE. The ethical approval for this study permitted 100 ml of blood to be taken. The study visit was supported by additional clinicians who were members of the NBR-BPD consortium (Acknowledgements, page 8).

Five participants were approached to volunteer as healthy controls, two for the analysis of platelet cDNA (Subsection 2.2.1, pages 76-79; Subsection 4.5.1, pages 181-185) and three to obtain PBMCs to derive ECFCs (Subsection 2.2.4, pages 84-88; Section 5.3, pages 199-209). The study into which they were recruited was named "Genetic analysis of platelets in healthy individuals" (REC 10/H0304/65) and allowed for medical history to be recorded and for up to 50ml of blood to be taken.

Plasma VWF:Ag measurements were carried out on NBR 1, C3, C4, and C5. The samples for analysis were prepared as follows. Citrate blood tubes were centrifuged at 2,500 g (acceleration [acc] 9; brake 3) for five minutes at room temperature (RT). The supernatant was then transferred to microcentrifuge tubes. These were then spun at 10,000 g in a microcentrifuge for 10 minutes at RT. The supernatant was then pipetted into cryovials in 500 µl aliquots and frozen at -80ºC. The samples were then transferred on dry ice to Maastricht University Medical Center for analysis (Acknowledgements, page 8).

2.2. Laboratory experiments

The list of reagents used are in tables in Appendix 7.3 (pages 249-256). Reagents used across experiments are in Table 7.4. Tables 7.5 - 7.12 then outline the reagents specific to each of the subsections as outlined below, with each table corresponding to a separate subsection.

2.2.1. Platelet cDNA

For platelet isolation and cDNA synthesis I was assisted by Ms Frances Burden (Acknowledgements, page 8). From NBR 1, 36 ml of blood was taken into citrate tubes. Platelet rich plasma (PRP) was generated by slow centrifugation. All centrifugations were carried out at 150 g. The blood was first centrifuged for 20 minutes (acc 9/brake 0). 14 ml of PRP was harvested to which 1ml of 0.1 M EDTA/sodium chloride solution was added. This was then centrifuged for 20 minutes (acc 9/brake 4). The supernatant was then transferred to a new 15 ml centrifuge tube and centrifuged for 10 minutes (acc 9, brake 4).

Whilst the supernatant was being centrifuged, leucodepleting CD45 beads were prepared. For every 2ml of PRP, 50µl of CD45 beads were required. Therefore, as there was 14.5 ml of PRP remaining, 362.5 µl of beads were prepared. This volume of beads was added to a 15 ml centrifuge tube containing 10 ml of Dulbecco's phosphate buffered saline (D-PBS) to which 10mM citrate solution was added which was then left at RT. Before the centrifugation of the PRP had stopped, the beads were magnetised for 2 minutes and the supernatant discarded. The PRP supernatant was then transferred to the centrifuge tube containing the beads. The PRP and bead mixture was then placed on a rotator for 20 minutes at RT and then magnetised for 2 minutes. The supernatant was then transferred to a new 15 ml centrifuge tube and magnetised again, from which a final volume of 13 ml of leucodepleted PRP was obtained, 8 ml of which was mixed with 1 ml of TRIzol™ and then frozen at -80ºC.

The platelet TRIzol™ solutions were subsequently thawed in order to extract RNA for reverse transcription. Two Maxtract tubes were used. 1 ml of platelet TRIzol solution was added to each phase lock tube, to which 200 µl of chloroform was then added. These were shaken for 15 seconds, incubated at RT for three minutes and then centrifuged at 12,000 g for 15 minutes at 4ºC. The upper aqueous layer was then transferred to a fresh microcentrifuge tube. 0.5µl glycogen at an initial concentration of 20 µg/ul and then 0.5 ml isopropanol were added to each tube. Each tube was then mixed, incubated at RT for 10 minutes, and then centrifuged at 12,000 g for 10 minutes to precipitate out the RNA. The supernatant was discarded and then RNA pellet was washed in 1 ml of 75% ethanol and then centrifuged at 8,000 g for 10 minutes. This step was repeated once. After removal of the ethanol, the RNA pellets were then dried for seven minutes at RT. Both pellets were then resuspended in nuclease free water and combined to a total volume of 20 µl. The RNA concentration of the solution was determined at 35.6 ng/µl using a Qubit™ RNA Assay Kit.

A High-Capacity cDNA Reverse Transcription Kit was used to convert RNA into cDNA. A 1.3 x master mix was made up using the kit ingredients. After mixing, a 10 ul aliquot of the master mix was then removed and then added to 200ng of RNA. Further nuclease free

water was added to bring the total volume to 20 µl and then mixed. This was then added to a thermal cycler with the following settings: 25ºC for 10 minutes, 37ºC for 120 minutes, 85ºC for 5 minutes, and then 4ºC overnight until the following morning. The concentration of cDNA was then measured using the NanoDrop™ spectrophotometer and diluted to 100 ng/µl. The same method was used, separately, to generate cDNA from frozen TRIzol™ solutions of platelets obtained from control participants, C1 and C2 (Subsection 2.1.6, pages 75-76). The steps below were then carried out simultaneously on the cDNA from NBR 1, C1, and C2. Oligonucleotide primers were designed by Dr Mattia Frontini (Acknowledgements, page 8), for amplification of *VWF*, *GAPDH*, and *ACTB* by polymerase chain reaction (PCR) (Table 2.1, below).

Gene	Target	Strand	Oligonucleotide sequence
VWF	Exon 49	Forward	GCCCACCCTTTGATGAACACAAGT
VWF	Exon 52	Reverse	GCACTCCATGGCATTGAGAACCTC
VWF	Exon 50/51	Forward	ACCTGCTGTGACACATGTGAGGAG
VWF	Exon 36	Forward	CCCCAAGACTTTTGCTTCAAAGAC
VWF	Exon 37	Reverse	CACAGAAATCAGGTGTCCTCCAGT
GAPDH	Exon 4	Forward	AATATGATTCCACCCATGGCAAAT
GAPDH	Exon 6	Reverse	CTGATGATCTTGAGGCTGTTGTCA
ACTB	Exon 4	Forward	GAAATCGTGCGTGACATTAAGGAG
ACTB	Exon 5	Reverse	CAGTGATCTCCTTCTGCATCCTGT

Table 2.1 | Primers used for the amplification of cDNA from NBR 1 and control participants.

The primers were reconstituted in nuclease free water to a concentration of 10 µM. PCR reactions were carried out using a Phusion™ HF DNA Polymerase, with 200 nM forward and reverse primers, 2 ng/ul cDNA template. Five different primer pairs were used: *VWF* exon 49 (forward)/exon 52 (reverse); *VWF* exon 50/51 (forward)/exon 52 (reverse); *VWF* exon 36 (forward)/exon 37 (reverse); *GAPDH* exon 4 (forward)/exon 6 (reverse); *ACTB* exon 4 (forward)/exon 5 (reverse). These were set up as separate reactions with the cDNA from NBR 1, C1, and C2. Once the PCR was complete the amplicons were analysed by gel electrophoresis. A 1.5% agarose gel was prepared in Tris-Borate-EDTA (TBE) buffer to which SYBR safe gel stain was added. A 100 bp DNA ladder was used and 6 x LD loading dye added to samples. The gel ran for 35 minutes at 90 volts and then

photographed on a UV transilluminator. The exon 52 primer, final concentration of 3.2 pmol/µl, was added to 5 µl of PCR product from NBR 1 and C1 in separate tubes. This was then sent to Source BioScience for Sanger sequencing. The .ab1 files were then aligned and analysed using Benchling (https://benchling.com/).

2.2.2. Site directed mutagenesis of p.Gly2706Ter

The 13.8 kb pcDNA3.1-WT-VWF plasmid (Figure 4.17, page 187) contained the ORF of VWF (Zhang et al., 2019). The plasmid was supplied as an agar stab. This contained NEB® Stable E.coli transformed with pcDNA3.1-WT-VWF. On arrival, the bacteria were aspirated and transferred to a 13 ml capped tube containing 5 ml of Lowenstein broth with 1:1000 ampicillin. The tube was then transferred to a shaker at 37ºC, 250 rpm for three hours. This 5 ml suspension was then transferred to a Erlenmeyer flask containing 150 ml Lowenstein broth and transferred back to the shaker and incubated at 37ºC, 250 rpm overnight. DNA was then extracted using a Maxiprep kit. The plasmid was eluted into 1.5 ml Tris-EDTA (TE) buffer and frozen at -20ºC.

Site-directed mutagenesis (SDM) was used to alter the first codon of exon 50 to a termination codon (p.Gly2706Ter). The sequence of pcDNA3.1-WT-VWF deposited in Addgene was downloaded. This was used to extract the sequence of 51 nucleotides containing the target codon to be altered (GGT corresponding to p.Gly2706) at its centre. This sequence was then used to design mutagenic primers. The online PrimerX tool (Lapid & Gao, 2006) was used. The parameters used in the design of the primers were: desired length of 25 - 45 nucleotides; melting temperature of 78 ºC; GC content between 40 and 60%; 5’ and 3’ flanking sequences between 11 and 21 bp; termination of primer sequences with G or C. These settings generated one primer pair with the desired mutation indicated (Table 2.2, page 80).

The mutagenic primers were then purchased from Merck, with the high performance liquid chromatography method of purification. These were resuspended in nuclease free water to a final concentration of 100 µM. The QuikChange XL Site-Directed Mutagenesis Kit was used. After thawing the kit reagents on ice, 50 µl suspensions were set up in thin-walled

Strand	Oligonucleotide sequence (5' to 3')
Forward	CACAAGTGTCTGGCTGAGGGATAAAAAATTATGAAAATTCCAGGC
Reverse	GCCTGGAATTTTCATAATTTTTTATCCCTCAGCCAGACACTTGTG

Table 2.2 | Mutagenic primers used in the site directed mutagenesis of pcDNA3.1-WT-VWF. The nucleotides which are underlined represent the nucleotide sequence to be altered. This change is GGT to TAA at the position indicated. The desired result was to create the p.Gly2706Ter variant.

PCR tubes. The suspensions contained: 5 µl reaction buffer; 2 µl (10 ng) of the maxiprep'd pcDNA3.1-WT-VWF template; 1.25 µl (125 ng) of the forward and reverse mutagenic primers; 1 µl dNTPs; 3 µl QuikSolution; 1 µl *PfuTurbo* DNA polymerase. And the remaining volume was made up to 50 µl with nuclease free water. A control reaction was also set up using the pWhitescript control plasmid and primers supplied with the QuikChange kit. Both reactions were then added to a thermal cycler with the settings as detailed in Table 2.3 (below) and left overnight.

Stage	Cycles	Temperature (ºC)	Time
1	1	95	1 minute
2	18	95	50 seconds
		60	50 seconds
		68	14 minutes[1]
3	1	68	7 minutes
4	1	4	To the following morning

Table 2.3 | Thermal cycling conditions used for SDM. [1]The QuikChange XL manufacturers recommend 1 minute/kb for this step, so I used 14 minutes based on pcDNA3.1-WT-VWF being 13.9 kb in size.

The following morning, 1 µl of DpnI was added to the product of each thermal cycling reaction. These were then incubated for two hours. DpnI digests methylated DNA. The unmutated pcDNA3.1-WT-VWF template DNA was methylated. In contrast, the mutant strand synthesised in the thermal cycling reaction (Table 2.3, above) is not dam methylated (Carey et al., 2013). Therefore, the *Dpn* I digestion step was used to select for the plasmid containing the site-directed mutant. The SDM plasmid and the pWhitescript control were

transformed into XL10-Gold E.coli. This was done in order to obtain sufficient DNA for sequencing to confirm whether the plasmid contained the site-directed mutant. In addition, the pUC18 vector (supplied with the QuikChange kit) was used as a control for transformation competence. The transformation steps were as follows. The XL10-Gold E.coli were thawed and three 13 ml capped tube tubes were chilled on ice. Once thawed 45 µl of the E.coli suspension and 2 µl of β-Mercaptoethanol was added to each tube. The mixtures were incubated on ice for 10 minutes. Two µl of the site-directed mutant plasmid was added to one of the tubes. This was then repeated for pWhitescript and pUC18 into each of the remaining tubes. All three mixtures were incubated on ice for 30 minutes. The tubes were heat-shocked at 42ºC for 30 seconds and then incubated on ice for 2 minutes. 500 ul SOC outgrowth medium was added to each tube. The suspensions were incubated on a shaker at 37ºC, 250 rpm for one hour. During the incubation step, six agar plates were poured using Amp Agar X-Gal mix and allowed to set. Each agar plate was spread with 250 ul of the transformation reactions and incubated overnight at 37ºC. Therefore, there were 2 plates for each of the three conditions. The following morning, one discrete colony was identified on one of the two plates spread with the site-directed mutant plasmid. This was transferred using a toothpick to a 13 ml capped tube containing three ml of Lowenstein Broth with 1:1000 ampicillin and cultured overnight on a shaker at 37ºC, 250 rpm overnight. The following morning, a miniprep extraction of DNA was performed and the DNA eluted into 20 µl nuclease free water and the concentration measured using the NanoDrop™ spectrophotometer. The WT and SDM plasmids were then analysed by Sanger sequencing using the Exon 52 primer (Table 2.1, pages 78). The method for this is as per page 79 except that 100 ng/µl of plasmid DNA was used. Subsequently, the full *VWF* cDNA sequence was analysed using Sanger sequencing with 13 overlapping primers (Table 2.4, page 82). These were designed using the NCBI Primer-Blast Tool (Ye et al., 2012), aiming for primers optimised for Sanger sequencing (18-23 bp, melting temperature 55-60 ºC, GC content 40-60%). I confirmed that site-directed mutant plasmid contained the p.Gly2706Ter variant (Figure 4.17, page 187). Thereafter it is referred to as pcDNA-3.1-Gly2706Ter-VWF. Both this and the pcDNA-3.1-WT-VWF vectors were then maxiprep'd in order to obtain sufficient DNA at a high enough concentration (> 1000 ng/ul) for transfection.

Sequence	Length	Melting Temperature (ºC)	GC content
TAATACGACTCACTATAGGGAG	22	54	41
TGTGTGAGAAGACTTTGTGT	20	56	40
CTGTGTGGGAATTACAATGG	20	60	45
CAGAACTATGACCTGGAGTG	20	57	50
ACCTGCCATAACAACATCAT	20	59	40
CTGAGTTTGAAGTGCTGAAG	20	58	45
TGCAGTACTCCTACATGGTG	20	59	50
GAATTGGAGATCGCTACGA	19	60	47
GATCTGTGATGAGAACGGAG	20	60	50
CAGTGCTGTGATGAGTATGA	20	57	45
CATTCAGCTAAGAGGAGGAC	20	58	50
CATCCAGCATACAGTGACG	19	60	53
ATCCAGAACAATGACCTCAC	20	59	45

Table 2.4 | Primers used to sequence the full *VWF* cDNA sequence.

2.2.3. p.Gly2706Ter transfection into HEK293T and western blotting

HEK293Ts and 1 X tandem affinity purification (TAP) lysis buffer were acquired from Dr Mayer. The pcDNA3.1-myc-his(+) and ER-mcherry (Malzer et al., 2013) plasmids were a gift from Dr Chambers and Professor Marciniak (Acknowledgements, page 8). Prior to use, the HEK293Ts were confirmed to be mycoplasma negative based on a PCR based assay carried out by the Stem Cell Institute, University of Cambridge. HEK293Ts were expanded on uncoated T75 flasks in 'HEK medium'. This comprised Dulbecco's Modified Eagle Medium - high glucose (DMEM-HG) supplemented with 10% fetal bovine serum (FBS), 1% minimal essential medium non-essential amino acids, 1% penicillin/streptomycin (pen/strep), and 0.1% tylosin. The cells were maintained at 37ºC at 5% CO_2 and cultured in a class 2 safety cabinet. Cell counts to estimate the number of cells were carried out by adding 10 µl trypan blue (0.4%) to a 10 µl single cell suspension in a chamber of a haemocytometer, manually counting using a x5 lens on a Leica DFC295 microscope, then calculating the total number of cells adjusting for the dilution factor and the total volume of the suspension. Once there were sufficient numbers of cells, transfections were carried out in a tissue culture treated 6 well plates. Into each well 1.8 ml of HEK293T suspension was added in HEK medium that contained 1.3 x 10^6 cells and the cells were allowed to become loosely attached for four hours. One hour before transfection two reactions were set up in 400 µl DMEM-HG in separate microcentrifuge tubes:

1) 2 µg pcDNA-3.1-Gly2706Ter-VWF, 2 µg pcDNA3.1-myc-his(+), and 2 µg ER-mcherry
2) 2 µg pcDNA-3.1-WT-VWF, 2 µg pcDNA3.1-myc-his(+), and 2 µg ER-mcherry

24 µl of 1mg/1ml polyethyleneimine (PEI) was then added to each of 1) and 2). The suspensions were vortexed for 15 seconds and then incubated for 20 minutes at RT to allow PEI/DNA complexes to form. A Pasteur pipette was used to add the suspension, one drop at a time, to the cell culture plates as prepared above. 200 µl of 1) was added to one well and then repeated in another. The same was done for 2). As an additional control, 200 µl of DMEM-HG with no PEI or plasmids was added to the final two wells. The plates were then returned to the incubator at 37ºC with 5% CO_2. The media was replaced with new HEK media the following day. Two days post transfection, transfection efficiency was assessed (Figure 4.18A, page 188). This was done by assessing red fluorescence from the co-transfected ER-mcherry plasmid by microscopy and flow cytometry.

Having confirmed the efficiency of transfection, cells from each condition were harvested using TrypLE and pellets frozen at -20ºC. The pellets contained ~ 3 x 10^6 cells. To lyse the cells, they were resuspended in 150 µl 1 X TAP buffer (0.1% NP- 40, 150 mM NaCl, 10 mM Tris with protease inhibitor cocktail tablets) and then centrifuged at 13,000 rpm for 15 minutes at 4ºC. The protein concentration was then determined using a Bradford assay using the BioRad protein assay and bovine serum albumin of known concentrations to calibrate the curve. The quantification was performed using an absorbance microplate reader. The lysates were then frozen at -20ºC.

To compare the glycosylation profile of the VWF in each of WT and p.Gly2706Ter conditions, digestion of 40 µg of lysate was performed with Endoglycosidase H (EndoH) and Peptide:N-glycosidase F (PNGaseF) using the supplied buffers and adding 2ul of each of the enzymes to a 40 µl reaction. This reaction was performed at both 37ºC for 12 hours (corresponding to the results shown in Figure 4.18D, page 188), and repeated at 4ºC for 15 minutes (the results in Figure 4.18E). A ‘no enzyme’ control was used for each reaction which went through exactly the same processing steps, but to which no enzyme was added. To reduce the protein in the lysates, 30 µg of the lysate solution from each of the WT,

p.Gly2706Ter, and no PEI/plasmid control was then mixed with 4 X Lithium dodecyl sulfate (LDS)-dithiothreitol (DTT) buffer and then heated for 70ºC for 10 minutes.

The samples were then analysed by western blot. A 15 well BisTris gel (4-12%) was placed in a tank to which 1 X 3-(N-morpholino)propanesulfonic acid (MOPS) sodium dodecyl sulfate (SDS) running buffer was added. In the inner chamber of the tank NuPAGE® antioxidant was added (500 µl for 200 ml of MOPS SDS buffer). 10.5 µg of the digested, reduced lysates were added to each well of the gel. 8 µl of Geneflow ladder was added to the first and last wells. Electrophoresis was carried out for 75 minutes at 200 V. For transfer, the gel was placed on top of a polyvinylidene fluoride (PVDF) membrane that had been pre-soaked in transfer buffer (50 ml 10 X Tris glycine [15.15 g Trizma base, 72 g glycine in 500ml distilled water], 85 ml methanol, 12.5 ml 20 X MOPS SDS buffer). This was then placed between two blot papers. Transfer was then carried out on a semi-dry transfer cell for three hours at 90mA. All blocking, antibody staining, and wash steps were carried out on an orbital shaker ensuring that the membrane was kept immersed using 10 ml of each solution. Blocking solution was added to the membrane for one hour. This was replaced with primary VWF antibody (1:1,000 polyclonal VWF antibody in 10ml of 1 X Tris-buffered saline (TBS) containing BSA at a final concentration of 0.05 g/ml). This was then incubated overnight at 4ºC. The following morning the membrane was washed three times, for five minutes each, with 1 x TBS, followed by secondary antibody staining (1:7,500 IRDye goat anti rabbit antibody 680 in blocking solution) for one hour at RT whilst covering the membrane from light. The membrane was then washed three times, for five minutes each, with 1 x TBS. The fluorescence signal from the secondary antibody was then detected using the 700 nm channel of the Odyssey® FC Imaging System.

2.2.4. ECFCs: derivation, flow cytometry, and imaging

The protocol used for deriving ECFC from NBR 1 and three control volunteers, C3, C4, and C5, was adapted from two sources (Ormiston et al., 2015; Blueprint Epigenome, 2016). Due to time constraints, blood samples from C3 and C4 were taken and processed on the day before samples were taken from NBR 1 and C5.

Prior to taking blood samples, T25 flasks were coated with collagen. First, a 15 ml 0.02 N solution of acetic acid was prepared in sterile embryo-transfer water, to which type 1 collagen was added to a final concentration of 50 ug/ml. Each T25 was then coated with 2.5 ml of this acidified type 1 collagen solution. ECFC medium was prepared by adding the supplements in the EGM™-2 MV Microvascular Endothelial Cell Growth Medium-2 SingleQuots™ kit to 500 ml of Lonza Endothelial Cell Growth Basal Medium-2, except for the FBS supplied with the kit. Instead, the latter was replaced with Hyclone™ Defined Fetal Bovine Serum added to obtain a final concentration of 16.7%. Blood samples were taken from NBR 1 (100 ml) and C3, C4, and C5 (50 ml each). The first step for deriving ECFCs was to first obtain PBMCs from each individual which had been collected in citrate blood tubes. The volume of whole blood used for the generation of ECFCs was: 54 ml (NBR 1); 45 ml (C3); 45 ml (C4); 40ml (C5). This volume of blood was then mixed with an equal volume of D-PBS. This was done by slowly pipetting ~ 25ml of the diluted blood along the side of a 50ml conical tube onto 12.5ml of density centrifugation medium (Ficoll® Paque Plus™) and then layered onto a density centrifugation medium, and repeated until all the diluted blood had been pipetted on top of the Ficoll. The conical tubes were then centrifuged at 800g (acc 4; brake 1) for 15 minutes at RT. Thereafter, a Pasteur pipette was used to extract the layer of cells (the buffy coat) caught between the blood and the Ficoll. The buffy coat was then transferred to a 50 ml conical tube, diluted 1:1 with D-PBS, mixed, and centrifuged at 300 g (acc 9; brake 9) for 20 minutes. The supernatant was then aspirated and the cell pellet resuspended in ECFC growth medium. Cells were counted in Türk's solution prior to seeding onto the pre-prepared collagen-coated T25 flasks. Circa 25 million cells were seeded into each T25 flask. Three flasks were seeded for NBR 1, C3, and C5, and two flasks for C4. These were then put into the incubator at 37ºC with 5% CO_2. ECFC medium was replaced every two to three days. The cells were checked regularly using a light microscope. When the cells became confluent they were passaged with trypsin/EDTA with further expansion on six well plates coated with type 1 collagen. Two different colonies of NBR 1, and one colony from each of C3 and C4 were passaged and expanded.

At Day (D) 36 post seeding of PBMCs, the ECFCs were evaluated using flow cytometry (FC). Before dissociating the cells, a solution of FC buffer was made by adding 0.5M EDTA to D-PBS to a final concentration of 0.02 mM, and 20% human serum albumin (HSA) solution was added to a final concentration of 0.5%. This was chilled to 4ºC. Polypropylene FC tubes for staining cells were prepared just before the cells were dissociated from the tissue culture vessels. To each tube 150 µl of FC buffer was added. Apart from the unstained control tube(s), fluorophore-conjugated antibodies were then added to the following tubes: 1:300 for the PECAM1-APC and 1:75 CDH5-FITC, 1:300 for APC isotype and 1:75 FITC isotype, 1:300 CD14-Alexa Fluor®(AF)700 and CD45-AF405, 1:300 AF700 and AF405 isotypes. Separate tubes were also set up with each of PECAM1-APC, CDH5-FITC, CD45-AF405, and CD14-AF700 for use with compensation beads. The tubes were then kept at 4ºC wrapped in foil. Following dissociation with trypsin-EDTA, the ECFCs the cells were pelleted by centrifugation at 300 g for 5 mins at RT. The supernatant was aspirated and then resuspended in cold FC buffer at a concentration of 20,000 cells per 50 µl. One l µl of FcR block was added for every 10 µl of cell suspension. Then 50 µl cell suspension was added to each of the prepared antibody tubes. After vortexing, one drop of the positive (antibody-binding) compbeads and one drop of negative compbeads was added to the pre-prepared tubes (as above). Once all compbeads and cells had been added to all tubes then the rack containing the tubes was incubated at 4ºC wrapped in foil for 10 minutes. Then a wash step was carried out to remove any unbound antibody: 1 ml of FC buffer was added to each FC tube and the tubes were centrifuged. The supernatant was then aspirated carefully avoiding the cell pellet and this was resuspended in 200 ul of FC buffer.

The samples were analysed on the cytoFLEX flow cytometer and fluorescence was detected on the most appropriate channel dependent on the emission spectrum of the four antibodies. Samples were aspirated with the aim of capturing 10,000 events per tube. The FC files were saved in .fcs format and exported for upload into FlowJo 10.8.1 for analysis. To take into account fluorescence overspill between different conjugated antibodies, a compensation matrix was created using FC samples prepared with a single antibody and compbeads as described above. This was then applied to the samples which used the

corresponding antibodies or their isotypes. To gate for cells, a pseudocolor dot plot of side scatter area (SSC-A) as a function of forward scatter area (FSC-A) was used. The 'cells' gate was drawn around the population with the highest event density and was subsequently checked on all samples being evaluated to ensure it did not truncate part of the population due to differences between the samples. Cell debris, which typically has low SSC-A was deliberately excluded. Endothelial cells can adhere to each other prior to analysis by FC, thus forming aggregates which can falsely increase the fluorescence signal attributable to an event. Therefore, a singlet gate was drawn using FSC-A versus FSC-H, excluding cells deviating from the correlation between the parameters for the bulk of the cells. Only events in the singlets gate were then used for comparison of fluorescence of populations from isotype versus stained controls. To gate isotype versus stained, a contour plot was drawn with 5% concentric circles and the quadrant (for dual colour stained cells) or the rectangular gate (for single colour stained cells) was set at so that the vertical (and horizontal, if applicable) lines touch the second outermost ring of the isotype control (as exemplified in Figure 5.3, page 204). Fluorescence intensity was downloaded from the events in the singlet gate and the calculation of the median and other analysis were carried out in R.

For the analysis of ECFCs by confocal microscopy, the cells were dissociated at D30 following two passages since colonies first emerged. They were seeded onto collagen coated Ibidi chamber slides at a density of ~ 12,000 cells/cm^2. ECFCs were washed twice in D-PBS and then fixed in 2% formaldehyde for 15 minutes, then washed twice in D-PBS. They were wrapped in parafilm and kept at 4^{o}C until being stained two days later. The following staining procedure was performed with 200 µl in each well, with removal of the previous solution prior to addition of the next. Note that between between all steps and at the end there were three sap-gel-PBS washes (50 ml of D-PBS with final w/v 0.05% sodium azide; 0.1% saponin from quillaja bark; 0.2% gelatin), each lasting five minutes.

1) 50mM ammonium chloride (five minutes)
2) Primary antibody staining solution (sap-gel-PBS with rabbit polyclonal VWF and mouse monoclonal CDH5 antibodies at final concentration of 1:200) (one hour)

3) Secondary antibody staining solution (sap-gel-PBS with goat anti-rabbit AF633 and anti-mouse AF488 at final concentration of 1:1000) (one hour)
4) 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) 1 µg/ml (three minutes)

The fixed cells were then mounted with a No 1.5 cover slide placed on top of the stained cells, adhered using Mowiol mounting media (4.4 g Mowiol®; 1.8 g glycerine; 29 ml distilled water; 4.4ml 1 M Tris), which was left overnight at RT to harden.

Immunofluorescent images were taken using a Leica Sp5 Confocal microscope with an oil immersion lens. The sample was scanned sequentially using three lasers (405 nm, 488 nm, and 633 nm). The parameters (laser power, frame averaging, pinhole, offset, and smart gain) set for each channel were kept constant for the imaging of the samples of NBR 1, C3, and C4. The images were saved in .tiff format. Creation of composite images and further analysis was performed in FIJI running ImageJ version 2.1.0/1.53c (Rasband, 1997-2018).

2.2.5. Differentiation of hiPSCs to iECs and flow cytometry

I differentiated three different human induced pluripotent stem cell (hiPSC) lines (Table 5.5, page 210) into endothelial cells (iECs):

- A1ATDc was obtained from the laboratory of Professor Ludovic Vallier (Stem Cell Institute);
- FFDK and QOLG_3 were derived by the HipSci consortium (Kilpinen et al., 2017) of which my supervisor, Professor Willem Ouwehand, was a co-Principal investigator (https://www.hipsci.org/about#investigators) and were retained in our laboratory from the time of derivation.

In addition, iECs derived from a different hiPSC line, C2, were obtained from the laboratory of Dr Amer Rana (Department of Medicine) for the RNA-seq analysis (Subsection 5.4.3, pages 216 - 218).

Both hiPSCs and iECs were cultured in feeder-free conditions in six well plates, incubated at 37°C and 5% CO_2, in a class 2 safety cabinet. All centrifugations to pellet cells were carried out at 300g for 5 minutes). Cell counts were as described in Subsection 2.2.3 (page

82). Both hiPSC and differentiation to iECs were carried out in tissue culture treated six well plates. For hiPSC expansion these plates were coated with vitronectin and the cells were cultivated in complete StemFlex (SF) media which was changed every 1-2 days. Cells were passaged every 3-5 days in small clumps using 0.5mM EDTA solution with a split ratio of 1:4 - 1:10. To minimise passages, when hiPSCs were not being maintained for a differentiation experiment they were frozen as clumps at -80°C or -150°C in KnockOut Serum Replacement (KOSR) containing 10% dimethyl sulfoxide (DMSO).

The outline of the iEC differentiation protocol I developed is in Figure 5.7B (page 211). The different media prepared at each step were put through a Stericup 250 ml 0.22 µm filter. On D-1, plates were placed on cold Lab Armor^{TM} beads. These were then coated with 0.33 mg/ml just-thawed matrigel using Iscove's Modified Dulbecco's Medium (IMDM) and incubated at RT for one hour. To obtain hiPSCs for seeding, they were dissociated from vitronectin coated plates using TryPLE for 5 minutes, centrifuged, and resuspended in complete SF media containing Y-27632 at a final concentration of 10 µM (hereafter SF + Y). When counting the cells, a check was made to ensure that the majority were single cells and that there were no large clumps. They were then seeded onto the matrigel-coated plates at ~ 2,000 - 4,000 cells/cm^2 in their regular maintenance (StemFlex [SF]) media. On D-1, the initial constituents of FCL were made: Poly(vinyl alcohol) (PVA) was added to 50ml complete F12 medium the evening before to make a F12-PVA suspension and put on a vertical rotator overnight to dissolve the PVA. On D0 further F12 was added to achieve a final PVA concentration of 2 mg/ml. F12-PVA was diluted with an equal volume of IMDM, then the following chemicals were added (final concentrations in parentheses): lipid concentrate (1:100), pen/strep (1:100), FGF2 (20 ng/ml), CHIR99021 (8 µM), LY294002 (10 µM), apo-transferrin (15 µg/ml). On D2, FCL was replaced with VFA media. This comprised complete StemPro-34 media to which the following chemicals were added (final concentrations in parentheses): L-glutamine (2 mM), forskolin (2 µM), VEGFA (200 ng/mL), and ascorbic acid (1 mM). VFA media was changed at least once before D5/6. At this point the emerging iECs were washed with D-PBS and dissociated using Accutase, warmed to 37ºC before use. They were then reseeded onto plates coated with a 20 µg/ml fibronectin solution in VFA media which was changed on

alternate days until the iECs were harvested and positively selected for using magnetic cell separation (MACS) with CDH5-coated microbeads, LS MACS columns, and FC buffer that was kept cold throughout. MACS was carried out in the class 2 cabinet. Following accutase dissociation, the iECs were passed through a 30 µm filter, suspended in FC buffer (page 86) at a concentration of 10^6 cells per 8 µl buffer. Two µl of microbeads were added for every 10^6 cells, and incubated for 15 minutes. They were then washed by adding 200 µl FC buffer, centrifuged and resuspended in 500 µl for 3 x 10^7 cells. Each LS column was attached to its corresponding sized magnetic MidiMACS™ separator and mounted on a MACS 'MultiStand'. Each column was washed with FC buffer, then the microbead-cell suspension was added. Following washes, the LS column was removed from the MidiMACS™ separator and the positively-labelled iECs were flushed out of the column with 5 ml FC buffer. These cells were then either frozen down in KOSR with 10% DMSO or expanded on fibronectin-coated plates.

As outlined in Chapter 5 (Subsection 5.4.1, pages 213-216) FC analysis was performed on D-1, D5, and D9 to evaluate both pluripotent and EC phenotypes. This was carried out as described on pages 86 - 87, with the following differences:

- The Beckman Coulter FC500 or Gallios flow cytometers were used for the data presented in Figure 5.7 (page 211), Figure 5.8 (page 212), and Figure 5.9 (page 215).
- If there were sufficient numbers of cells, then 50,000 were added to each FC tube prepared.
- Additional antibodies were used (with the final dilutions indicated in parentheses): CD34 (1:1500), TRA-1-60 FITC (1:150), SSEA-4 PE (1:7.5), with their corresponding isotypes used at the same dilution (except for the SSEA-4 isotype which was used at a 1:998 dilution).

2.2.6. RNA-seq analysis of WT QOLG_3 iECs

I carried out RNA-seq on both QOLG_3 iECs and human lung microvascular endothelial cells (HMVECs). 500,000 iECs were obtained, post MACS, from experimental replicates 2, 4, 5, and 6 (Table 5.5, page 210). These were pelleted by centrifugation in a

microcentrifuge tube, the supernatant removed and mixed with 700 µl of TRIzol™ and then frozen at -80ºC. Cryopreserved human lung microvascular endothelial cells (HMVECs) were cultured in EGM™-2 MV media prepared as per page 85, except that the FBS supplied with the kit was used. HMVECs were cultured in a class 2 safety cabinet. They were seeded on tissue culture treated T25 flasks but no additional extracellular matrix was added and passaged with trypsin/EDTA. At passage number 4 post initial seeding, the cells were dissociated and 2 pellets prepared in TRIzol™ as above.

Initial RNA extraction from the six samples (four iEC, two HMVEC) was performed as described on pages 77-78. Concentrations of RNA obtained were 180 - 490 ng/µl. The RNA integrity number was determined on the Agilent BioAnalyzer using a Pico kit and was between 7.6 and 9.0 for all samples. Stranded RNA-seq libraries were prepared with 200 ng of RNA using the KAPA stranded RNA sequencing kit with RiboErase and RNA clean XP beads. This was following the manufacturers instructions except for the modifications outlined in Sims et al., 2020 (Supplemental file 6). The libraries were quantified KAPA Illumina SYBR Universal Lib Q. Kit. They were then pooled with equimolar concentrations for a final concentration of 5nM. They were sequenced on a single lane of an Illumina HiSeq 4000. Sequencing was paired-end with a read length of 150 bp. Dr Dinan (Acknowledgements, page 8) processed the samples using our group's pipeline (Seyres et al., 2022), including gene expression quantification and differential expression analysis.

2.2.7. QOLG_3 iEC VWF release and agonist response

Post MACS iECs from replicate five (Table 5.5, page 210) were seeded at ~ 10,000 cells/cm^2 on fibronectin coated 12 well plates. They were cultured overnight in VFA media, one aliquot of which was frozen down and was not used to culture cells. Supernatant was collected from one well the next day. For the agonist release assay, the VFA in 3 wells was removed. This was replaced with 1ml of VFA media containing (final concentration in parentheses): thrombin (2.5 U/ml) or adrenaline (100 µM), or (as a negative control) D-PBS with the same volume used to add thrombin and adrenaline (20 µl). After 30 minutes incubation, the cells were dissociated with Accutase, then pelleted by

centrifugation at 300 g for 5 minutes at RT. The supernatant was then aspirated and the pellet snap frozen on dry ice at -80ºC. Both lysates and supernatants were analysed by western blotting as described on pages 83-84. The only additional modification was that the day following staining for VWF in lysates, the membrane was re-probed using rabbit monoclonal anti-GAPDH at 1:1000 final dilution. This meant that semi-quantitative analysis of fluorescence intensity could be carried out using Image Studio Lite as supplied with the Odyssey® FC Imaging System.

Electric cell-substrate impedance sensing (ECIS) was carried out by seeding post MACS iECs from replicate five onto a fibronectin coated 8 well array with 10 electrodes per well (8W10E) and were cultured in VFA media. On a separate, vitronectin coated 8W10E array, QOLG_3 hiPSCs were seeded in complete SF media. Both hiPSCs and iECs were seeded at 40,000 cells/cm^2. The array was screwed into the holder that had a cord linking it to the ECIS Z theta machine with an attached laptop running the proprietary ECIS software. The array holder was then placed in an incubator at 37ºC with 5% CO_2 whilst remaining connected to the computer. Once the resistance profile of the cells had stabilised the following day, agonist stimulation was carried out. This was done by setting the software to rapid time course (RTC) mode and then adding thrombin 1 U/ml in 10 µl water to the 200 µl media in one well and then straight afterwards adding 10 µl water to the immediately adjacent well. The resistance profile was then measured for 15 minutes afterwards. This was then repeated for hiPSCs.

2.2.8. CRISPR/Cas9 of QOLG_3 hiPSCs

CRISPR guides targeting exons 50 (to remove the CK domain) and exon 2 (to KO VWF) were designed using the Wellcome Trust Sanger Institute Genome Editing tool (Hodgkins et al., 2015). Only three sgRNAs were found that overlapped exon 50, so all three were chosen. Four potential sgRNAs were found targeting exon 2. The three closest to the ATG codon of exon 2 were selected. The guides are shown in Table 2.5 (page 93). Desalted oligonucleotides corresponding to the complementary forward and reverse sequences of each sgRNA were ordered from Sigma-Aldrich. These were then ligated and cloned into the PX458 plasmid (Figure 5.12B, page 222) (Ran et al. 2013). The maxiprep of PX458

had been carried out by Dr Lambourne, and the cloning of each sgRNA into this plasmid was done by Dr Phill North (Acknowledgements, page 8).

Target exon	sgRNA name	WTS[1] ID	Strand	Sequence (including PAM)[2]
50	6	1092109851	Forward	CATGTGTCACAGCAGGTGCCTGG
50	7	1092109850	Forward	GCACTCACATGTGTCACAGCAGG
50	8	1092109852	Reverse	GGTAAAATTATGAAAATTCCAGG
2	2	1092132593	Forward	AAGCAGCACCCCGGCAAATCTGG
2	4	1092132594	Forward	AGCACCCCGGCAAATCTGGCAGG
2	5	1092132597	Reverse	ATGATTCCTGCCAGATTTGCCGG

Table 2.5 | sgRNAs used for CRISPR. [1]WTS = Wellcome Trust Sanger CRISPR ID. [2]The PAM is underlined.

The same batch of QOLG_3 (passage number 30) was used for all nucleofections, which was mycoplasma free and had demonstrable capacity to differentiate to iECs based on prior experiments. For nucleofection, 4 µg of the PX458 plasmid containing one of the six ligated sgRNAs was prepared in 100 µl Human Stem Cell Nucleofector™ Kit 2 solutions (82 µl solution 2; 18 µl Supplement1). This DNA master mix was then added to ~ 35,000 QOLG_3 hiPSCs dissociated using TryPLE and transferred to an electroporation cuvette. Nucleofection was carried out using the Amaxa Nucleofector II machine using B-016 cycle. Following nucleofection the hiPSCs were suspended in 200 µl SF + Y media. This step was repeated for the rest of the sgRNA-containing plasmids as well as the pmaxGFP plasmid supplied with the Nucleofector™ Kit which was a positive control. Two days post nucleofection cells were dissociated using TryPLE, and resuspended in SF + Y in microcentrifuge tubes. Fluorescence assisted cell sorting into 96 well plates was performed on the BD FACSAria™ Fusion flow cytometer at the NIHR Cambridge BRC Cell Phenotyping Hub. Two to three 96 well plates of GFP-positive cells were obtained for each sgRNA nucleofected. Genomic DNA was extracted from single cell clones using the QIAGEN DNeasy Blood & Tissue kit, eluted into nuclease free water and the concentration determined using the NanoDrop™ spectrophotometer. Primers were designed that were 763 and 825 bp apart, that placed exon 50 and exon 2 respectively, in the centre of the amplicon (Table 2.6, page 94). The primers were reconstituted in nuclease free water to a concentration of 10 µM. PCR reactions were then carried out using

Phusion™ HF DNA Polymerase with 500 nM forward and reverse primers and 50 ng gDNA template and analysed using agarose gel electrophoresis (page 78-79).

Target	Strand	Oligonucleotide sequence
Exon 2	Forward	CCCAGGAAGACTGACCTCTG
Exon 2	Reverse	GTTAGCTCCCAGCTGCTTCA
Exon 50	Forward	AAGCTGGAGACAGGAGGGAG
Exon 50	Forward	TCCCAGCTCCGGCATAATTT

Table 2.6 | Primers used to sequence CRISPR/Cas9 edited clones.

The post PCR DNA was then cleaned up using Zymo columns and eluted into ~ 20 nuclease free water. The eluted DNA from 14 potential ΔCK and nine potential KO clones was then A-tailed and cloned into the pGEM® -T-easy plasmid in order to carry out allele-specific sequencing. To A-tail the amplicons, 18 µl of DNA eluate was added to 7.5 µl 10 X terminal transferase reaction buffer, 1.5 mm dATP, 7.5 µl 2.5 mM cobalt dichloride ($CoCl_2$), 6 µl terminal transferase, and made up to 75 µl with nuclease free water. This was then incubated in a thermal cycler at 37ºC for 90 minutes and the reaction cleaned up using Zymo columns. To ligate the A-tailed amplicons into the pGEM®-T-easy vector, an overnight incubation at 4ºC was performed in which the insert and vector were added in an 8:1 molar ratio with 2 X rapid ligation buffer, and T4 DNA ligase. The following morning, the whole ligation reaction (~ 10 µl) was mixed with 45 µl JM109 highly-efficient competent *E. coli* and incubated on ice for 20 minutes. These were then heat shocked for 45 seconds at 42ºC and then placed back on ice for 2 minutes. After this, 950 µl of SOC medium was added and put on a shaker at 150 rpm for 90 minutes. The bacteria were then pelleted by centrifugation and resuspended in 200 µl SOC medium. The transformation reaction was then plated on Amp Agar X-Gal platelets for blue-white selection. White colonies were then selected using a toothpick aiming for at least 10 colonies for each potential clone. These were cultured overnight, underwent miniprep DNA extraction, and were analysed by Sanger sequencing using the method described on page 79, except that the T7 forward primer was used (as supplied by Source Bioscience).

The initial sequencing reads from the successful ΔCK clone all included the same c.8139_8140insA variant. This was confirmed by sequencing 38 further colonies, of which 25 also confirmed the presence of c.8139_8140insA (the 13 remaining sequencing reactions failed). The successful KO clone (biallelic for c.3_13del and c.12_13insA) needed to be subcloned. This was because, although both of these variants were found in 10 and 7 out of A-tailed amplicons sequenced, a third less common allele was identified, suggesting that there was not a single clonal population. Subcloning was done by doing a single cell sort using the FACSAria™ as above but no fluorescence was required. On resequencing, a clone was found which contained only the desired two alleles in compound heterozygosity (Figure 5.14, page 225).

2.2.9. Differentiation of ΔCK and KO iECs and characterisation

Differentiation of ΔCK, KO, WT, and the nucleofection control (Figure 5.15, page 227; Subsection 5.5.2, pages 221-223), were following the protocol in Subsection 2.2.5 (pages 88-90). The FC was carried out on the CytoFLEX and only CDH5-FITC (page 86) and SSEA-4 PE (page 90) were stained for on D-1 hiPSCs and D9 iECs post MACS. Post MACS iECs were frozen in 90% KOSR/10% DMSO in vials of 500,000. One vial of each of ΔCK, KO, WT was thawed. Each was seeded at 25,000 cells/cm^2 into two wells of a fibronectin coated six well plate. The media used to expand the cells was EGM-2 supplemented with 10% FBS in order to encourage proliferation. Four days post initial seeding they were 100% confluent, so were dissociated using Accutase and seeded on fibronectin coated Ibidi chamber slides at a density of ~ 30,000 cells/cm^2. After four days the cells had been confluent for at least 48 hours and they were formaldehyde fixed. The fixing and staining procedures were as described on pages 87-88 but with the following modifications to try and improve the preservation of the monolayer of cells pre-imaging: 4% methanol-free formaldehyde as a fixative, D-PBS was used for washes, and the tip end of the pipette was cut to reduce shear when pipetting into the wells. Immunofluorescent imaging and processing was performed as described on page 88 with the following improvements. First, in order to try and increase resolution of intracellular VWF localisation, all images were taken at x 63. Second, to try and reduce photobleaching from the UV laser, the sample was initially scanned, simultaneously, with the 488 nm and 633

nm lasers and each laser was set up to be detected by a separate photomultiplier tube (PMT). Subsequently, a second scan was performed with the UV 405 nm laser onto a different PMT. Third, as a large number of images (37) were taken, all data was saved in a single .lif file and then imported into FIJI using the Bio-Formats Importer plugin version 6.6.1 (Linkert et al., 2010). Fourth, in order to process, in bulk, all images using a consistent approach, three ImageJ macros were written to carry out the following tasks:

1) Merge the frames taken on different channels (blue, green, red) to create a composite image, which was then converted to RGB format, the brightness/contrast was set to have minimum and maximum displayed values of 0 and 167 respectively.
2) Merge blue and red channel images only but set brightness/contrast to a minimum of 50 and maximum 147 respectively to help reduce the background noise observed in antibody stained cells in the red channel and facilitate the analysis of VWF localisation (Figures 5.17 D, E, and F, page 230).
3) Apply the Li auto threshold method (Li & Tam, 1998) to count the number of cell nuclei in each of the images, using a minimum size cut off of 5 μm^2.

Composite images were exported by the macro in .tiff format for further analysis, and nuclei threshold images were exported in .jpeg format. For the semi-quantitative analysis of VWF localisation outlined in Figure 5.18 (page 232), I reviewed all 37 iEC images, unblinded to genotype, and using the blue-red composite image, cellular localisation was adjudicated to be one of four categories - perinuclear and punctate, perinuclear, punctate, and absent - and then assigned to each cell in the image for which a nucleus was detected using the auto threshold method. Perinuclear localisation was defined as a circumferential band of diffuse or more confluent red fluorescence that - in total - encompassed more than half of the visible circumference of the visible nucleus. Punctate localisation was defined as the presence of ≥ 5 discrete puncta of red fluorescence. The perinuclear and punctate category was used when cells met the perinuclear definition but there were also ≥ 5 puncta distinct from the perinuclear fluorescence. Absent was defined as no visible red fluorescence or < 5 puncta. In 7 images, VWF was detected in some parts of the image which did not meet the defined criteria. For instance, oversaturated blobs of red fluorescence also present on the blue and/or green channel likely to represent debris, or

cloudy amorphous smudges of red fluorescence that appeared to be in a different plane to the rest of the image. Such artefacts were disregarded during the classification.

3. Results: reappraisal of the genetic basis of VWD

3.1. Background

The genetic analysis of VWD in clinical genetics laboratories is increasingly moving towards the use of short-read high-throughput sequencing (HTS) (Baz et al., 2021; Megy et al., 2021), which is effective at detecting short nucleotide and insertion-deletion (indel) variants ≤ 50 nucleotides in length in *VWF* (Turro et al., 2020). However, what is still lacking is reliable and comprehensive information against which the detected variants can be evaluated in order to quickly prioritise pathogenic candidates. Reasons for this include:

1) The fact that the first variants causally attributed to VWD (Shelton-Inloes et al., 1987) were described nearly 20 years before the first large scale reference human population was sequenced (International HapMap Consortium, 2005). As a result, the majority of *VWF* variants reported to be potentially causal of VWD were reported either in comparison to small reference populations, mainly of European ancestry, with limited accuracy of MAF estimation, or this was not considered at all.
2) The inconsistent and heterogenous terminology historically used to describe VWD variants (Goodeve, 2010) making it onerous to compare recent findings to those described using legacy cDNA and/or protein nomenclature.

In this chapter I demonstrate how whole exome sequences generated from hundreds of thousands of individuals in UK Biobank (UKB) (Bycroft et al., 2018), and supported by data from the Genome Aggregation Database (gnomAD) (Karczewski et al., 2020), can be leveraged to comprehensively evaluate the MAFs of *VWF* variants brought together into a single database, 'VWDbase'. This is then used to critically reappraise the pathogenicity of the assembled variants, particularly when evaluating against up-to-date diagnostic criteria for VWD (James et al., 2021), taking into account in silico predictors of variant deleteriousness (Kircher et al., 2014), and considering disparities in MAFs between different ancestral populations.

3.2. Aims

1) Build 'VWDbase', a resource of SNVs and indels (≤ 50 bp), which includes a summary pathogenicity for each *VWF* variant with respect to VWD.

2) Define a subset of variants in VWDbase - 'Putatively Aetiological VWD Variants' (PAVVs) - for further analysis in UKB and the National Institute for Health Research (NIHR) BioResource Rare Diseases, in both this chapter and Chapter 4.

3) Determine which PAVVs are carried by UKB participants (hereafter 'UKB PAVVs'), and use the MAF determined from their whole exome sequences to establish a high confidence ('accepted') set of PAVVs that have credible published evidence supporting a causal relationship with the laboratory phenotype of VWD.

4) Understand how the in silico Combined Annotation-Dependent Depletion (CADD) score can be used to predict which PAVVs were accepted or rejected as pathogenic in the UKB analysis.

5) Explore whether the ancestral diversity of the UKB whole exome sequencing (WES) data can be used to identify clinically relevant discordance in the MAF of a given PAVV between different ancestry groups and how this impacts on the likelihood of the given variant being pathogenic for VWD.

3.3. VWDbase

The steps taken to create VWDbase are summarised in Figure 3.1 (page 100). The methods used are outlined in Subsection 2.1.3 (pages 70-73).

Figure 3.1

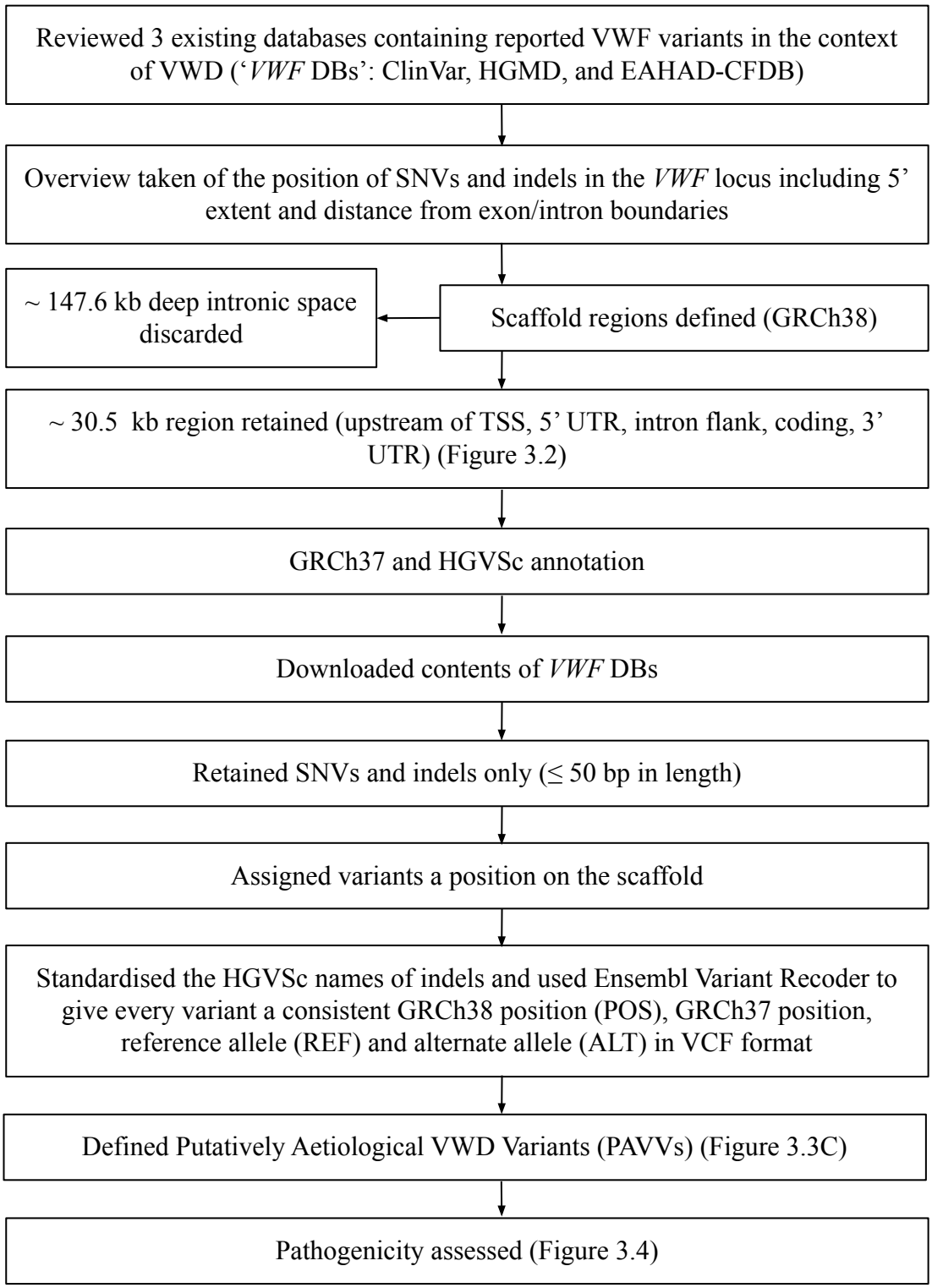


Figure 3.1 | VWDbase generation and subsequent evaluation. Flowchart representing the steps taken to generate VWDbase.

3.3.1. Scope of VWDbase and the definition of a *VWF* scaffold

VWDbase was built to provide an accurately labelled resource in Variant Call File (VCF) format (Danecek et al., 2011) suitable for the identification and prioritisation of potentially causal VWD variants using short read sequencing. The objective was to collate variants from ClinVar, EAHAD-CFDB, and HGMD (Subsection 1.2.5, page 61). These three databases - hereafter collectively referred to as '*VWF* DBs' - were chosen because they are the largest repositories of *VWF* variants in which there is a) the aim of asserting the clinical relevance of the deposited variant to VWD, and b) regular updates continue to be made.

Aggregation of variants from the three *VWF* DB sources was complicated by differences in nomenclature used to describe the position of the variant. ClinVar was most comprehensive with regards to the information that was available: variant position was detailed using the coordinates of both GRCh37 and GRCh38, reference (REF) and alternate (ALT) alleles in VCF format, and HGVS nomenclature (den Dunnen et al., 2016) was used. In the summary HGMD pages used for variant extraction, only HGVS terms were provided. In EAHAD-CFDB, only HGVS and GRCh37 terms were consistently available for all variants. To overcome these challenges, a nucleotide scaffold was built to facilitate merging of variants across the three *VWF* DBs.

Based on an initial overview of the variants contained within *VWF* DBs it was apparent that they occurred within a limited space within the *VWF* locus. The majority were in the coding sequence. The remainder were in the 5' region upstream of the TSS or in an intronic position close to an exon. The range of positions of the intronic variants identified was between 1 nucleotide from the exon/intron junction through to 177 nucleotides away. Therefore, the nucleotide scaffold of the *VWF* locus was confined to:

- The canonical *VWF* transcript (coding sequence of 8442 nucleotides in exons 2-52 plus the 5' and 3' UTR);
- 2305 nucleotides upstream of the TSS, as this corresponded to the position of the most proximal reported variant in the *VWF* DBs (c.-2555A>G in ClinVar);
- Exon flanking regions of 200 bp length at both the 5' and 3' ends of each intron.

The scaffold was built by initially downloading the sequence and corresponding GRCh38 coordinates from Ensembl, which extended from the position 2305 nucleotides upstream of the TSS through to the last nucleotide of the 3' UTR (Figure 3.1, page 100). This was 178.1 kb in length. Following the removal of 147.6 kb of intronic space outside of the exon flanking regions, the final scaffold comprised 30,513 nucleotides categorised as follows: upstream of TSS; 5' UTR; exon flank; coding; and, 3' UTR (Figure 3.2, page 103). Each position was then annotated with its corresponding root HGVSc term (e.g. c.1A) and GRCh37 coordinate in order to permit variants from all three DBs to be mapped to the scaffold.

3.3.2. The spectrum of variants in VWDbase

After downloading variants from each of the three *VWF* DBs the following filtering steps were taken: 214 structural variants were removed, as well as those which were only listed in relation to a non-VWD phenotype (e.g. haemolytic uraemic syndrome). The remaining SNVs and indels were then mapped to the scaffold. Finally, indel names were standardised against the latest HGVSc nomenclature (den Dunnen et al., 2016; HGVS, 2020; Hong, 2020) in order to remove redundancy. A total of 1455 unique variants were identified, 194/1455 (13.3%) of which were indels (Figure 3.3A, page 104). Less than 20% (281/1455) VWDbase variants were present in all three *VWF* DBs and 843/1455 (57.9%) were identified in one DB source only (Figure 3.3B).

3.3.3. Putatively Aetiological VWD Variants (PAVVs) in VWDbase

Each of the downloaded tables of variants from the three *VWF* DBs contained a field which recorded the pathogenicity assertion of the variant made by the given source and this tag was incorporated into VWDbase. However, the pathogenicity adjudication of each of the three DB sources varied both in the method of classification and the degree to which it was summarised.

Figure 3.2

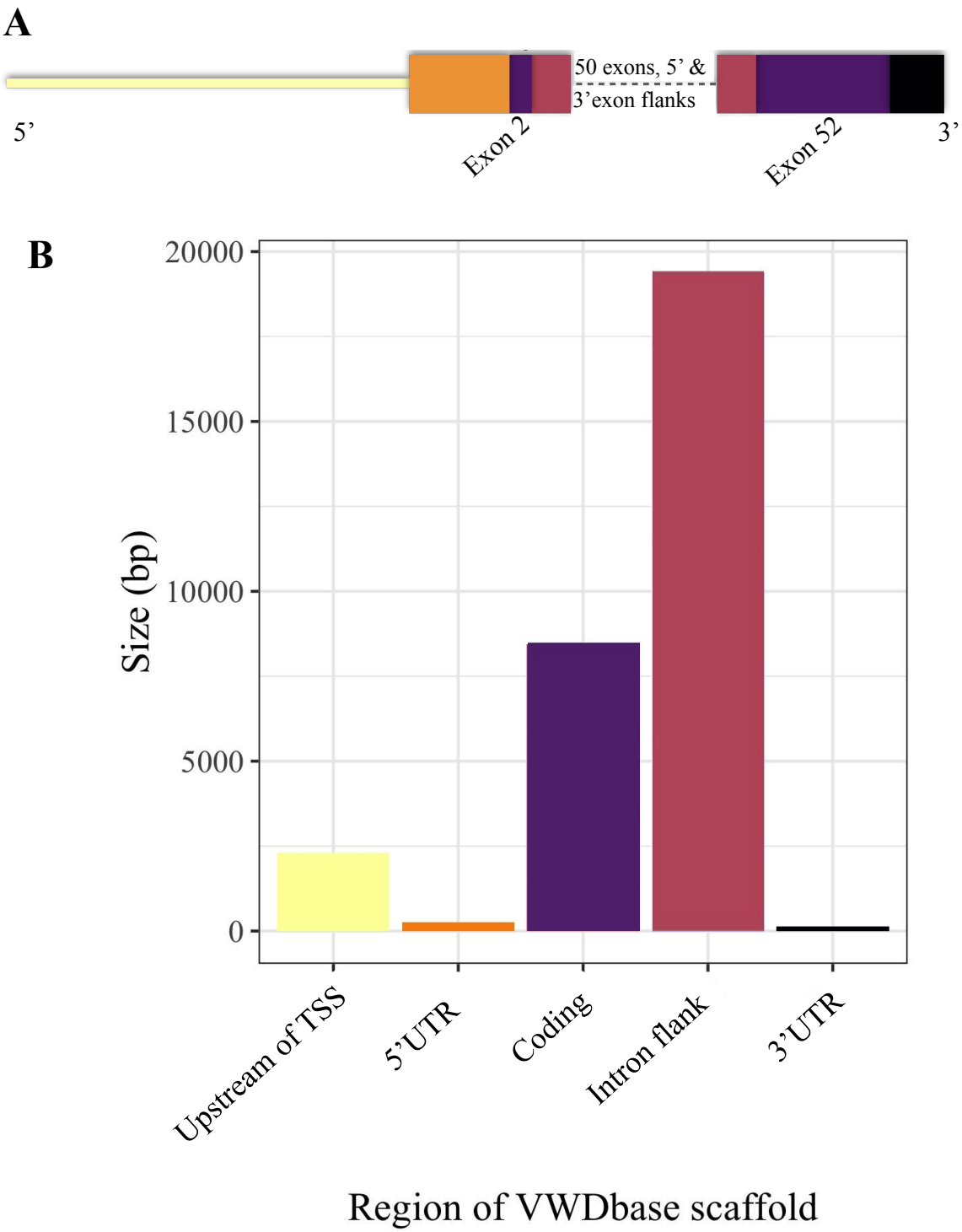


Figure 3.2 | VWDbase scaffold. (A) Representation of the five regions of the 30,513 nucleotide *VWF* scaffold which were retained as a means for the accurate annotation of the position of retrieved variants from the three *VWF* DBs. N.B. this is not to scale. (B) A bar chart showing the relative sizes of the annotated regions of the *VWF* scaffold.

Figure 3.3

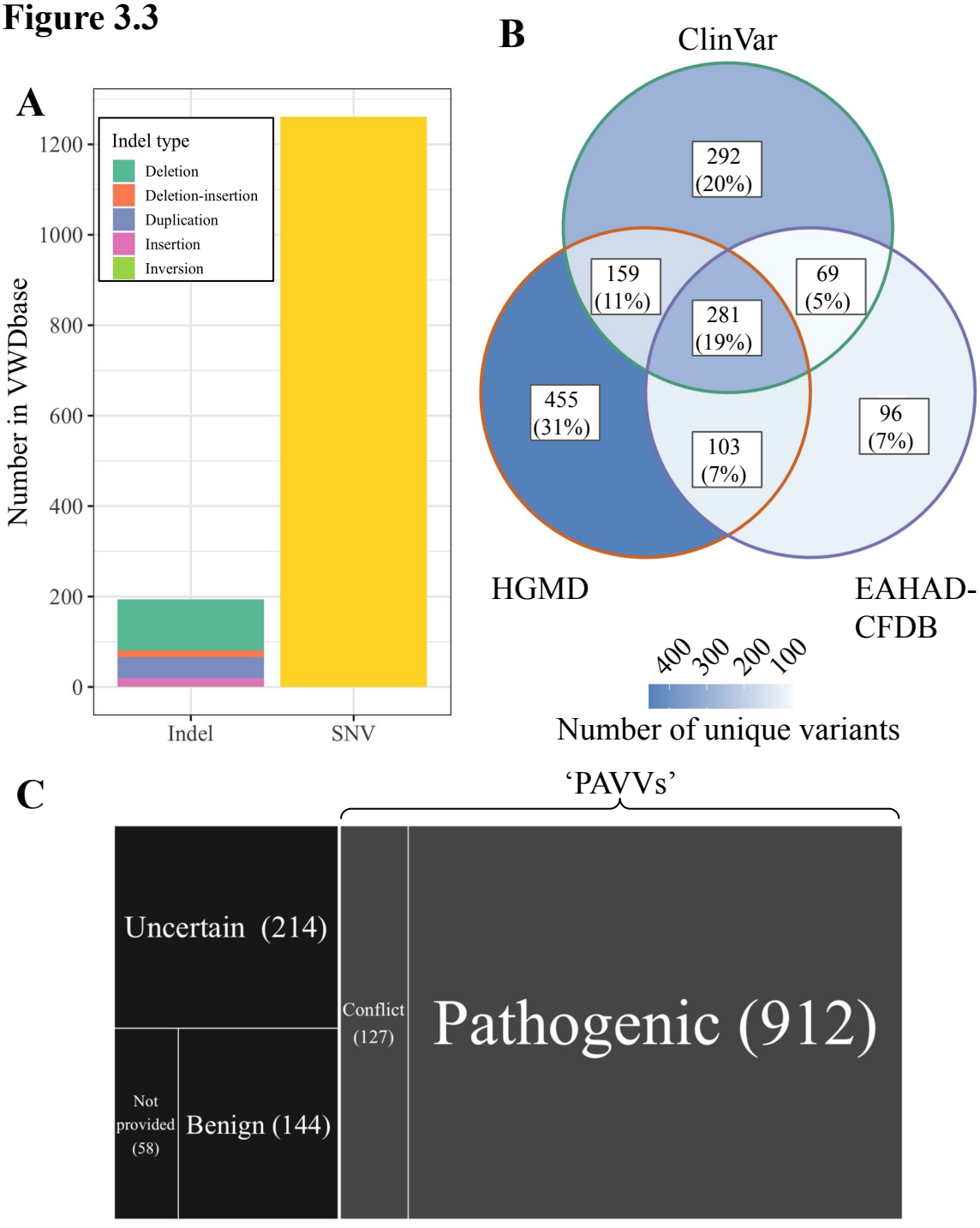


Figure 3.3 | The spectrum of VWDbase variants. (A) A bar chart representing the unique variants in VWDbase. The colour of the indels represents HGVS sub-type. (B) A Venn diagram representing the overlap of the 1455 unique variants in VWDbase between each of the *VWF* DBs. The darkness of the blue colour positively correlates with the number of unique variants. (C) A treemap showing the summary pathogenicity assertions made for each variant in VWDbase. The size of each rectangle is proportional to the number of variants that fall into the category (with the number also shown in parentheses). The clusters on the right in grey are the Putatively Aetiological VWD Variants (PAVVs). N.B 'Conflict' = 'conflicting interpretations of pathogenicity', 'Uncertain' = 'uncertain significance'.

The five-tier system for pathogenicity classification compiled by the ACMG/AMP (Richards et al., 2015) was introduced in Subsection 1.2.4 (page 55). This is used by ClinVar (Landrum & Kattman, 2018). Hence, the data downloaded from ClinVar included the clinical significance assertion of the submitter using the ACMG/AMP system. If the variant had been classified it was rated as pathogenic, likely pathogenic, uncertain significance, likely benign, or benign. If a pathogenicity assessment was 'not provided' then it was recorded as such. If all submitters agreed in their clinical significance interpretation, then this was mirrored in the summary-level interpretation. If there was discordance between submitters in their interpretations, then the summary-level interpretation was recorded as 'conflicting interpretations of pathogenicity'. This only applied if the disagreement was between one of three groups: 1) benign/likely benign; 2) uncertain significance; or 3) likely pathogenic/pathogenic (Landrum & Kattman, 2018). However, if the difference was only based on likelihood (of being benign or pathogenic) then the summary interpretation was given as 'benign/likely benign' or 'pathogenic/likely pathogenic'.

The pathogenicity interpretation downloaded from EAHAD-CFDB was the most heterogeneous, principally because two different systems were adopted. One assessment provided was that of the DB curator, using the 5-tier LOVD system, where the assessment conclusion was represented by a symbol. The assessment made was whether the variant affected protein function. The symbols used were '+', '+?', '?', '-?', '-', corresponding to an effect on function, probable effect, unknown effect, probable absence of an effect, and no effect on function, respectively (Fokkema & Asscheman, 2021). The symbol, '.', was used to represent the absence of a classification being made. In addition a EAHAD-CFDB assessment using the ACMG/AMP system was also provided. An additional reason for the complexity of the EAHAD-CFDB pathogenicity ascription was that no summary-level interpretation was available, so that all the pathogenicity assessments linked to each individual submission were concatenated. For example, c.3835 G>A was labelled with three tags: 'uncertain significance', '.', and '+?'.

HGMD provided a single interpretation of a variant based on a curated assessment of the published literature about it (Stenson et al., 2014). The *VWF* variants from this database were already labelled with one of five attributions: 'disease-causing mutation' (DM), 'probable/possible disease-causing mutation' (DM?), 'disease-associated polymorphism' (DP), 'functional polymorphism' (FP), and 'retired' (R) (Stenson et al., 2017). Curators used the DM? to indicate where caution was advised regarding variant-disease causation. There is not a published definition of how HGMD adjudges what is categorised as a polymorphism. However, the reference to "polymorphic frequency" and the "[general expectation of a] lack of clinical concern" with respect to these variants (Stenson et al., 2020) suggests that a higher-than-expected MAF of the variant compared with the disease of interest and a weak association of the variant with the given disease are important factors taken into consideration when HGMD curators label a variant as a polymorphism. The label DP was used when there was an association with a disease and there was additional (functional and/or in vitro) supportive evidence. The label FP was used where the data supporting pathogenicity was confined to laboratory experiments only and no direct link with disease had been established.

In an effort to understand the level of agreement between *VWF* DBs, and ascribe a simplified pathogenicity assertion to each variant in VWDbase, each unique variant was categorised using the criteria outlined in Table 3.1 (page 107).

One of the purposes of VWDbase was to identify a group of variants in *VWF* thought to be causal of VWD for further scrutiny in UKB. Just over 60% (912/1455) variants met the VWDbase criteria for being classified as pathogenic (Figure 3.3C, page 104). Although 127/1455 (8.7%) VWDbase variants had a summary assertion that there were 'conflicting interpretation of pathogenicity', 110/127 (86.6%) were labelled with at least one of the following tags: 'pathogenic', 'likely pathogenic', 'pathogenic/likely pathogenic (ClinVar and EAHAD-CFDB); DM, DM? (HGMD); and '+', '+?' (EAHAD-CFDB). Therefore, these were also deemed an interesting group for further evaluation utilising the UKB. A total of 1,039 variants labelled as 'pathogenic' and 'conflicting interpretation of

VWDbase assertion	Criteria		
	ClinVar	**HGMD**	**EAHAD-CFDB**
Not provided	Not provided *or* NA	NA	'.' *and/or* NA
Benign	Benign *or* Likely benign *or* Benign/Likely benign *or* NA	R *or* FP *or* NA	Benign *and/or* Likely benign *and/or* '-?' *and/or* '.' *and/or* NA
Uncertain significance	Uncertain significance *or* NA	DP *or* NA	Uncertain significance *and/or* '?' *and/or* '.' *and/or* NA
Pathogenic	Pathogenic *or* Likely pathogenic *or* Pathogenic/Likely pathogenic *or* Not provided *or* NA	DM *or* DM? *or* NA	Pathogenic *and/or* '+' *and/or* Likely pathogenic *and/or* '+?' *and/or* '.' *and/or* NA
Conflicting interpretation	Either: "Conflicting interpretations of pathogenicity" categorisation by ClinVar; or, does not meet any of the criteria above.		

Table 3.1 | Aggregation of pathogenicity assessments in VWDbase. Note that NA stands for not available, i.e. variant not present in the given *VWF* DB. As a variant had to be in one of the 3 *VWF* DBs in order to be included in VWDbase, there were no variants in which NA applied across all 3 *VWF* DBs.

pathogenicity' were grouped together under the heading 'Putatively Aetiological VWD Variants (PAVVs)' for further exploration.

3.4. Evaluation of PAVVs using UKB and gnomAD

WES data were obtained from 140,327 unrelated UKB participants (Subsection 2.1.4, pages 73-74). These data were then used to identify participants who had a PAVV. This was for the purpose of tackling the following two issues identified when appraising *VWF* DBs to create VWDbase which posed challenges for the interpretation of the variants they contain:

1) There is limited evidence to support the assertion of pathogenicity for the majority of VWD variants (Subsection 1.2.4, page 53-58). VWD is defined by a reduced plasma VWF antigen (VWF:Ag) and/or platelet-binding activity (VWF:Act) assays (Table 1.2, page 40). However, VWF levels were only routinely captured in EAHAD-CFDB, but only a third of VWDbase variants had an entry from this

database (Figure 3.3B, page 104). Information about co-segregation of VWD phenotype with a *VWF* variant is one of the factors taken into account when assessing pathogenicity. While this was not detailed in HGMD, it was captured by ClinVar and EAHAD-CFDB. On reviewing the PAVVs from these sources, only 363/1039 (34.9%) PAVVs recorded an assertion regarding co-segregation and for 246/363 (67.8%) the assertion was '?' (i.e. unknown), thus leaving only 117 PAVVs with clear information on co-segregation. Finally, MAF is important in adjudicating the likelihood of a *VWF* variant being pathogenic. There is consensus that VWD-causing variants are rare (Swystun & Lillicrap, 2018), but *how rare is rare*? This is a question about which there is ongoing debate given the wide estimates of the true prevalence of VWD (Subsection 1.2.3, pages 43-48). EAHAD-CFDB contained MAF information, however this estimate was only based on the population of the sampling laboratory. In contrast, HGMD recorded MAF in gnomAD for some variants but the field was blank for others, in which case it was difficult to determine whether the variant was unobserved in gnomAD (minor allele count of 0), the region had inadequate sequence coverage, or the information had not been used to annotate the variant. Furthermore, it was unclear how, if at all, MAF was factored into the assessment of DM and DM? categorisation in HGMD, which was relevant given that 455 of 1455 VWDbase variants were only identified in HGMD (Figure 3.3B, page 104).

2) There is increasing awareness that some variants, which are rare in individuals of European ancestry and labelled as being causal of VWD, have common MAFs in individuals of non-European ancestry. For example, Bellisimo et al. (2012) reported that p.Met740Ile was found in 12/66 (18.2%) African Americans, all of whom had normal VWF levels, but was absent from 118 American controls of European ancestry. None of the *VWF* DBs annotated their variants with ancestry specific MAFs. Despite this, the free text comments made by several ClinVar depositors from clinical testing laboratories demonstrate that some submitters are taking this into consideration. For example, in the deposition of c.7390C>T, the

submitter stated that it “was not observed in 6500 individuals of European and African American ancestry in the NHLBI Exome Sequencing Project”.

The UKB provides a large prospective population resource with which to accurately estimate the MAF of PAVVs. Leveraging published and database-deposited data, the shortlist of PAVVs identified in at least one UKB individual (‘UKB PAVVs’) was then systematically curated using MAF in order to stratify variants into those which could be accepted or rejected as pathogenic, as outlined in the following five subsections. The size of the populations in different ancestry groups within UKB meant that, when combined with WES from gnomAD v2.1, a comparison could be made not only between the MAF in those of European compared to African ancestry but - for the first time - also included a sufficiently large number of participants with South Asian, East Asian, and Admixed American ancestry to robustly challenge assumptions about the pathogenicity of PAVVs that have for too long focussed on the variant being rare in individuals of European ancestry (Subsection 1.2.5, pages 59-61).

3.4.1. Pathogenicity of PAVVs found in UKB participants (UKB PAVVs)

Of the 1039 PAVVs identified in VWDbase (Figure 3.3C, page 104), 1003 were taken forward for analysis in UKB WES data. This discrepancy was because an earlier version of VWDbase (frozen October 2020) was used for the filtering of UKB WES data and selection of variants for analysis in the MDT, prior to my final completion of VWDbase. And hence this earlier version of VWDbase contained fewer variants.

The 1,003 PAVVs were then searched for in the WES data of 140,327 UKB participants, representing 280,654 alleles. PAVVs were retained for downstream analysis if the sequencing reads were of sufficient quality and depth (Figure 3.4A, page 110) and were identified in at least one UKB participant. When strictly defined according to current international consensus guidelines, a diagnosis of VWD requires that the VWF:Ag and/or VWF:Act level is below 0.30 IU/ml (James et al., 2021). If this definition is used, the estimated population prevalence of VWD is 0.22%, i.e. ~ 1 in 500 (Table 1.3, page 45-46). Given that the majority of VWD is inherited in an autosomal dominant manner

Figure 3.4

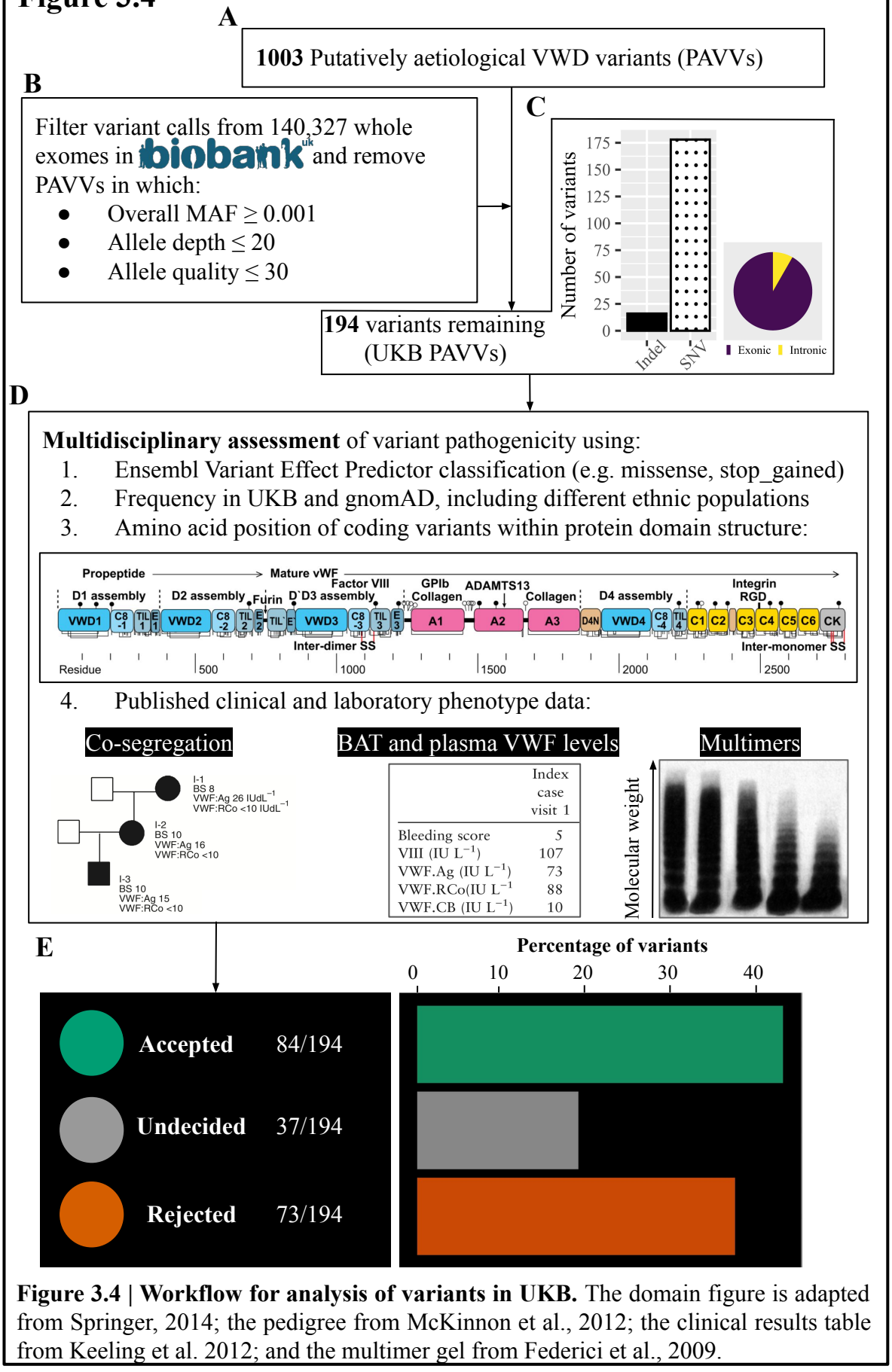


Figure 3.4 | Workflow for analysis of variants in UKB. The domain figure is adapted from Springer, 2014; the pedigree from McKinnon et al., 2012; the clinical results table from Keeling et al. 2012; and the multimer gel from Federici et al., 2009.

(Subsection 1.2.4, pages 53-54), this suggests that 1 in 1000 *VWF* alleles should harbour a pathogenic variant. Therefore, PAVVs with an MAF ≥ 0.001 in UKB were filtered out (Figure 3.4A, page 110). Of the initial 1,003 PAVVs, 194 fulfilled the above criteria, hereafter termed 'UKB PAVVs', of which 16 were indels and 178 were SNVs (Figure 3.4A-C, page 110). All UKB PAVVs were situated in the coding exons (numbered 2-52), or intervening introns and none were in the UTRs or in the region upstream of the TSS.

To assist in the evaluation of the pathogenicity of the PAVVs, I convened a multidisciplinary team (MDT). This group comprised myself assisted by one clinical data scientist and two academic consultant haematologists specialised in genetics and haemostasis (Acknowledgements, page 7). This group had prior experience of adjudging pathogenicity of variants obtained by high throughput sequencing (HTS) of several thousands of patients with congenital bleeding, thrombotic, and platelet disorders (Downes et al., 2019; Sims et al., 2020, Turro et al., 2020). The MDT opined on the pathogenicity of each UKB PAVV, using data about the variant from published articles, the *VWF* DBs from which they were retrieved, and the UKB MAF. The aim was to assess the likelihood that a PAVV was pathogenic. Based on the evidence, the MDT evaluated the effect of a PAVV in isolation, i.e. its effect in heterozygosity and/or homozygosity, but did not consider compound heterozygous effects with other *VWF* variants. When assessing each UKB PAVV, the key question asked by the MDT was: *does this variant cause the laboratory phenotype of VWD?* If the response to this question was yes, then the variant was given an outcome of 'accepted', if no, then the outcome was 'rejected', and if a decision could not be made, then the outcome was 'undecided'. This simple three tier system was used in preference to trying to assess each variant using the far more complex ACMG/AMP system (Richards et al., 2016). An additional reason for adopting this approach was the paucity of information available for many variants. If the decision was to accept the variant, then a further decision was made as to whether the pathogenicity of the variant was with respect to autosomal dominant or recessive VWD. Four main questions were asked when assessing UKB PAVV pathogenicity (Figure 3.4D, page 110) :

1) What was the (predicted) consequence of the UKB PAVV? To determine this, all 194 UKB PAVVs were annotated using the Variant Effect Predictor (VEP) tool

from Ensembl (McLaren et al., 2016). Using this method, eight categories of PAVVs were identified, as outlined in Table 3.2 (below). Only 16/194 (8.24%) PAVVs were located in introns, with the remainder situated in the coding sequence of *VWF* (Figure 3.4C, page 110). The VEP categories were further clustered into three types: predicted loss-of-function (pLoF) (Emdin et al., 2018); protein-altering; and, deep intronic (Table 3.2, below).

VEP classifications	Definition[1]	Number of PAVVs[2]	Intronic or exonic	Super-classification
missense_variant	≥1 base is changed to a different base, leading to change to amino acid sequence but no change to the protein length	141	Exonic	Protein-altering
stop_gained	≥1 base is changed to a different base, leading to a premature stop codon	16	Exonic	pLoF
frameshift_ variant	≥ 1 base insertion or deletion that results in an alteration to translational reading frame	12	Exonic	pLoF
intron_variant	Located within intron of transcript, but not within 8 bases of a boundary between intron and exon	9	Intronic	Deep intronic
missense_variant, splice_region_ variant	As for missense_variant but within 3 bases of 5' or 3' end of exon	7	Exonic	Protein-altering
splice_acceptor_ _variant	A change to the 2 bases at the 3' end of an intron	4	Intronic	pLoF
splice_donor_ _variant	A change to the 2 bases at the 5' end of an intron	3	Intronic	pLoF
inframe_deletion	A deletion of bases from the coding sequence but without disruption to the reading frame	2	Exonic	Protein-altering

Table 3.2 | VEP classification of UKB PAVVs in descending order of frequency. [1]Definitions adapted from https://www.ensembl.org/info/genome/variation/prediction/predicted_data.html (European Bioinformatics Institute (EMBL-EBI), 2021). [2]From a total of 194 PAVVs identified in UKB participants (Figure 3.4D, page 101).

2) What was the MAF both in the 280,654 alleles of UKB participants and the 251,496 alleles of gnomAD v2.1 participants? Both datasets were used to provide independent verification of MAFs in different overall populations. In addition, the MAF of the variant in specific, genetically-determined, ancestry super-populations

(Table 3.3, below) in both UKB and gnomAD v2.1 (Karczewski et al., 2020) was considered.

Population (abbreviation)	Allele number	
	UKB	gnomAD v2.1
African/African-American (AFR)	6932	16,256
Ashkenazi Jewish (ASJ)	Not determined	10,080
Latino/Admixed American (AMR)	1458	34,592
East Asian (EAS)	2302	18,394
European (EUR)	262,044	Not determined
Finnish (FIN)	Not determined	21,648
Non-Finnish European (NFE)	Not determined	113,770
South Asian (SAS)	7918	30,616
Other (population not assigned)	Not determined	6140
TOTAL	280,654	251,496

Table 3.3 | Allele numbers, stratified by ancestry super-populations in UKB and gnomAD v2.1 exomes.

3) If this was a protein-altering UKB PAVV, was it situated in a phenotypically-relevant domain? This distinction was most important for VWD2 subtypes, where there is known enrichment of variants aetiological of VWD2B and VWD2M in the A1 domain, VWD2M in the A3 domain, and VWD2N in the proximal three domains of the D'D3 assembly (de Jong & Eikenboom, 2017) (Figure 1.5, page 50).

4) What additional clinical and laboratory data were available to facilitate assessment of the UKB PAVV? Sources of these data were publications, ClinVar, EAHAD-CFDB, and HGMD. The focus was on ascertaining mode of inheritance (using details about pedigree consanguinity and co-segregation of phenotype with genotype) and evaluating the results of VWF assays. The latter were assessed in the context of the subtype(s) of VWD being considered. For example, when p.Ile1416Asn was reviewed, both its position in the A1 domain and the pedigree tree depicted in Figure 3.4D (page 110) were taken into account (McKinnon et al., 2012). The three pedigree members shown were heterozygous for the variant and

there was clear multi-generational co-segregation of low VWF:RCo/Ag in an autosomal dominant mode of inheritance, consistent with heterozygosity for this variant resulting in VWD2. In another example, the published pedigree who were heterozygous for the UKB PAVV, p.Met1761Lys (Keeling et al., 2012) were reviewed. This variant is situated in the A3 domain. The isolated, but marked, reduction in VWF:CB (Figure 3.4D, page 110) supported the assertion that this variant resulted in VWD2M. Plasma VWF multimer gels were available in some of the publications describing patients with types 2A, 2B, and 2M. The image shown on the bottom right of Figure 3.4D (page 110) shows the result of plasma gel electrophoresis, adapted from a case series of 67 patients with VWD2B (Federici et al., 2009). The lane on the far left of this blot shows the full range of VWF multimers of different molecular weights in plasma from individuals without VWD. The subsequent four lanes show the multimer profiles of VWD2B patients in the case series, revealing that such patients can have multimer profiles varying from normal (2nd lane) through to the complete loss of high and intermediate molecular weight multimers (5th lane).

Although the decision regarding some UKB PAVVs was unambiguous, for some of them, the data retrieved were conflicting. Therefore, a consensus based approach was adopted following a review of all available data sources. This incorporated clinical gestalt into the decision making. Although this is hard to define, its utility in informing the relationship between a variant and disease has been recognised for the diagnosis of paediatric congenital syndromes (Cianci & Selicorni, 2015) and with respect to the interpretation of variants identified by WES (Yehia & Eng, 2019).

In order to accept only variants where there was high confidence in pathogenicity, UKB PAVVs were interpreted using a conservative definition of VWD. Unless there was strong evidence supporting pathogenicity from other sources (e.g. co-segregation with phenotype across multiple pedigree members with supporting functional experiments), UKB PAVVs were generally only accepted if individual-level data could be found demonstrating a VWF:Act or VWF:Ag level < 0.30 IU/ml in the published source(s) and if the MAF was <

0.0001 (1 in 10,000) in both UKB and gnomAD. The rationale for the 0.30 IU/ml threshold was that a) monogenic VWD is more likely below this threshold (Subsection 1.2.4, pages 55-57), b) autosomal dominant variants are more likely to be phenotypically penetrant below this level (James & Goodeve, 2011) and c) it's below the lower-end of the histo-group O VWF reference range of 0.36 - 1.57 IU/ml (O'Donnell et al., 2002). Therefore, using this threshold should increase the likelihood that the reduced VWF:Act or VWF:Ag result is related to the *VWF* variant rather than group O. The MAF threshold of 0.0001 was based on the fact that the referral-based prevalence of VWD in the UK is 0.017%, equivalent to ~ 1 in 5,900 (Appendix 7.2, pages 243-249; Figure 1.4, page 47) and the majority of VWD cases are autosomal dominant. Therefore, no more than 1 in 10,000 *VWF* alleles would be expected to harbour a VWD-causing *VWF* variant that results in referral to a UK haemophilia centre, and hence this served as a pragmatic threshold for the MDT to use. Following on from this, a UKB PAVV was generally rejected by the MDT under the following circumstances: if it occurred at an MAF of ≥ 0.0001 in the overall population or an ancestry super-population of either UKB or gnomAD, and/or if the reported individual(s) had a VWF:Act and VWF:Ag ≥ 0.30 IU/ml, or had another *VWF* variant potentially aetiological for VWD. Less common reasons for rejection were recorded as 'other', with an explanation provided as to why the UKB PAVV was rejected.

Finally, a couple of other factors were taken into account by the MDT when reaching a decision. Clinical and laboratory results took precedence over in vitro functional experiments, particularly when the results were discordant. In addition, the sequencing strategy detailed in the article(s) reporting the variant was scrutinised, focussing on coverage (normally-targeted exon(s) or all 52 exons +/- promoter +/- intronic flanking sequence). Furthermore, a check was made of whether the authors had used a technique to detect structural variants, most commonly multiplex ligation-dependent probe amplification (MLPA) (Schouten et al., 2002) as if a structural variant was missed this could lead to the false attribution of pathogenicity to a SNV or indel.

Following MDT adjudication, 84/194 (43.3%) UK PAVVs were accepted as pathogenic, 73/194 (37.6%) were rejected, and 37/194 (19.1%) were classified as undecided (Figure 3.4E, page 104). One of the purposes of the MDT assessment was to critically appraise the summary pathogenicity assertions made for each UKB PAVV in VWDbase (Figure 3.3C, page 104; Table 3.1, page 107). Compared to all PAVVs, in which variants with 'conflicting interpretations of pathogenicity' only made up 127/1039 (12.2%) of variants, these were significantly overrepresented in the UKB PAVVs, comprising 54/194 (27.8%) of this subset (OR = 2.28, $P = 1.37 \times 10^{-5}$). Thirty eight out of 54 (70.4%) UK PAVVs with a prior VWDbase assertion of 'conflicting interpretation' were rejected, in contrast to 35/140 (25.0%) of those with a prior assertion of 'pathogenic'. However, only 76/140 (54.3%) of this group were accepted as pathogenic following the MDT reappraisal (Figure 3.5A, page 117), which suggests that the criteria used for adjudication of pathogenicity by the MDT were more stringent than that used by *VWF* DBs. Thirty two out of 35 (91.43%) pLoF variants were considered as pathogenic following review by the MDT (Figure 3.5B, page 117). To assess how UKB PAVVs were topologically distributed by MDT decision, these were plotted according to their cDNA position (Figure 3.6, page 118). In all three MDT decision categories, UKB PAVVs were distributed across the entire length of the transcript. There was a significantly different distribution of variants between those accepted by the MDT compared with those which were rejected (Table 3.4, below). As can be observed in Figure 3.6 (page 118), there is a proximal skewing of accepted UK PAVVs: 82% occur in the first two-thirds of the transcript (c.1-5625, corresponding to AA residues 1-1875), with significant overrepresentation of protein-altering variants in the A1 domain (OR 3.05, CI 1.46-5.92, P 1.82×10^{-3}) but not in the D'D3 assembly or A3 domains.

MDT outcome categories compared	D test statistic	*P* value
Accepted and undecided	0.18	0.37
Undecided and rejected	0.14	0.76
Accepted and rejected	0.23	0.04

Table 3.4 | Two-sample two-sided Kolmogorov-Smirnov test comparing the cDNA position distribution of UKB PAVVs in each MDT decision category.

Figure 3.5

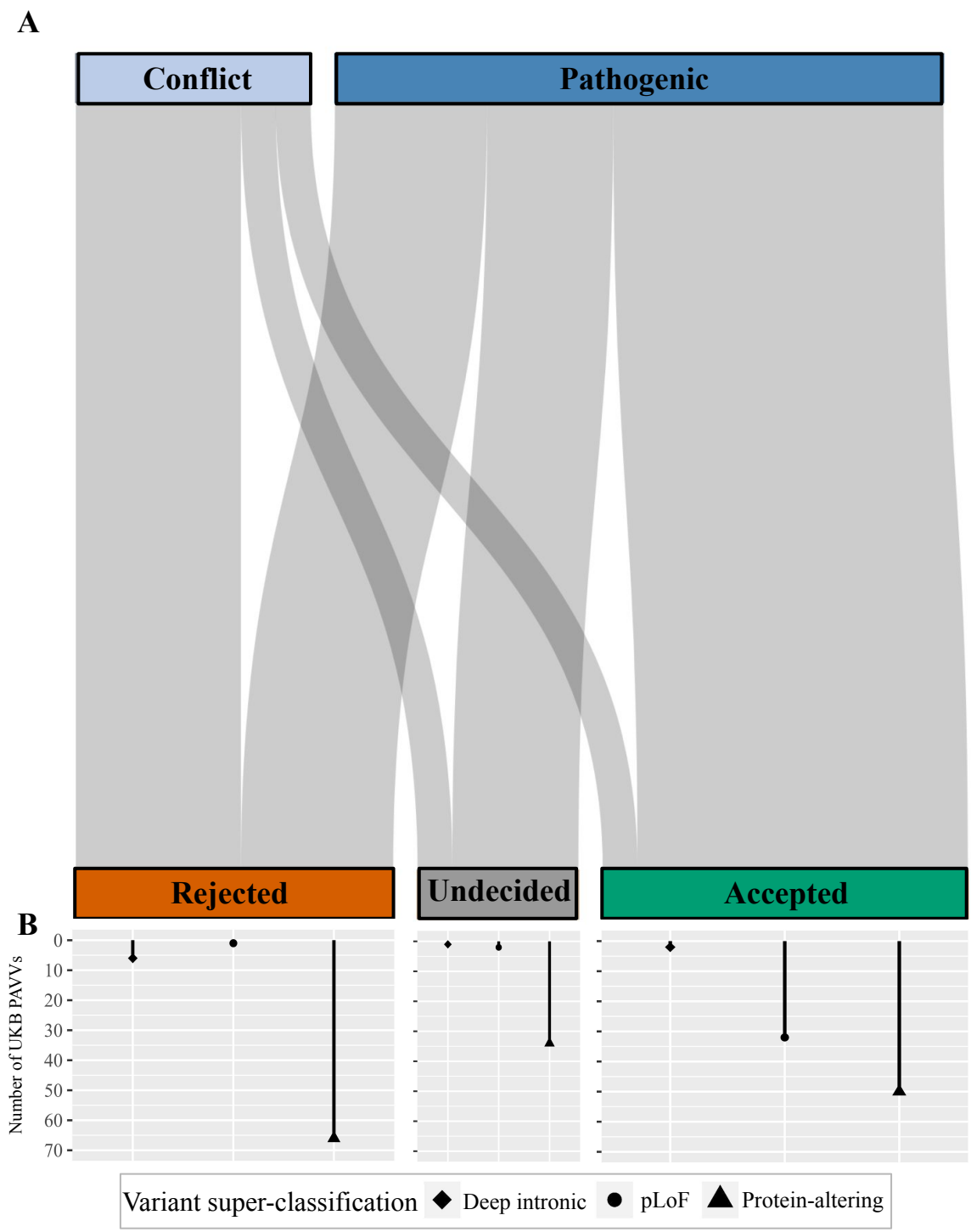


Figure 3.5 | Reappraising UKB PAVVs based on MDT outcome. (A) A Sankey diagram illustrating the summary pathogenicity assertions made for each UKB PAVV in VWDbase at the top and the MDT decision regarding variant pathogenicity at the bottom. Conflict is shorthand for 'conflicting interpretations of pathogenicity'. The width of each of the connecting grey links is proportional to the number of UKB PAVVs within the given category. (B) Lollipop plot representing the number of UKB PAVVs in each variant super-classification.

Figure 3.6

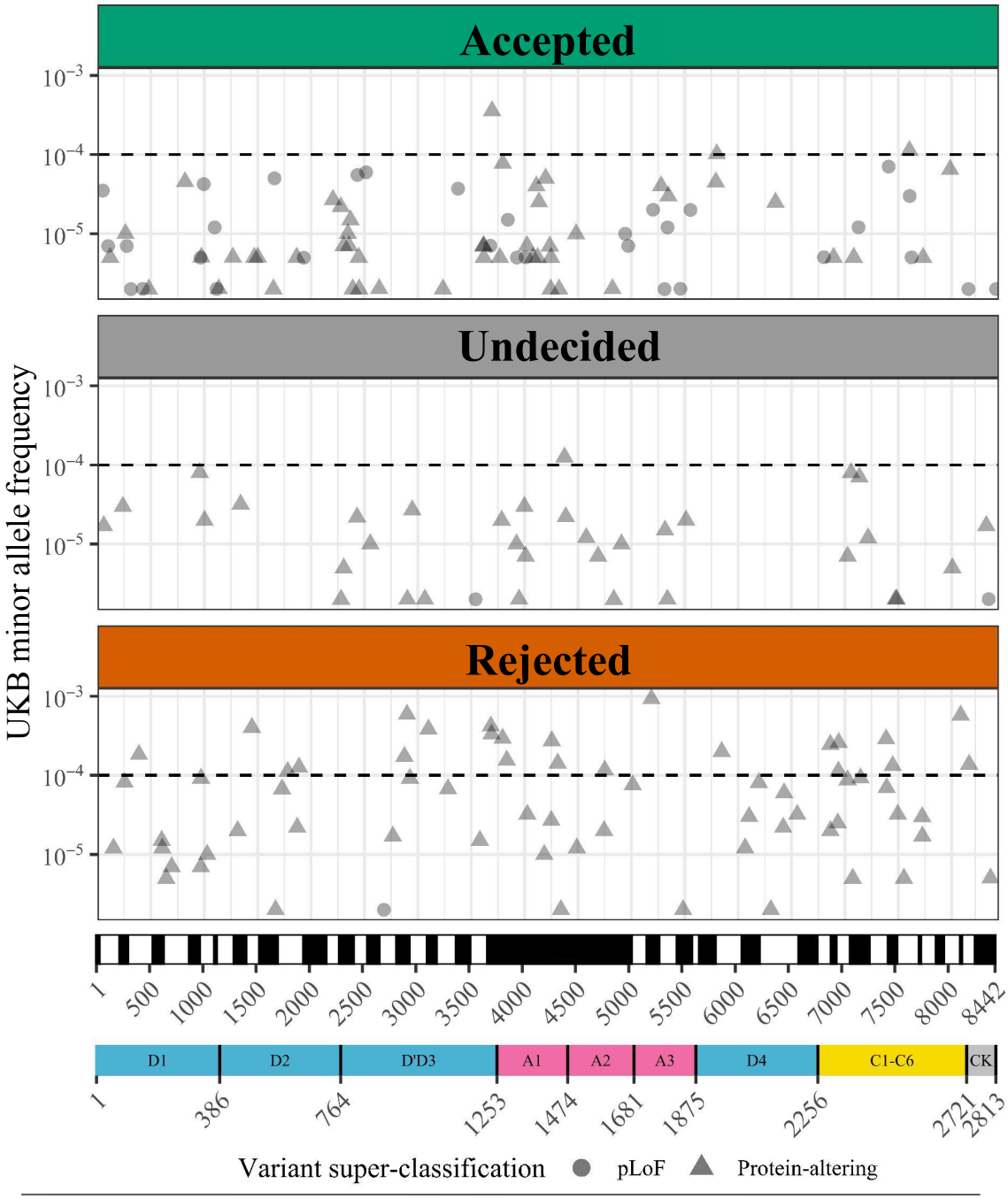


Figure 3.6 | The spectrum of UKB PAVVs. Each dot represents a UKB PAVV, at an x-axis position corresponding to its *VWF* cDNA nucleotide and VWF amino acid residue. Splice acceptor and donor variants are positioned at their nearest exonic nucleotide. Exons 2-52 are shown in alternating black and white. Key functional assemblies (D1, D2, D'D3, D4) and domains (A1, A2, A3, C1-C6, CK) of the VWF protein are shown using the same colour scheme as Figure 3.4. The y-axis represents the allele frequency in UKB using a base 10 logarithmic scale. The three horizontal facets stratify UKB PAVVs by MDT decision category. Note that the UKB MAFs of 10^{-3}, 10^{-4}, 10^{-5} correspond to 1 in 1,000, 1 in 10,000, and 1 in 100,000 alleles respectively and the dashed horizontal line represents the frequency cut off of 1 in 10,000 used by the MDT.

3.4.2. Heterogeneous phenotype of pLoF heterozygotes

Thirty two of the accepted UK PAVVs were pLoFs and two were deep intronic (Figure 3.5B, page 117): c.1730-10C>A and c.2968-14A>G. The former was reported in homozygosity in one individual who had a VWF:Ag of 0.03 IU/ml, and in another individual in compound heterozygosity with a pLoF, p.Gln1931Ter, who had a VWF:Ag of 0.04 IU/ml (Mohl et al., 2011). The latter was reported in compound heterozygosity with the frameshifting indel (c.1534–13_c.1551delinsCA, p.Leu512ThrfsTer138) in a patient with a VWF:Ag of 0.02 IU/ml (Liang et al., 2017). Therefore, given both of these deep intronic variants appeared to be behaving as null alleles, they were re-categorised as pLoFs for onward analysis (34 accepted pLoFs in total).

A contentious and unresolved issue is whether there is phenotypic expression of VWD in individuals heterozygous for pLoF variants (Subsection 1.2.4, page 54). For 22 of the accepted pLoFs there were individuals in whom VWF:Ag levels had been published (Figure 3.7, pages 120-121; Subsection 2.1.4, page 74). As illustrated in this figure, there is a much wider range of VWF:Ag levels (0.11 - 1.32 IU/ml) in the 45 published individuals who were heterozygous for pLoF variants, compared with the 55 published individuals who were homozygous for pLoFs (0.00 - 0.10 IU/ml). However, the pLoF heterozygotes had a median VWF:Ag, 0.39 IU/ml, which was below the lower end of the typical laboratory reference range. Furthermore, in 39 out of 45 pLoF heterozygotes, the publications included the Bleeding Assessment Tool (BAT) score or information about haemorrhagic symptoms. Based on this information, 31% were adjudicated to have a bleeding propensity. Therefore, of the 45 pLoF heterozygotes, 11 met criteria for VWD based on a VWF:Ag level < 0.30 IU/ml and six based on a VWF:Ag level between 0.30 and 0.49 IU/ml in the presence of a bleeding history (James et al., 2021). This data does suggest that one-third of individuals carrying an accepted pLoF variant in the literature have a VWD phenotype. Therefore, UKB participants who were pLoF heterozygotes were a subgroup that were focussed on in the analysis of bleeding symptoms in Subsection 4.3.3 (pages 163-166).

Figure 3.7

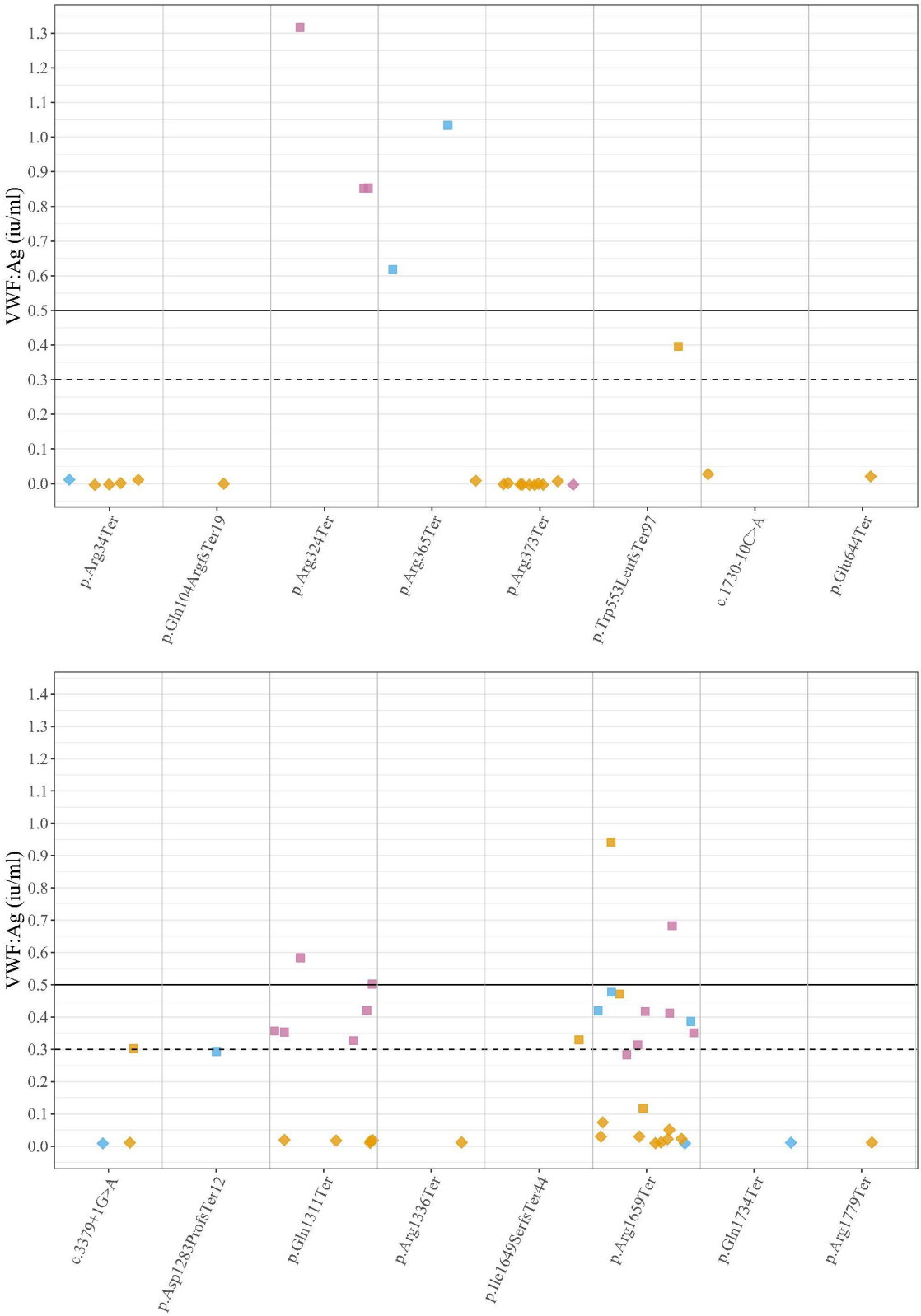

VWF:Ag (iu/ml)
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
p.Arg34Ter
p.Gln104ArgfsTer19
p.Arg324Ter
p.Arg365Ter
p.Arg373Ter
p.Trp553LeufsTer97
c.1730-10C>A
p.Glu644Ter
VWF:Ag (iu/ml)
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
c.3379+1G>A
p.Asp1283ProfsTer12
p.Gln1311Ter
p.Arg1336Ter
p.Ile1649SerfsTer44
p.Arg1659Ter
p.Gln1734Ter
p.Arg1779Ter
Bleeding propensity
Yes
NA
No
Zygosity
Heterozygous
Homozygous

Figure 3.7 (cont.)

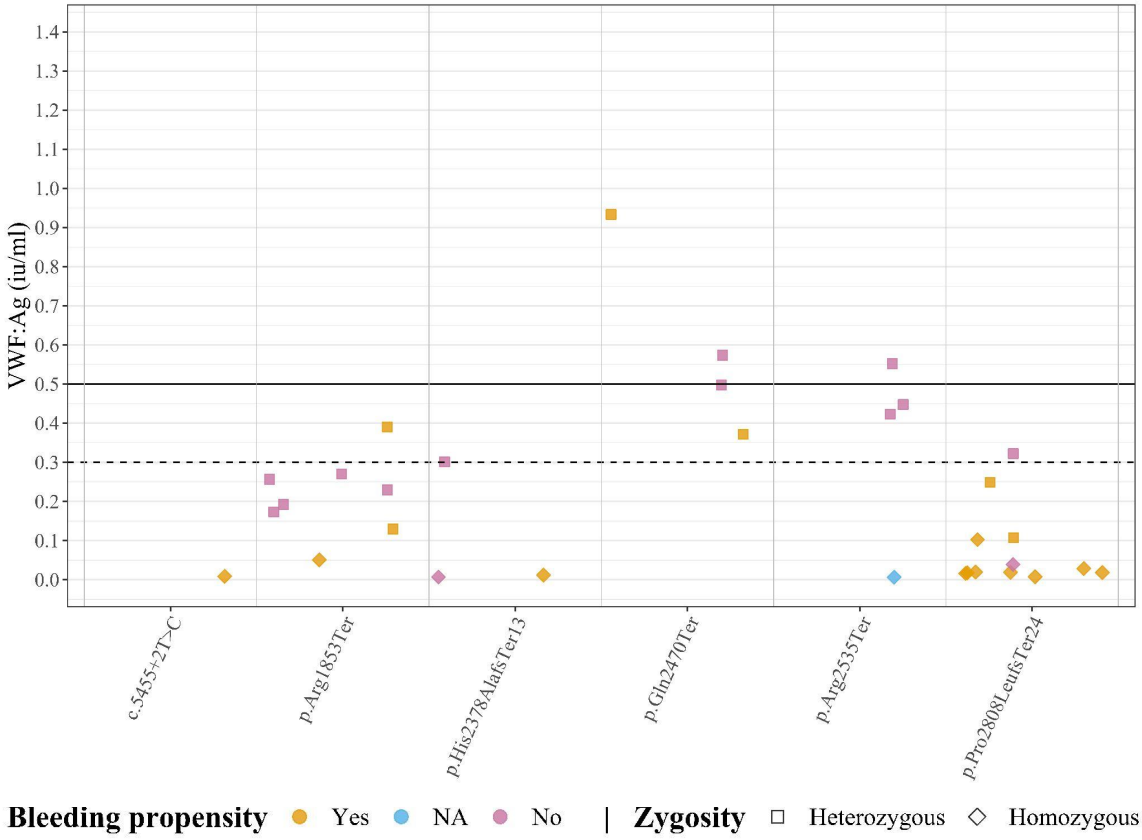


Figure 3.7 | The published phenotype of UKB accepted pLoFs. Each dot represents a published individual confirmed to be heterozygous (square) or homozygous (diamond) for a predicted loss of function (pLoF) variant accepted as pathogenic by the MDT. Individuals with an orange dot have a bleeding propensity, those without have a dot with a reddish purple colour, and dots with a sky blue colour indicate that the bleeding history was either not reported, nor valid because of the presence of another bleeding disorder (NA). The published phenotypes were extracted from: Ahmad et al., 2010; Ahmad et al., 2013; Bahnak et al., 1991; Baronciani et al. 2000; Baronciani et al. 2003; Borras et al., 2017; Bowman et al., 2013; Casaña et al., 2000; Corrales et al., 2009; Cumming et al., 2006; Eikenboom et al., 1998; Elayaperumal et al., 2018; Goodeve et al., 2007; Hampshire et al. 2013; Hilbert et al., 2002; Jokela et al., 2013; Kasatkar et al., 2014; Lethagen et al. 2002; Mohl et al., 2011; Robertson et al., 2011; Schneppenheim et al., 1994; and Zhang et al., 1992. Only the phenotype details provided in the first reported publication of a given individual was used to avoid duplication. The x-axis represents the pLoF variants, in ascending order along the transcript, using HGVSp nomenclature, except for those in introns which are in HGVSc nomenclature. The y-axis represents plasma VWF antigen (VWF:Ag). Where VWF:Ag was reported as being less than a specific value, e.g. <0.02 iu/ml, because of the known lower limits of detection associated with its measurement (Verfaillie et al., 2013), the measurement was rounded to the limit of detection, e.g. <0.02 to 0.02, so the results could be visualised on a continuous scale. Individuals with a VWF:Ag measurement below the dashed horizontal line (0.30 IU/ml) had levels consistent with VWD, and if between the dashed and solid line (0.50 IU/ml) consistent with VWD if bleeding symptoms were also present (James et al., 2021).

3.4.3. Using the CADD score to predict pathogenicity

In ascertaining the genetic basis of VWD, one of the major hurdles is the prioritisation of variants identified within *VWF* (Freson & Turro, 2017). There are several reasons why this is challenging. First, the *VWF* open reading frame (ORF) is in the upper quartile of the ORF distribution of all human proteins (Vanderperre et al., 2013) and the *VWF* gene has a low 'probability of being loss-of-function intolerant (pLI)' score (Lek et al., 2016) of 0 (range 0-1). As a consequence, *VWF* acquires and retains mutations at the normal background rate. Second, because of the long ORF and low pLI the gene has acquired a high burden of rare variants. Third, under normal evolutionary considerations the vast majority of these rare variants are expected to be function neutral (Dudley et al., 2012). Fourth, in VWDbase, there were 1,455 *VWF* variants in which a variant-VWD pairing had been previously suggested (Figure 3.3, page 104). Finally, in the majority of VWD cases, a search for causal variants needs to be made in all protein-coding exons and their immediate flanking regions (Subsection 3.3.1, pages 101-102). This is because domain-specific enrichment of variants only applies to VWD2, which makes up the minority of total VWD cases (Subsection 1.2.2, page 41-42). Given these challenges, there is a need to develop approaches for the efficient prioritisation of rare variants in *VWF* generated from the short read sequencing of patients with VWD.

Given the systematic nature of the MDT appraisal of the 194 UKB PAVVs, the outcome decisions provided a gold standard against which to determine whether there was a simpler and faster method of arriving at the same conclusion. The metric already used, population MAF, was helpful for the filtering of PAVVs in the UKB WES data in order to establish a collection of variants sufficiently rare to be potentially consistent with a diagnosis of VWD (Figure 3.4, page 110). However, a low MAF by itself was not enough to establish pathogenicity as highlighted by the fact that 48/73 (65.8%) rejected UKB PAVVs had an overall MAF in UKB of < 1 in 10,000 (Figure 3.6, page 118). This is consistent with the notion that a low MAF must not be considered synonymous with deleteriousness (Richards et al. 2016).

Figure 3.8

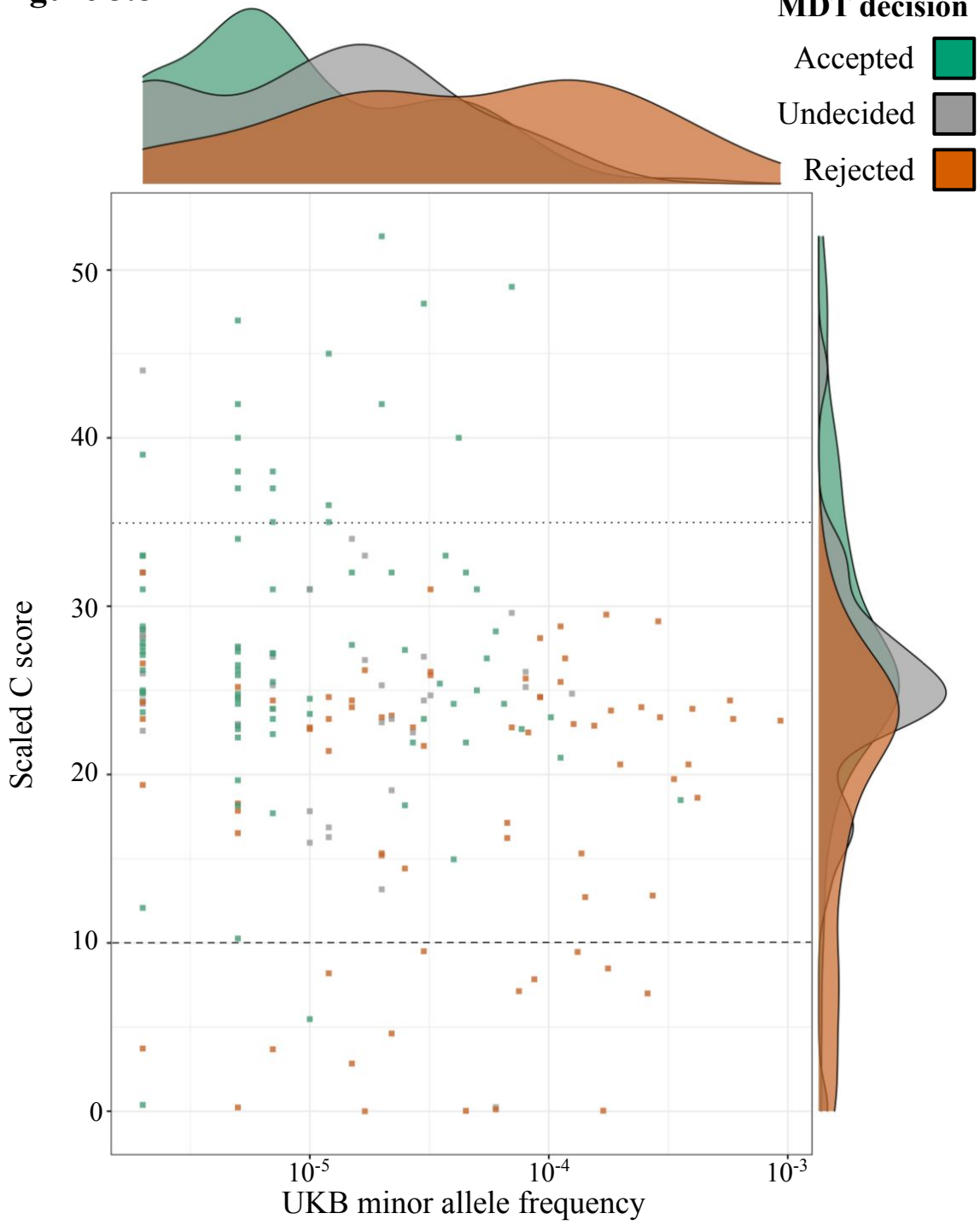


Figure 3.8 | The distribution of Combined Annotation-Dependent Depletion (CADD) scores of UKB PAVVs. Each square on the scatter plot represents one of the 194 UKB PAVVs. The x-axis represents the allele frequency in UKB using a base 10 logarithmic scale: 10^{-5}, 10^{-4}, 10^{-3} correspond to 1 in 100,000, 10,000, and 1,000 alleles respectively. The y-axis represents the scaled C scores (Rentzsch et al., 2021). The marginal plots represent the density distributions. The colours represent the MDT decision on the pathogenicity of the UKB PAVV. The dotted and dashed lines at scaled C scores of 35 and 10 represent proposed cut-offs for filtering *VWF* variants with a high and low probability, respectively, of being pathogenic for VWD.

Therefore, I turned to the Combined Annotation-Dependent Depletion (CADD) score as an estimate of deleteriousness (Kircher et al., 2014). Two types of CADD score are available, the raw, and the scaled. To facilitate comparison of deleteriousness between variants the scaled CADD (C) score is more useful. The scaled score represents the rank of the raw score on a $\log_{10}$ scale from 1 to 99 (Kircher et al., 2014), such that a score of 10 corresponds to the 10% least frequently seen alleles, 20 to 1%, 30 to 0.1%, and so on (Rentzsch et al., 2019). Higher scaled C scores (hereafter abbreviated to just 'C scores') are associated with both greater deleteriousness and reduced frequency amongst observed human alleles compared to simulated common ancestor alleles. Each of the 194 UKB PAVVs was annotated with the latest C score (Subsection 2.1.4, page 74).

A scatterplot of the C scores of the variants in each of the MDT outcome categories is shown in Figure 3.8 (page 123). Although 112/194 (58%) of all UKB PAVVs had a C score between 20 and 30 inclusive, there was a difference in the distribution of the scores between the three MDT outcome groups (Kruskal test, $\chi^2 = 40.09$; $P = 1.97 \times 10^{-9}$). Indeed, the median C scores of the accepted and rejected UKB PAVVs were 27.0 and 22.8 respectively. Furthermore, the positive Fisher-Pearson moment coefficient of skewness of 0.14 for accepted variants was consistent with their visible tail towards higher C scores, whereas for rejected variants the skewness was -0.86 consistent with the tail towards lower C scores. Based on this information, and visual inspection of the density plots, two different C score cutoffs were chosen: 35 or greater, and 10 or less. The former predicted a UK PAVV being accepted as pathogenic in 94% of cases, and the latter predicted a UK PAVV being rejected as pathogenic in 84% of cases.

3.4.4. Reason for rejecting the pathogenicity of UKB PAVVs

Having established that rejected PAVVs have a different C score distribution to accepted UKB PAVVs, the reasons for rejection were further explored to establish if this could inform additional variant prioritisation decisions. To understand whether there was a specific type of variant that had a C score of 10 or below, all 73 rejected UKB PAVVs were plotted in descending order (Figure 3.9, page 126, which revealed that there were 16 variants with C scores less than 10. There were six deep intronic variants (defined as per

Table 3.2, page 112) which were rejected UKB PAVVs, and all six had C scores less than 10. In comparison, c.1730-10C>A and c.2968-14A>G, which were both accepted as pathogenic, had C scores of 17.7 and 12.1 respectively, suggesting that deep intronic variants should only be considered as potentially relevant if the C score is greater than 10.

There were four reasons why UKB PAVVs were rejected. The most common reason was due to VWF levels (Figure 3.9, page 126), i.e. if the VWF:Ag and VWF:Act levels were greater than or equal to 0.30 IU/ml in the majority of published individuals with the given variant (Bellissimo et al., 2012; Borras et al., 2017; Boylan et al., 2015, Castaman et al., 2008; Corrales et al., 2009; Goodeve et al., 2007; Hampshire et al., 2010; Hampshire et al., 2013; Hickson et al., 2010; James et al., 2007; Melo-Nava et al., 2007; Schneppenheim et al. 2010; Ribba et al., 2001; Robertson et al., 2011; Vangenechten et al., 2019). This was not unexpected, as the VWF level threshold of 0.30 IU/ml used as a criterion for accepting a PAVV as pathogenic was a deliberately strict definition (as outlined in Subsection 3.4.1, pages 114-115). The varied eligibility criteria adopted by different studies (Table 1.5, pages 56-57) is one potential explanation for the high number of variants which were rejected based on VWF levels. Individuals with the rejected UKB PAVVs, c.2686-1G>C, p.Ile1094Thr, p.Arg1583Trp, and p.Arg1830Cys, who had VWF:Act levels of 0.42, 0.74, 0.54, and 0.52 - 0.73 IU/ml respectively, were identified in the MCMDM1-VWD (Goodeve et al., 2007) and BRNO-VWD (Vangenechten et al., 2019) studies. These individuals were enrolled into these studies if they were “historically diagnosed” with VWD. Subsequent age-related rise in VWF levels (Gill et al., 1987) from when the patient was initially diagnosed compared to when they entered the study may be one of the reasons why three out of four of the individuals had VWF:Act levels which were now within the reference range. Seven of the 30 ‘VWF level ≥ 0.30 IU/ml’ UKB PAVVs were rejected based on data obtained from patients published in the PCM-EVW-ES study, which included patients in the “presence of VWF candidate mutations” (which was not defined) without the necessity for the presence of a laboratory or bleeding phenotype (Borras et al., 2017).

Figure 3.9

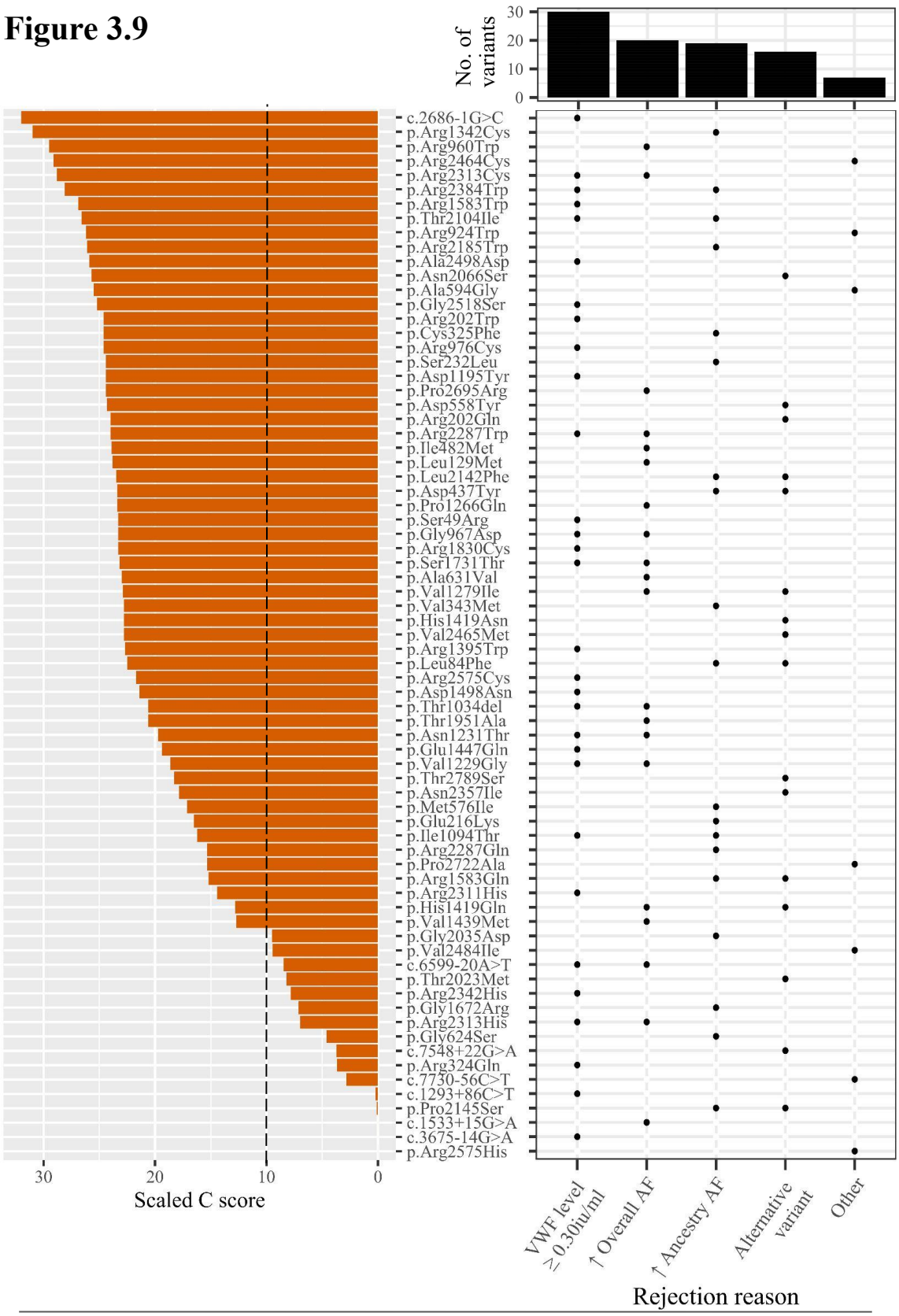


Figure 3.9 | The reasons for UKB PAVV rejection. The 73 rejected UKB PAVVs are listed in ascending order of C score. The dot plot shows rejection reasons. The number in each rejection category is shown by the bar graph. The dashed line represents the C score threshold (10), below which 84% of UKB PAVVs were rejected as pathogenic.

Sixteen PAVVs were published only in individuals where the authors had identified another potentially-causal variant (in cis or trans). The latter was defined as a UKB PAVV that the MDT had accepted, or if it wasn't in the list of 194 UKB PAVVs, then if the alternative variant was reported as a PV or LPV in ClinVar or as DM in HGMD, or was a deletion > 50 bp. If there was no publication in which the UK PAVV was reported without an alternative causal variant, then the published level was not taken into consideration such that rejection reasons, 'VWF level ≥ 0.30 iu/ml' and 'Alternative variant', were mutually exclusive categories.

Twenty UKB PAVVs were rejected based on the overall MAF being too high, i.e. if the MAF in both the 280,654 alleles of UKB participants, and 251,496 alleles of gnomAD participants was > 0.0001. Four major non-European ancestry super-populations were defined because they were common to both UKB and gnomAD exomes (Table 3.3, page 113): African/African-American (AFR); East Asian (EAS); Latino/Admixed American (AMR); and South Asian (SAS) (Subsection 2.1.4, page 73)

If the overall MAF was < 0.0001 in at least one of UKB and gnomAD but the MAF in at least one of these four ancestry super-populations was ≥ 0.0001 in both UKB and gnomAD, then the variant was rejected using the category '↑ ancestry AF', which applied to 19 UKB PAVVs (Figure 3.9, page 126). Therefore, 53.0% of variants were rejected on the basis that their MAF, either in the total population or in a specific ancestry super-population, was higher than that which would be expected for a referral-based prevalence of VWD of ~ 1 in 5,900 (Subsection 3.4.1, pages 114-115). There was no significant difference in the distribution of the C scores of the variants in each of the five rejection categories (Kruskal test, $\chi^2 = 1.04$; $P = 0.90$).

3.4.5. The ancestry specific MAF of some UKB PAVVs is too high for VWD

Only three previous analyses have investigated the discrepancy in MAF of potentially causal VWD variants between different ancestry groups (Bellissimo et al., 2012; Johnsen et al., 2013; Wang et al., 2013). The focus of the two former papers was on individuals of African ancestry, with an ability to detect alleles with frequencies down to 0.0003 (as 1455

individuals were sequenced by Johnsen et al., 2013). However, in other non-European ancestry groups the lower limit of the MAF was only 0.002 (as 286 individuals of Asian ancestry were studied in Wang et al., 2013). In contrast, the data aggregation across the four non-European ancestry super-populations common to both UKB and gnomAD increased the number of alleles available for analysis in AFR tenfold compared to previous publications, and by 100 fold for EAS, AMR, and SAS as shown in Table 3.5 (below).

Ancestry group	Number of combined alleles
European (EUR)	*375,812*
African/African-American (AFR)	23,188
Admixed American/Latino (AMR)	36,050
East Asian (EAS)	20,696
South Asian (SAS)	38,534

Table 3.5 | Total alleles in each of the four non-European ancestry super-populations. Following aggregation of both UKB and gnomAD v2.1 exomes, the total number of combined alleles in each of the four non-European super-populations is shown. For comparison the number of alleles in those of European ancestry is shown in italics.

Nineteen UKB PAVVs were rejected because they were sufficiently rare in European ancestry (EUR) participants in UKB and gnomAD to be compatible with VWD (overall MAF < 0.0001) but had a MAF > 0.0001 in at least one of AFR, AMR, EAS, and SAS ancestry super-populations (Figure 3.9, page 126). For each of these variants the aggregated MAF in each of the five ancestry super-populations was then determined.

This data is depicted in the heatmap in Figure 3.10A (page 130). This shows that there are two main types of '↑ Ancestry AF' UKB PAVVs: those in whom the variant can be detected in four or five different ancestries ('pan-ancestry' group) and those confined to one or two different ancestries whilst absent from the rest ('oligo-ancestry' group). p.Met576Ile, p.Ile1094Thr, p.Arg1342Cys, p.Arg1583Gln, p.Gly1672Arg, p.Arg2287Gln, and p.Arg2384Trp are in the pan-ancestry group. The C scores of these variants are 17, 16, 31, 15, and 7, 15, and 28 respectively. Therefore, with the exception of p.Arg1342Cys and p.Arg2384Trp, the pan-ancestry variants are in the lowest third of C scores of the UKB PAVVs which were rejected (Figure 3.9, page 126). This could be because these variants arose in a common ancestor but have been under weak purifying selection or that these are

recent, recurrent, mutations that have occurred independently in different ethnic backgrounds as has also been observed in β-thalassaemia (Filon et al., 1994).

The UKB PAVVs which were categorised into the oligo-ancestry group were p.Leu84Phe, p.Glu216Lys, p.Ser232Leu, p.Asp437Tyr, p.Gly624Ser, p.Thr2104Ile, and p.Leu2142Phe. Four of these variants - Glu216Lys, Gly624Ser, Thr2104Ile, and Leu2142Phe - and three from the pan-ancestry group, Gly1672Arg, Gly2035Asp, and Arg2287Gln, had a MAF which was highest in East Asians, EAS (mean 3.0×10^{-3}, corresponding to 1 in 329 alleles; range 1.5×10^{-4} - 5.5×10^{-3} corresponding to 1 in 183 - 1 in 6,900 alleles). Of these seven variants, the six with the highest EAS AF were initially described in publications in which the proband was of an ethnicity (Chinese or Japanese) consistent with the genetic definition of EAS ancestry used. These six variants are indicated by the circles on the heatmap of Figure 3.10A (page 130). All of these six variants had an EAS MAF > 0.001 (1×10^{-3}), meaning that more than 1 in 500 East Asian individuals would be expected to be heterozygous for the given variant. This is not only higher than the population-based prevalence of VWD of ~ 1 in 500 (Subsection 1.2.3, page 48), it is also over 100-fold higher than the referral-based VWD prevalence of 1 in ~ 91,000 in Japan (Appendix 7.2, pages 245; Figure 1.4, page 47). The evidence that any of these six, 'EAS-frequent' variants can be considered pathogenic for VWD is now scrutinised in turn.

Glu216Lys was first identified in a 4-year-old boy from Shanghai (Qin et al., 2014). He was reported as blood group O. Their VWF:Act results are shown in Figure 3.10B (page 130), showing the boy's low VWF:Act of 0.23 IU/ml. However, when the same boy was reported again in a later publication by the same group, the VWF:Act and all other VWF parameters were normal (Liang et al., 2017). Glu216Lys was the only variant identified despite a comprehensive sequencing strategy that included all *VWF* exons and exon/intron boundaries (Liang et al., 2017; Qin et al., 2014).

Gly624Ser has only been reported in one Chinese individual (P44) with VWD2A who had a VWF:Act of 0.18 IU/ml, VWF:Ag of 0.43 IU/ml, corresponding to an Act/Ag ratio of 0.4 (Liang et al., 2017). Gly624Ser is located in the D2 domain, where variants causing

Figure 3.10

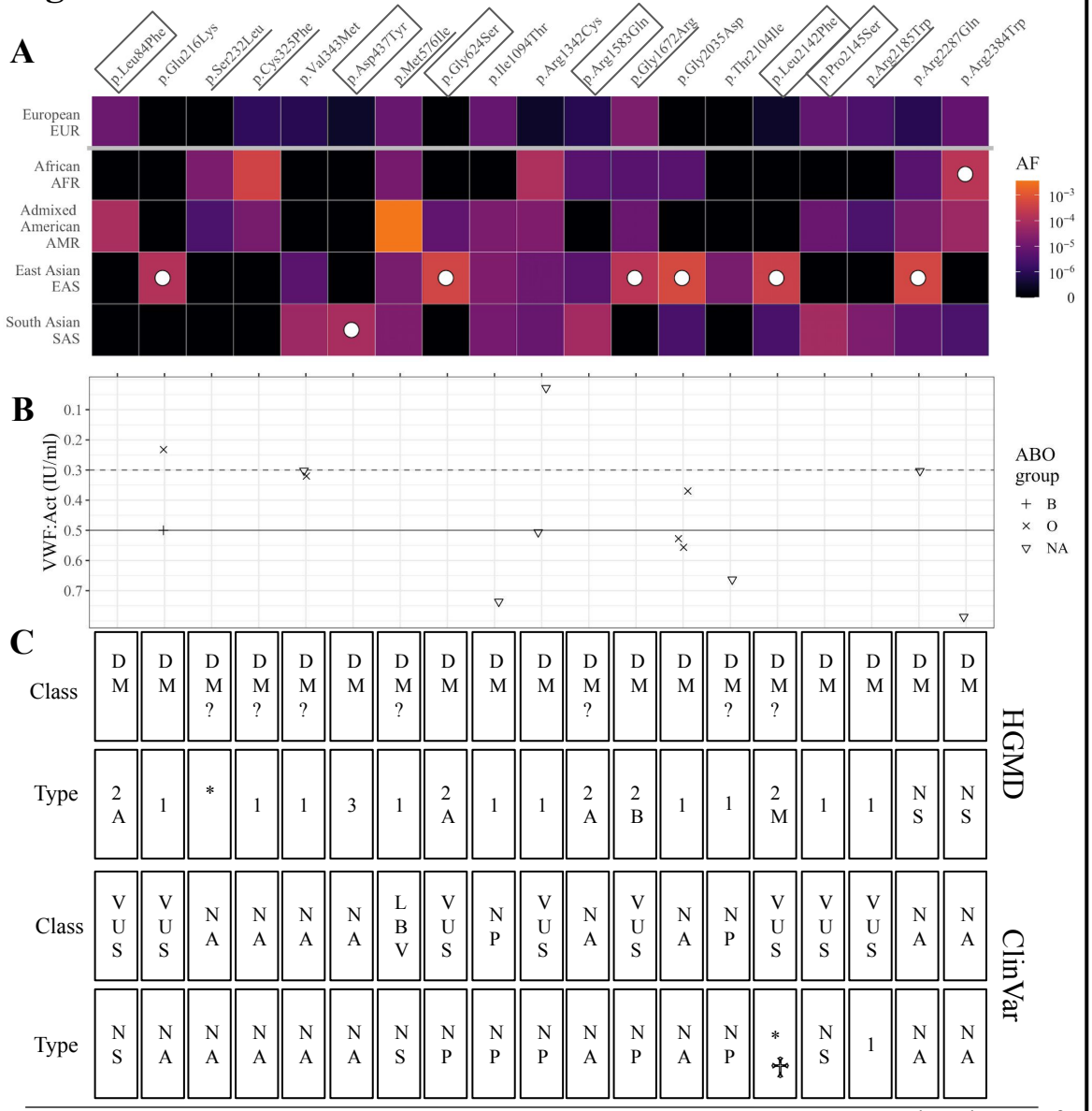


Figure 3.10 | Rejected UKB PAVVs with high ancestry specific AFs. Each column of (A), (B), and (C) represents one 19 UKB PAVV. (A) A heatmap representing the MAF of the variant aggregated across both UKB and gnomAD v2.1 exomes. This is stratified by the four non-European ancestry super-populations with Europeans shown above the grey horizontal line for comparison. A circle is placed in a tile of the heatmap if the individual(s) in the publication in which the UKB PAVV was first published was of an ethnicity consistent with the corresponding ancestry group. (B) The VWF:Act levels of published individuals are shown in the dot plot below the heatmap for those who do not have an alternative causative variant, with the shape of the dot indicating ABO group. Where the UKB PAVV x-axis label is boxed there are no published individuals without an additional causative variant, so the VWF level is not on the graph; where it is underlined there are no published levels for the given individual. (C) A table showing the classification in HGMD and ClinVar: NS denotes not specified, i.e. listed as VWD variant but sub-type not given; NA denotes that variant is not in the database; NP indicates that the information wasn't provided. *Denotes a phenotype of thrombocytopenia; ✝denotes phenotype of abnormal bleeding.

VWD2A have previously been reported (Figure 1.5B, page 50). However, P44 was reported as having the additional, much rarer p.Ile1628Thr variant. Ile1628Thr co-segregated across four generations in a pedigree of 39 individuals with VWD2A (Iannuzzi et al., 1991), and has subsequently been identified in two additional pedigrees with the same phenotype (Ahmad et al., 2014; Melo-Nava et al., 2007). Furthermore, Ile1628Thr occurs in the distal A2 domain, close to the ADAMTS13 cleavage site (Dent et al., 1990) and through functional work has been shown to result in enhanced ADAMTS13 proteolysis (Interlandi et al., 2012), a known mechanism for the depletion of HMWMs resulting in VWD2A. Therefore, there is much stronger evidence that P44's type 2A VWD is caused by the rare, functionally-deleterious Ile1628Thr variant than Gly624Ser.

Gly1672Arg was first described in a Japanese individual reported to have type 2A VWD but no VWF levels were reported in the manuscript (Hagiwara et al., 1996). Gly1672Arg did co-segregate with the VWD2A phenotype in both the proband and her affected father and was absent from the unaffected mother. As genetic sequencing was limited to exon 28, other potentially causal VWD2A variants may have been overlooked. Further weight in support of this argument is that the EAS MAF of this variant, 1.8×10^{-3}, determined from the aggregated UKB and gnomAD v2.1 exomes (Figure 3.10A, page 130) is close to that calculated in 8,300 Japanese alleles, 2.3×10^{-3} (Tadaka et al., 2021). This highlights the limitations of using small control populations (Hagiwara and colleagues sequenced 50 healthy Japanese individuals) to estimate the MAF of a given variant.

Out of the six EAS-frequent variants, Gly2035Asp was the most common with an aggregated MAF of 5.5×10^{-3} and in different datasets it was found at similar frequencies: 1.6×10^{-2} in Kinh Vietnamese and 1.3×10^{-3} in Japanese respectively (Le et al., 2019; Tadaka et al., 2021). Gly2035Asp was reported in three unrelated Chinese individuals (Liang et al., 2017). In two of the individuals, the VWF:Act levels were normal and one had a VWF:Act of 0.37 IU/ml which could be explained by blood group O alone (Figure 3.10B, page 130). Furthermore, the ISTH-BAT scores for the two female patients (2 and 4), and one male patient (3), were within the range considered normal (Elbatarny et al.,

2014); taken alongside the laboratory results there is very weak phenotypic evidence to suggest that these patients have VWD.

Leu2142Phe was reported as a novel variant in an individual with a VWD2M phenotype (VWF:Ag 0.16 iu/ml; VWF:Act 0.03 iu/ml) in a Chinese individual, P82 (Liang et al., 2017), and is not published elsewhere. However, the authors note that on the same allele as Leu2142Phe, the rare c.3897del variant was also identified. The consequence of this deletion is a frameshift and introduction of a premature termination codon (p.Phe1299LeufsTer5), 5' of Leu2142Phe. Phe1299LeufsTer5 was suspected to be a causal variant of VWD3 in a child who also had the compound heterozygous p.Cys827Tyr variant and the child's father had Phe1299LeufsTer5 in heterozygosity and had a phenotype of type 1 VWD (Ouyang et al., 2014). Therefore Leu2142Phe is irrelevant to the phenotype of P82, and the reason the authors of Liang et al., 2017 have reported this variant in the case of P82 is unclear. Taken together with its high EAS MAF and lack of supportive evidence in any other publications, Leu2142Phe is not a credible pathogenic VWD variant.

Arg2287Gln had an aggregated MAF of 4.8×10^{-3} (Figure 3.10A, page 130), comparable to the 1.5×10^{-3} calculated from 8,300 Japanese alleles (Tadaka et al., 2021). There is only one publication reporting the variant in a Chinese female (P1) (Qin et al., 2011). Despite a comprehensive sequencing strategy including all 52 exons and flanking sequences only Arg2287Gln and the Asp1472His were identified. The latter is a known, common, benign variant in *VWF* (Flood et al., 2013b) known to cause an in vitro reduction in platelet-dependent VWF activity when measured by the ristocetin cofactor activity (VWF:RCo) that is not functionally relevant (Subsection 1.2.2, pages 42-43). Therefore, this may explain the VWF:Act of 0.30 IU/ml in P1 (Figure 3.10B, page 130).

In summary, by combining WES data from both UKB and gnomAD v2.1 in order to estimate an aggregated MAF for each of four different ancestry super-populations, 19 of the UKB PAVVs were found to have a MAF ≥ 1 in 10,000 in at least one non-European ancestry group. This is in contrast to their MAF of < 1 in 10,000 in the (majority European) overall populations of both UKB and gnomAD v2.1. Based on a population

prevalence of VWD of ~ 1 in 500, it can be extrapolated that approximately 1 in 1000 alleles contain a pathogenic *VWF* variant. Six of the 19 UKB PAVVs (Glu216Lys, Gly624Ser, Gly1672Arg, Gly2035Asp, Leu2142Phe, and Arg2287Gln) occurred at a MAF in EAS higher than this, which suggests that they are not causal of VWD. Three of these variants - Gly216Lys, Gly624Ser, and Gly2035Asp - were absent from 375,812 aggregated alleles of individuals of EUR ancestry (Figure 3.10A, page 130), and the other 3 are 10 - 1000 times rarer in EUR than EAS populations. This illustrates the risk of solely relying on the overall MAF of large reference datasets such as gnomAD when filtering potentially-aetiological VWD variants because of the European bias of the total populations. In the case of the six EAS-frequent variants, these are likely to be "coincidental bystanders". That is, the patients in the studies reporting the variants are of EAS ancestry (Hagiwara et al., 1996; Liang et al., 2017; Qin et al., 2011; Qin et al., 2014), but, because of the lack of an adequately-sized control dataset to estimate MAF in their local population, a previously unreported *VWF* variant has been found - by chance - because the variant is relatively common in this population. And then, because of the reasons outlined above, including poor variant prioritisation and the inclusion of patients with very mild or absent VWD phenotypes, these variants have been attributed as the cause of the published individual's VWD, when it fact should have either been discarded in favour of alternative, more plausible causal VWD variants, or the individual shouldn't have been included at all (because they don't meet criteria for VWD). Unfortunately, the mislabelling (as pathogenic) has then been carried forward into HGMD which rated five out of six of these EAS-frequent variants as disease-causing mutations (DMs) (Figure 3.10C, page 130), a problem that has been highlighted with respect to HGMD and ancestry-specific alleles in other diseases (Kessler et al., 2016). However, the pathogenicity classification for these six EAS-frequent variants in ClinVar was more circumspect, with the four out of six that had a classification recorded being adjudicated as VUS.

3.5. Conclusions

For too long, the variants in *VWF* potentially causal of VWD have been siloed in disparate repositories, termed here as *VWF* DBs (ClinVar, EAHAD-CFDB, and HGMD), with

differing terminology, inclusion strategies, and means for adjudicating whether the variants they contain are likely to be pathogenic of VWD or not. The absence of a comprehensive resource has stymied progress in understanding the genetic basis of VWD in several different ways. Firstly, failure to consider all relevant *VWF* variants could impact on the estimation of their collective effect on a phenotype of interest. For example, in a case-control comparison of individuals with plasma VWF:Ag levels between 0.30-0.50 IU/ml and heavy menstrual bleeding, *VWF* variants in HGMD were overlooked in the authors' gene burden analysis (Sadler et al., 2022). This is despite 31% of *VWF* variants previously associated with VWD being unique to HGMD (Figure 3.3B, page 104). Secondly, not utilising all *VWF* DBs may mean that variants are missed: in the BRNO-VWD study (Vangenechten et al., 2019), the authors cross-referenced the *VWF* variants they identified against EAHAD-CFDB which represents only 38% of those reported (Figure 3.3B, page 104). Thirdly, without due consideration being taken of the contents of all *VWF* DBs, variants may falsely be described as being newly discovered when in fact they have previously been published. This means that the opportunity to build on established knowledge about a variant has been missed. This happened in the report of patient P82 with VWD2M, in whom c.3897del was purported to be novel (Liang et al., 2017). In fact it had been previously published three years earlier (Ouyang et al., 2014) and had been curated into HGMD.

In an effort to solve these shortcomings, VWDbase was collated to create an accessible community resource (Appendix 7.4, pages 256-304) in which previously reported variants in all three *VWF* DBs were unified. In order to accomplish this, a 30.5kb region of the *VWF* locus was defined, to cover the space in which VWD variants were known to occur (Figure 3.2, page 103). Annotating each nucleotide in the scaffold with the position and reference sequence from the latest genome builds (Church et al., 2011, Schneider et al., 2017) and HGVS terms (den Dunnen et al., 2016), meant that the scaffold was equipped with a "lingua franca" to facilitate the merging of variants across all *VWF* DBs, each of which used a different nomenclature. This process also removed misleading redundancy due to several indels being notated differently in different *VWF* DBs. A total of 1455 SNVs and indels were identified (Figure 3.3A, page 104). The output was rendered in VCF format so that VWDbase can be used for the rapid filtering of variants from high

throughput short read sequencing obtained in both clinical and research settings (Figure 3.1, page 100). Seventy one percent of the VWDbase variants were classified as PAVVs (Figure 3.3C, page 104), defined either because they were consistently called as pathogenic by the *VWF* DB(s) from which they were sourced, or because conflicting interpretations of pathogenicity between different sources meant they warranted further consideration. Concerned about the lack of evidence supporting these pathogenicity assertions, particularly the lack of consistent utilisation of MAF reaching these decisions, the VWDbase VCF was used to filter the variants called in 140,327 unrelated whole exome sequences of individuals enrolled in UKB (Bycroft et al., 2018; Van Hout et al., 2020). The 194 PAVVs present in at least one UKB participant were then subjected to systematic multidisciplinary scrutiny (Figure 3.4, page 110) with the purpose of defining a high confidence set of PAVVs with a solid evidence base supporting a causal relationship with VWD as defined by a plasma VWF level less than 0.30 IU/ml (James et al., 2021) and an MAF of < 1 in 10,000 consistent with a UK referral-based prevalence of VWD of 1 in 5,900, equivalent to 0.017% (Appendix 7.2, page 248; Figure 1.4, page 47). This reappraisal revealed that only 76 out of 140 PAVVs with a prior VWDbase label of 'Pathogenic' were accepted as such following MDT adjudication (Figure 3.5, page 117), with a significant overrepresentation of accepted PAVVs in the A1 domain (Figure 3.6, page 118). Accepted PAVVs were also enriched in pLoF variants convincingly linked to the phenotype of autosomal recessive VWD3. This included two variants (c.1730-10C>A and c.2968-14A>G), initially categorised as deep intronic, but recategorised as pLoFs following the MDT process. Both are likely to disrupt splicing (Liang et al., 2017; Mohl et al., 2011).

In an effort to prioritise *VWF* variants most likely to be pathogenic, the authors of large sequencing studies of individuals with VWD have often annotated *VWF* variants with the output of in silico prediction tools. Examples of the tools used include 'Sifting Intolerant From Tolerant' (SIFT) (Ng and Henikoff, 2003), which is based primarily on sequence conservation, and PolyPhen (Polymorphism Phenotyping; Ramensky et al., 2002), which also includes this information, but additionally incorporates biochemical and structural parameters. However, in the context of VWD, this annotation is then often provided

without further aggregation of the in silico tools' outcomes (James et al., 2007). Alternatively, a majority principle (Tennessen et al., 2012; Fu et al., 2013) is used, in which the prediction from each in silico tool is binarised as 0 (non-deleterious) or 1 (deleterious). This score is then summed and considered more relevant if the sum is higher than half the total number of tools used. For example, in the PCM-EVW-ES study, which sequenced 480 Spanish patients with VWD (Borras et al., 2017), an 'in silico global score' was determined from five prediction algorithms with the authors considering the variant deleterious if the score was ≥ 3. Although this approach has the advantage of being straightforward to calculate, it is problematic as it implies an equal weight of each feature and does not take into account the overlapping parameters used by the algorithms used to derive their outcome (Niroula & Vihinen, 2016). An alternative approach is CADD which has the advantage that it provides a single continuous metric of deleteriousness (the C score). This is because the machine-learning approach utilised takes into account 63 existing annotations (Kircher et al., 2014), including aforementioned SIFT and Polyphen. However, in order for CADD to be utilised effectively, gene-specific thresholds need to be developed as a C score implying pathogenicity in one gene does not necessarily translate to another gene (Chen et al., 2016; Meyts et al., 2016; van der Velde et al., 2015). Furthermore, there is a need for external validation of CADD outcomes (Grimm et al., 2015). The MDT assessment of the 194 PAVVs against predefined strict VWD criteria meant that this dataset provided a benchmark against which to assess the performance of the latest version of CADD (Rentzsch et al., 2021) in the adjudication of the pathogenicity of *VWF* variants. This analysis (Figure 3.8, page 123) demonstrated that C score thresholds of 10 or less and 35 or greater have a predictive value of 84% and 94% for rejecting or accepting PAVVs as pathogenic, respectively. These cutoffs are further explored in Chapter 4, where they are used to filter and prioritise whole genome sequencing (WGS) data from hitherto unexplained VWD patients (Subsection 4.4.1, pages 166-181). The C score cut off of 35 or greater is higher than the cut off of 20 used as a surrogate of pathogenicity for VWD as used in a recent analysis associating rare variant burden with VWF:Ag (Sadler et al., 2021). Using a different approach, a mutation significance cut off of 8.7 and 18.1 were proposed, depending on whether the assessment was made using HGMD or ClinVar respectively (Itan et al. 2016). The lower HGMD value in this analysis possibly relates to a

higher rate of false positive ascriptions of pathogenicity in this database compared with ClinVar, as has been suggested for haemostasis genes in general (Freson & Turro, 2017).

There are a number of limitations of my analysis. With regard to VWDbase, there is the possibility that potentially relevant VWD-causing variants were missed by focussing on the three *VWF* DBs described. Of particular concern are variants published prior to 2001 where cDNA coordinates were often numbered from the TSS rather than the first nucleotide of the start codon. Older publications also tended to number amino acid residues relative to mature VWF discounting the propeptide (Goodeve, 2010). Furthermore, I deliberately excluded structural variants (SVs) from VWDbase as it was designed as a resource for filtering short read, high throughput sequencing and, at present, SVs are not reliably identified using this technology (Linderman et al., 2021).

An additional major caveat is that the pathogenicity assessment of each UKB PAVV was with respect to its laboratory as opposed to clinical phenotype, despite bleeding (and its clinical assessment) being one of the top concerns of patients living with VWD (Kalot et al., 2020; Ragni et al., 1999). However, information about the relationship between bleeding and a given variant was not systematically captured in the *VWF* DBs and did not consistently feature in the publications reporting variants. One of the strengths of the UKB resource is that the relationship between genotype and phenotype can be analysed on an individual basis in contrast with gnomAD where only aggregated data is available. Therefore, an analysis of the effect of PAVVs on haemorrhagic risk in UKB participants was undertaken and this is outlined in Chapter 4.

4. Results: clinical relevance of PAVVs

4.1. Background

Bleeding is one of the main concerns of patients living with VWD (Kalot et al., 2020; Ragni et al., 1999). The development of Bleeding Assessment Tools (BATs), such as the ISTH-BAT (Rodeghiero et al., 2010) has helped to standardise the way bleeding histories are taken (Subsection 1.2.1, pages 31-32). The most common format is a questionnaire, in which a trained nurse or physician asks an individual about their bleeding symptoms across a number of different domains (Rydz & James, 2012). The main utility of BATs is as a triage tool for clinicians working in haemophilia centres (Elbaz & Sholzberg, 2020). If a referred patient has a negative BAT score this helps to rule out a diagnosis of VWD and avoids unnecessary laboratory tests (Fasulo et al., 2018) whereas a positive score increases the likelihood of a VWD diagnosis (Bowman et al., 2008; Bowman et al., 2009; Elbatarny et al., 2014; Rodeghiero et al., 2005; Rodeghiero et al., 2010). There are several limitations of the BAT approach and the design of the studies that created them.

First, the thresholds used for an abnormal BAT score (greater than 3 in males and 5 in females; Elbatarny et al., 2014) are not calibrated for the lifetime accumulation of haemostatic challenges and bleeding events with advancing age.

Second, scores of 3 and 4 are - for most categories - defined by what was done to the individual as opposed to a quality related to the bleeding itself. Such measures include surgery, infusion of a pro-haemostatic medicine (such as recombinant factor VII), or blood transfusion. However, the thresholds and accepted indications for instigating these measures have altered over time and vary between individuals, hospitals, and countries (Chandraharan & Krishna, 2017; Goodnough et al., 2013; Levi et al., 2010; Matteson et al., 2012).

Third, haemostatic challenges and bleeding events may not be accurately recollected (Cohen & Java, 1995) or indeed remembered at all compared with what is documented in the individual's medical notes (Eze-Nliam et al., 2012).

Fourth, the control populations used in the development and validation of BATs were biased towards those with an increased prior chance of a low score. For instance in the Vicenza BAT study the 215 controls "had never been referred for haemostasis evaluation" (Rodeghiero et al., 2005), and the 1040 individuals used to develop a normal range for the ISTH-BAT were chosen because they did not have a diagnosis of a bleeding disorder, or a "known problem with bleeding or bruising" (Elbatarny et al., 2014). This is problematic because both the selection method and small sample size mean that the controls used did not accurately represent the background prevalence of past bleeding symptomatology. Indeed there is a wide range of estimates of different bleeding events in apparently healthy individuals (as summarised in Mauer et al., 2011).

Fifth, the individuals with VWD on whom the BATs were developed were those that had been referred to haemophilia centres (Bowman et al., 2009; Rodeghiero et al., 2005; Tosetto et al., 2006). However, as highlighted previously (Subsection 1.2.3, page 48), referral-based prevalence of VWD is at least six-times lower than population-based estimates, potentially due to underdiagnosis of VWD (Sidonio Jr et al., 2020). Therefore, the utility of BAT scores for the 'never-seen' individuals who have VWD, but who have not been referred to a haemophilia centre is less certain.

In the first section of this chapter, I demonstrate an alternative approach to the evaluation of bleeding in VWD utilising UKB. Although plasma VWF measurements have not been made on UKB participants, hospital inpatient data is accessible. Furthermore, as shown in the previous chapter, of the 1,039 PAVVs identified in VWDbase (Figure 3.3, page 104), 194 were found in UKB participants (Figure 3.4, page 110). Therefore, I used this data to make my approach variant-centric. That is, the identification of a PAVV in a UKB individual is used as a proxy for a diagnosis of VWD, and those meeting this criterion are then analysed as a separate group. Their risk of hospital-defined bleeding episodes over a

23.5 year period is examined and compared to the rest of the participants in UKB. Therefore, by using this method I challenge the pitfalls identified with BATs. This is because bleeding episodes are defined by objective hospital data as opposed to subjective recollection. Furthermore, the control population identified is 100-times larger than any previously considered, and individuals with a bleeding history have not been removed. Finally, defining VWD genetically may provide a better approximation of the VWD population as a whole (both never-seen individuals and those with a known diagnosis).

In a minority of patients with a phenotype consistent with VWD, a molecular diagnosis cannot (initially) be established (Baronciani et al., 2017). This may be due to the lack of conformity of some cases of VWD1 and low VWF (Subsection 1.2.4, pages 55-57) to Mendelian inheritance (Ng et al., 2015), because they result from oligogenic (Downes et al., 2019) or polygenic effects (Collins et al., 2008), some of which is driven by (mainly common) variants outside of the *VWF* locus (Figure 1.5A, page 50). Other factors that limit diagnostic yield include the design of genotyping test used, in particular the coverage and type of sequencing and the strategy for variant prioritisation.

The NIHR BioResource Rare Diseases (NBR-RD) study included 10 individuals with VWD (VWF:Act < 0.30 IU/ml), deliberately recruited because analysis of their DNA did not identify a molecular explanation. These are hereafter referred to as the NBR-VWD participants. As for the other participants in NBR-RD, the DNA from these 10 NBR-VWD individuals was analysed by WGS. In the second section of this chapter (4.4, pages 166-181), I explore the use of VWDbase for variant prioritisation in the analysis of the WGS data from the NBR-VWD group.

4.2. Aims

1) Design a bleeding phenotype scoring system to assess the background longitudinal risk of bleeding in the UKB.

2) Define a group of UKB individuals who have PAVVs (genetically defined VWD). Establish whether this group has an increased risk of bleeding when compared to

the rest of the UKB, taking into account the MDT outcome (accepted, undecided, rejected) of the PAVV(s) that the individuals carry.

3) Utilise VWDbase to facilitate the prioritisation of *VWF* variants identified in the WGS data of patients with VWD for whom no molecular explanation has been identified.

4.3. Effect of PAVVs on bleeding risk

4.3.1. UKB bleeding assessment cohort

Before focussing on the bleeding risk conferred by PAVVs, the first step was to define a subpopulation within UKB in which confounding variables affecting this risk were minimised. The initial population consisted of 140,327 unrelated UKB individuals for whom WES data were available at the time of the analysis and had been used for the assessment of PAVV pathogenicity (Figure 3.4, page 110; Subsection 2.1.4, pages 73-75). This initial population is hereafter referred to as the 'UKB population'.

To define the subpopulation, individuals with confounding variables for bleeding risk were removed from the UKB population. The flow chart in Figure 4.1A (page 142) summarises the criteria used. Based on genotypically defined ancestral super-populations, the majority of the UKB population were of European ancestry (EUR) (Table 3.3, page 113). Given ancestry is reported to affect bleeding symptoms (Daniel et al., 2006; Huerta-Franco et al., 2018; Mauer et al., 2011), only UKB participants of EUR ancestry were retained. This resulted in a population of 131,022 participants, genetically defined as EUR, which was 98.8% concordant with self-declared ethnicity (white British, white Irish, or white other). As detailed below, the analysis of bleeding in UKB individuals relied on information coded by hospital episode statistics (HES). However, because of methodological disparities in hospital episode coding between the home nations of Great Britain direct comparison of hospital inpatient data between individuals in England, Wales, and Scotland was not possible (UKB, 2020b). However, the majority of UKB individuals were enrolled in England. Therefore, the analysis was limited to the 117,919 participants enrolled at the 17 UKB assessment centres in England (Figure 4.1B, page 142). The final criterion for

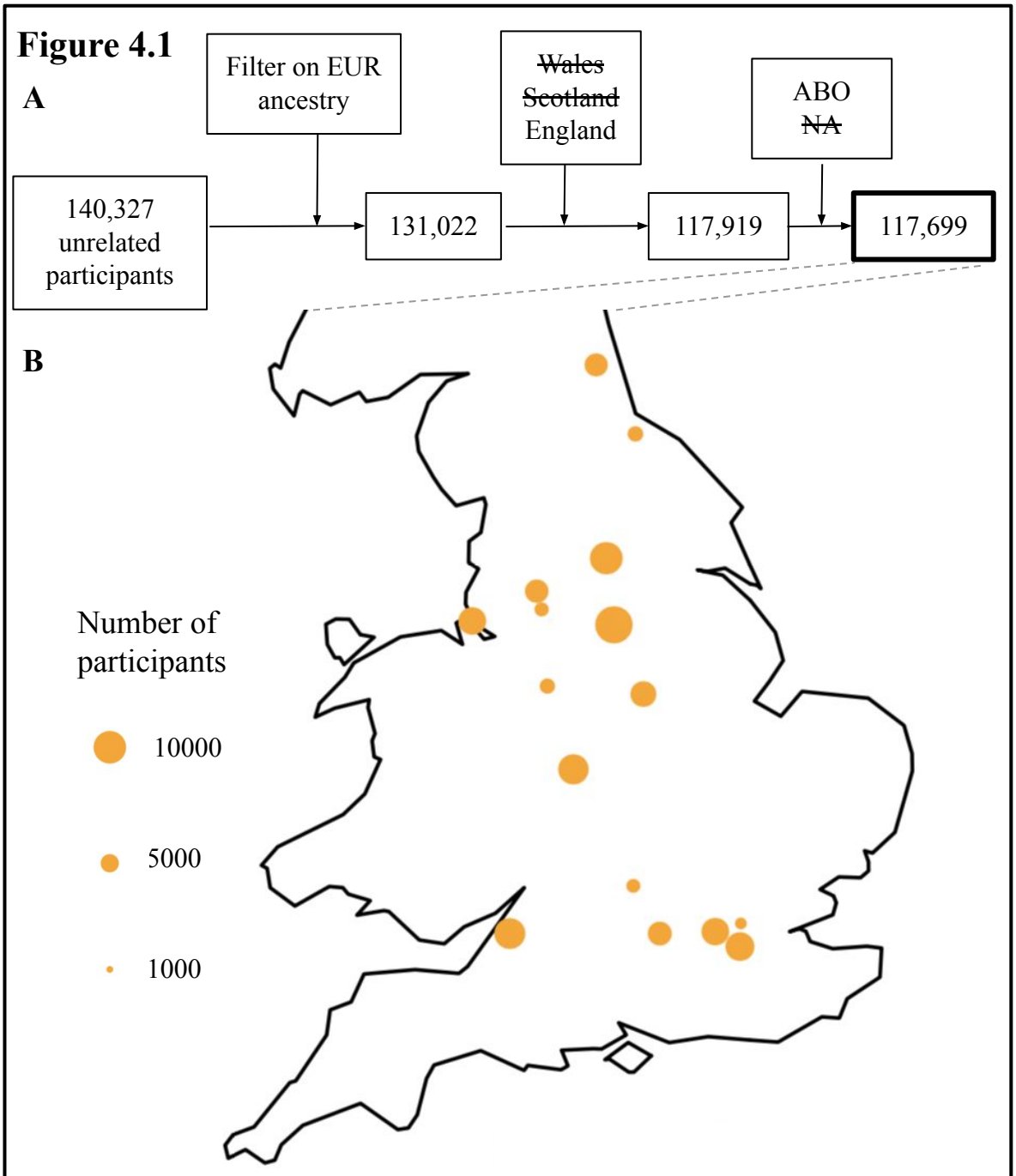


Figure 4.1 | Defining the UKB Bleeding Assessment Cohort. (A) Of the unrelated UKB individuals from which WES data was available, 117,699 were retained for the bleeding analysis after consecutive filters were applied. This subpopulation was referred to as the 'UKB bleeding assessment cohort' (UKB BAC), as shown in the box with the bold outline on the far right of the flow chart. (B) A dot plot showing 16 of the 17 assessment centres at which participants were recruited in England. Each centre is marked with an orange circle. The centres were from north to south: Newcastle, Middlesbrough, Leeds, Manchester, Bury, Liverpool, Sheffield, Stoke-on-Trent, Nottingham, Birmingham, Oxford, Barts, Hounslow, Bristol, Reading, and Croydon. The area of the circle is proportional to the number of the UK BAC individuals recruited at the centre. Only fifty one UKB BAC individuals were recruited from the pilot centre in Stockport, and so the dot is not shown (as too small to be visible).

selection was based on *ABO* histo-groups, because of the strong trans-acting effect of common variants at *ABO* on plasma VWF levels (Subsection 1.2.4, pages 51-52). The most likely ABO phenotype was determined by applying the bloodTyper algorithm (Lane et al., 2016; Gleadall et al., 2020) to the UKB genotyping array (Bycroft et al., 2018). Reliable inference of the ABO histo-group phenotype was not possible because of the inadequate quality of the array genotypes at the *ABO* locus for 220 participants. After removal of these participants, a cohort of 117,699 participants remained for the final analysis, hereafter referred to as the 'UKB bleeding assessment cohort' (UKB BAC) (Figure 4.1A, page 142).

HES were available for each individual in UKB through linkage to their Electronic Health Record (EHR). HES data summarise unstructured, principally free text, clinical entries into a structured coded format. The principal purpose of HES collection is to ensure hospitals are reimbursed for the services they provide, although there has been increasing interest in the last decade in the use of HES for longitudinal research studies enabled by linkage to external datasets on overlapping participants (Boyd et al., 2018). The HES data was first made available to UKB in January 2019.

Each 'episode' of care reflected in the HES data represents an inpatient hospital admission which is coded with a single primary and up to 19 secondary diagnoses (Davis et al., 2018; UKB, 2020b). Inpatient in this context refers to any stay when a patient occupies a bed. Hence, day cases where patients are discharged the same day are counted as an inpatient episode but outpatient clinic visits and primary health records are not included in the HES dataset (UKB, 2020b). All episodes were coded by the NHS using the World Health Organisation's International Statistical Classification of Diseases and Related Health Problems (ICD) version 10 (ICD-10) terms (World Health Organisation [WHO], 2019).

I used the primary and secondary ICD-10 diagnosis used to code each hospital episode as the basis for developing an 'ICD bleeding assessment tool' ('ICD-BAT'). This was then used to categorise the inpatient bleeding symptoms experienced by individuals in the UKB BAC. Firstly, each of the 13 components of the ISTH bleeding assessment tool

(ISTH-BAT) score (Rodeghiero et al., 2010) (Appendix 7.1, pages 241-243), were mapped to ICD-10 using a manual search of its online portal (https://icd.who.int/browse10/2010/en). For example, epistaxis is the first listed code in the ISTH-BAT. When 'epistaxis' was searched for, it mapped to a single ICD-10 code, R04.0, and a search using 'nose bleeding' brought up the same code. Of the categories in the ISTH-BAT , there were two - 'muscle haematomas' and 'bleeding from minor wounds' - for which closely matching ICD-10 codes could not be found. This reflects the lack of direct correspondence between different phenotyping coding systems, which is a well recognised challenge (Kafkas et al., 2021). Between one and 26 ICD-10 codes were mapped to each of the remaining 11 ISTH-BAT categories, with bleeding after surgery and dental extractions being combined to a single bleeding category, called 'procedural bleeding', based on the mapping to the ICD-10 code, T81.0 "Haemorrhage and haematoma complicating a procedure, not elsewhere classified" (Appendix 7.5, pages 304-308; Figure 4.2A, page 145). In addition to the ISTH-BAT categories, it was evident that there were bleeding episodes that are coded by ICD-10 that were not captured in the design of the ISTH-BAT. These included obstetric and gynaecological bleeding other than the narrowly defined menorrhagia, and post-partum bleeding in the ISTH-BAT, as well as bleeding from the heart, respiratory tract, eye, and that related to trauma. Therefore, these ICD-10 categories were clustered under six different additional bleeding categories (Figure 4.2B, page 145). In total, 16 different ICD-10 BAT categories were defined.

HES data was downloaded for the 117,699 individuals from UKB between 01 April 1997 and 30 September 2020 inclusive (Figure 4.3A, page 146). The CALIBER phenotyping algorithm (Denaxas et al., 2012) (Subsection 2.1.4, page 74) was used to select hospital episodes during this period that had one of the 97 ICD-10 codes used to create the ICD-BAT score (Figure 4.2B, page 145) and then assign it to the parent ICD-BAT category. As an example, any hospital episode coded with retinal haemorrhage (ICD-10 code H35.6) and/or vitreous haemorrhage (H43.1), was annotated with the ICD-BAT category 'Eye bleeding'. Furthermore, the date corresponding to this hospital episode was also recorded. However, for each UKB BAC individual, only the first instance of a

Figure 4.2

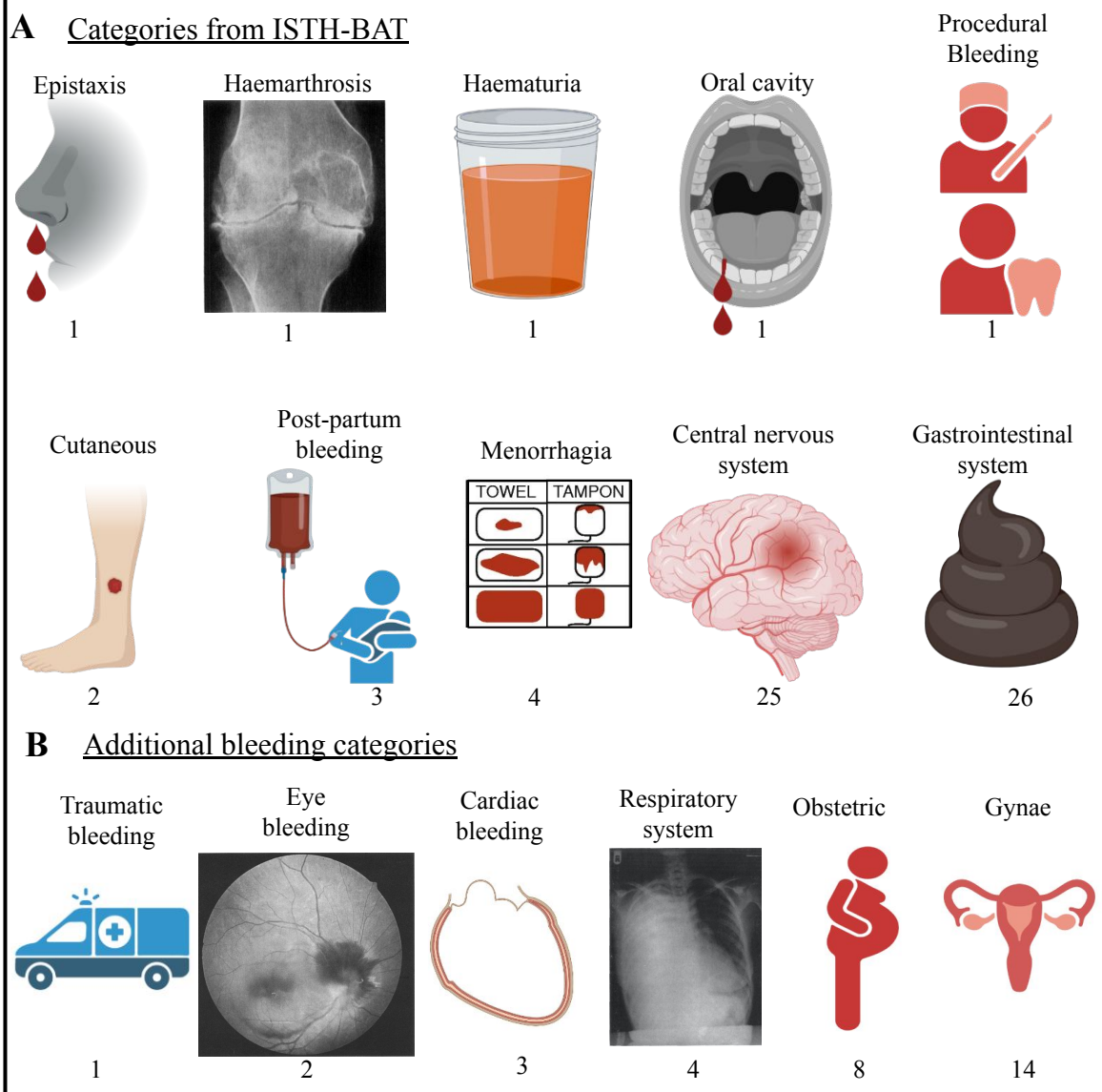


Figure 4.2 | The ICD-Bleeding Assessment Tool (ICD-BAT). Images in both (A) and (B) are in ascending order of the number of ICD-10 codes assigned to that symptom (represented by the number below the picture). The panels in (A), represent the categories of the ISTH-BAT score (Rodeghiero et al., 2010). The radiograph used to represent haemarthrosis shows the joint space narrowing in the knee of patient with VWD3 due to bleeding into the joint. (Ahlberg and Silwer, 1970). The icon used to represent menorrhagia is the pictorial blood loss assessment chart (PBAC), a means for qualitatively assessing the volume of menstrual blood loss (image adapted from Higham et al., 1990). (B) Additional ICD-10 codes reflective of bleeding, but not mappable to the ISTH-BAT were clustered under 6 additional groups. The image used to represent respiratory system bleeding is a chest radiograph of a patient with VWD who had a haemothorax (Bowes et al., 1969). The photograph of the fundus that represents eye bleeding shows retinal, subretinal, and vitreous bleeding in a teenager newly diagnosed with VWD (Shiono et al., 1992).

Figure 4.3

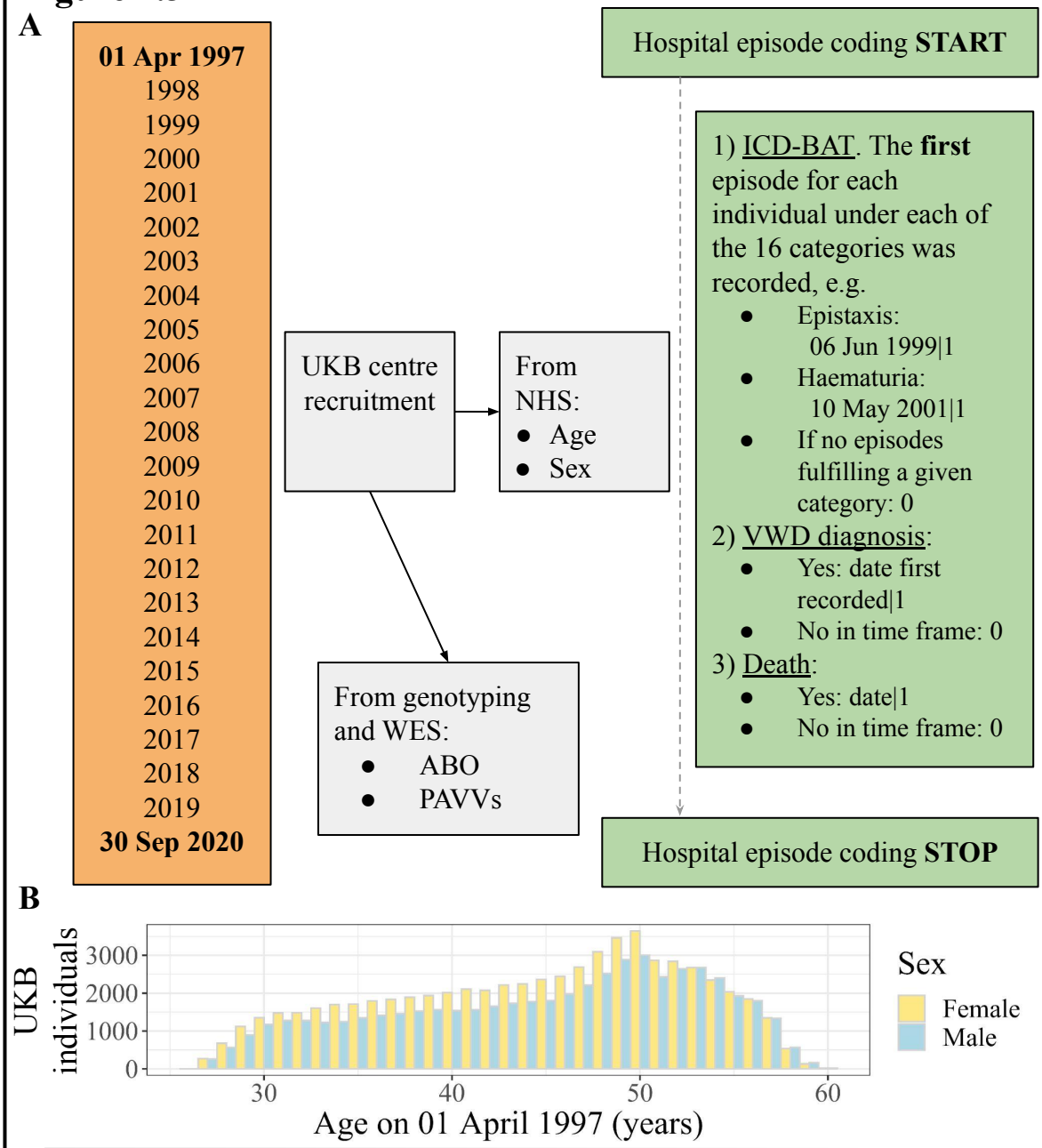


Figure 4.3 | Parameters obtained from UKB for use in the bleeding analysis. (A) From left to right: the orange box on the left shows the 23.5 year period in which hospital episode data for the 117,699 UKB BAC individuals was evaluated. The middle boxes in light grey show the key demographic data obtained and that extracted from the DNA analysis. Age and biological sex were acquired, prior to enrolment, from the NHS primary care registry. Following extraction of DNA, genotyping data were released from 2015 onwards meaning that ABO histo-group could be determined. Putatively aetiological VWD variants (PAVVs) were identified in UKB participants using the whole exome sequencing (WES) data first released in October 2020. The green boxes on the right hand side explain how the ICD-BAT categories and death were captured both with date and 1/0 to indicate whether these events did/did not occur during the 23.5 year period. (B) Histogram of the number of UKB individuals by their year of age on 01 Apr 1997, stratified by biological sex.

bleeding episode within a given ICD-BAT category was captured. Therefore, to continue the example above, if the first time eye bleeding was recorded was 01 April 1999, then no further episodes coded with H35.6 or H43.1 were captured after this time point.

In addition to the ICD-BAT categorisation, two further fields were obtained from HES: whether the participant was coded with a diagnosis of VWD (ICD-10 code D68.0) and whether they died during the time period considered in the analysis. Both were also recorded using the same method as for bleeding episodes (Figure 4.3A, page 146).

Based on the demographics on 01 April 1997, the UKB BAC consisted of 63,855 females and 53,844 males (54% and 46%, respectively), with an over-representation of females prior to the age of 53 but not afterwards (Figure 4.3B, page 146). The median age of participants on 01 April 1997 was 46 years, with a range from 26 to 60 years. As highlighted in the background to this chapter (Subsection 4.1, pages 138-140), there is wide variation in the estimates of the prevalence of bleeding symptoms in the overall population (Mauer et al., 2011). Therefore, prior to the analysis of the effect of PAVVs on bleeding risk, the number of individuals in the UKB BAC who had bleeding episodes within each of the 16 ICD-BAT categories was first evaluated. The first observation was that the majority - 95,917/117,699 UKB individuals - did not experience a hospital bleeding episode (Figure 4.4A, page 148).

Of the non-sex specific bleeding episodes, five occurred in more than 1000 participants: gastrointestinal (GI) bleeding (n = 8831), haematuria (n = 5260), procedural bleeding (n = 2220), central nervous system (CNS) bleeding (n = 1199), and epistaxis (n = 1078) (Figure 4.4C, page 148). Three of these symptoms have been previously investigated in questionnaire-based studies of bleeding in the general population. Eight percent of UKB BAC individuals had a GI bleeding episode. This is similar to the mean of 7% who reported “blood in stools” (Mauer et al., 2011) as calculated from seven studies (Drews et al., 2002; Friberg et al., 2006; Nosek-Cenkowska et al., 1991; Quiroga et al., 2007; Šrámek et al., 1995; Wahlberg et al., 1980; Wahlberg, 1984). Likewise haematuria affected 4% of

Figure 4.4

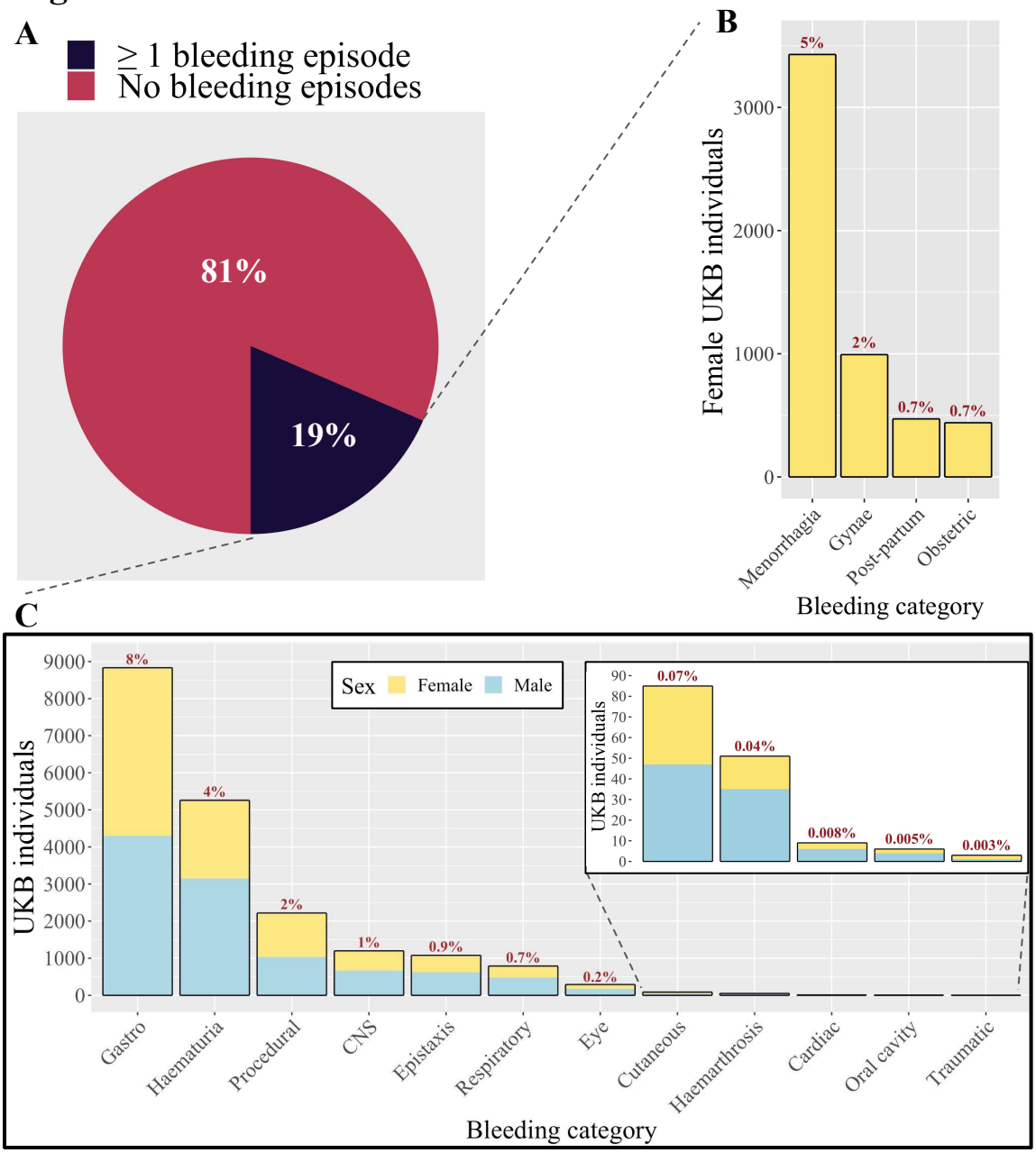


Figure 4.4 | Profile of bleeding episodes in the UKB BAC. (A) Pie chart representing the number of UKB BAC participants with at least one hospital episode categorised as a bleeding event using the ICD-BAT classification. (B) A bar chart showing the number of participants with female-specific bleeding episodes ordered on the x-axis, from left to right, in descending order of frequency, with annotated percentages given a denominator of 63,855 female UKB individuals. (C) A bar chart showing the number of UKB participants with bleeding episodes that are not sex specific. The number of participants is ordered, on the x-axis, from left to right, in descending order of frequency. The annotated percentages are based on a denominator of 117,699 participants. The inset shows the bleeding episodes affecting less than 100 individuals on a larger y-axis scale. In both (B) and (C), females are represented by yellow bars, males by light blue bars. All percentages are to rounded to whole integers (if ≥ 1), or to 1 significant figure (if ≤ 1).

UKB participants compared with a mean of 5% in previous studies (Friberg et al., 2006; McKay et al., 2004; Quiroga et al., 2007; Wahlberg et al., 1980; Wahlberg, 1984). In contrast, epistaxis only affected 0.9% of UKB participants set against a mean estimate of 23% of the population reporting this symptom (Drews et al., 2002; Dilley et al., 2001; Mauser Bunschoten et al., 1988; Wahlberg et al., 1980; Wahlberg, 1984). Furthermore, easy bruising and gum bleeding are reported by around one-quarter of the general population (Mauer et al., 2011), but were identified in less than 100 participants of the UKB BAC (Figure 4.4C, page 148).

Of the female-specific ICD-BAT categories, menorrhagia was the most common, affecting 3429/63,855 (5.4%) UKB BAC females (Figure 4.4B, page 148). This is seven-fold lower than the mean of 35% calculated from 7 previous studies (Friberg et al., 2006; Plug et al., 2006; Mauser Bunschoten et al., 1988; McKay et al., 2004; Šrámek et al., 1995; Quiroga et al., 2007; Wahlberg, 1984).

One potential explanation for the relative comparability of the GI and haematuria bleeding rates to self-reported estimates from previous publications are that these symptoms are more likely to warrant hospital-level care whereas the majority of individuals affected by menorrhagia, epistaxis, cutaneous bleeding, and oral cavity bleeding may present either to general practice, alternative healthcare providers (such as dentists), or not come to medical attention at all, and hence were not captured by the ICD-BAT analysis.

4.3.2. ICD-BAT score

In 2,212 (1.9%) of UKB BAC individuals at least one of the 194 PAVVs were identified at being present, and not a single participant with the same PAVV on both alleles was identified. Of these 2212 participants, 1974, 235, and 3 participants had one, two, and three PAVVs, respectively. The remaining UKB BAC individuals - 115,487 - were categorised as the no PAVV group (Figure 4.5A, page 150). The MDT outcome regarding the pathogenicity of the PAVV(s) which the individual carried (Figure 3.4, page 110), was used to determine each participant's genetically defined VWD status in order to categorise the PAVV heterozygotes into one of three groups.

Figure 4.5

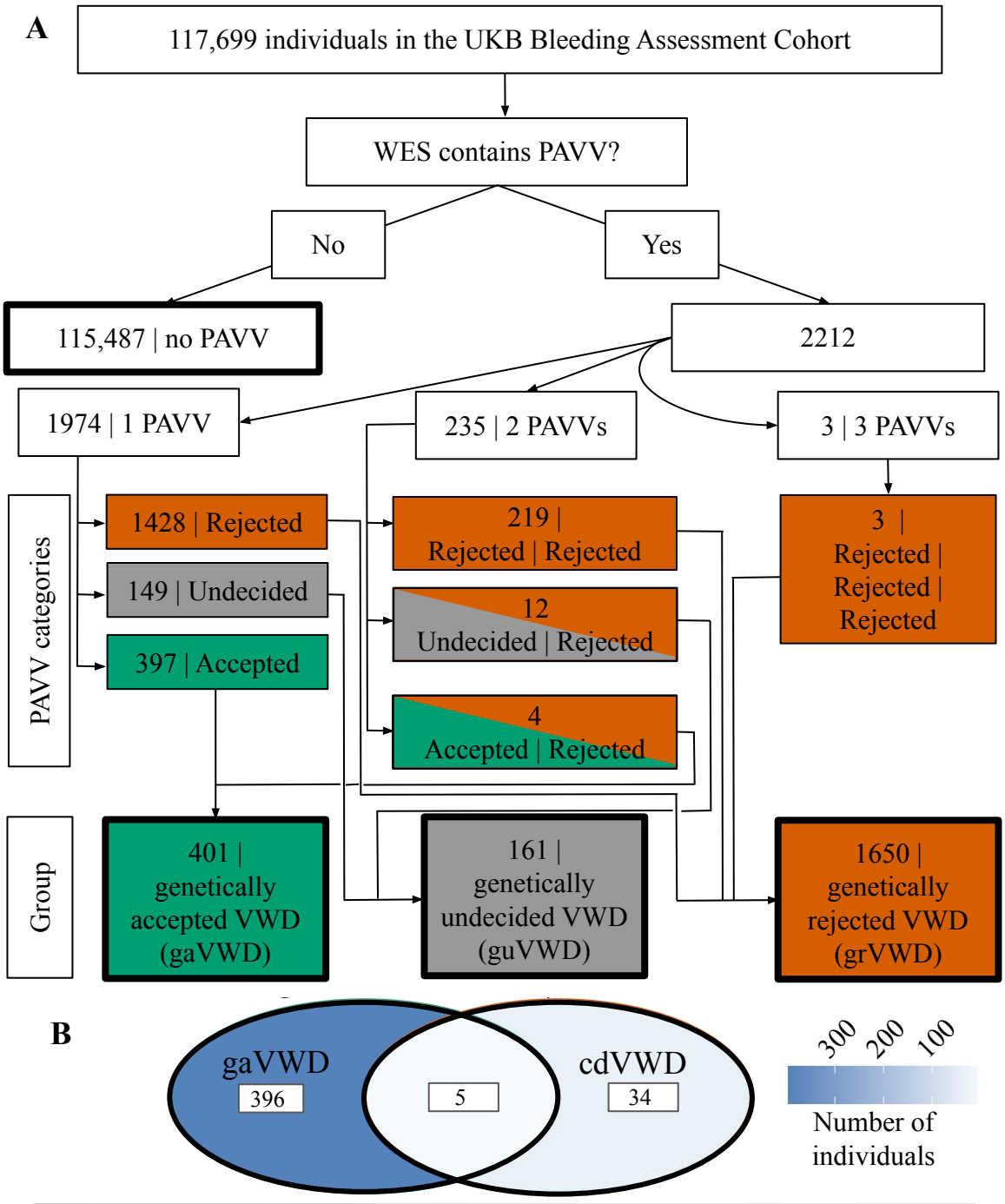


Figure 4.5 | Defining case and control groups based on heterozygosity for PAVVs in the UKB BAC. (A) A flow chart illustrating how the 117,699 participants in the UKB BAC were categorised into four groups (no PAVV, and genetically accepted [ga], undecided [gu], and rejected [gr] VWD, respectively), based on the MDT categorisation of the putatively aetiological VWD variant (PAVV) that they have in heterozygosity. The numbers prior to the '|' symbol represent the number of individuals in the given group. (B) A Venn diagram representing the overlap between the 401 individuals with gaVWD and the 39 in whom a diagnosis of VWD was recorded during a hospital episode, termed 'clinically determined VWD' (cdVWD). The darkness of the blue colour correlates positively with the number of individuals.

Individuals heterozygous for an accepted PAVV were allocated to the genetically accepted (ga)VWD group (Figure 4.5A, page 150), whereas those heterozygous for PAVVs classified as undecided or rejected by the MDT, were allocated to the genetically undecided (gu) and genetically rejected (gr)VWD groups, respectively. In the 238 participants with more than one PAVV, MDT outcomes were ranked such that accepted PAVVs took precedence over those classified as undecided or rejected, and undecided PAVVs took precedence over those classified as rejected. Therefore, the four individuals who were heterozygous for both accepted and rejected PAVVs were put into the gaVWD group and the 12 individuals with undecided and rejected PAVVs were put into the guVWD group. After completion of the categorisation, there were 401 participants in the gaVWD group, 161 in the guVWD group, and 1650 in the grVWD group. The assertion that heterozygosity, in itself, for an accepted PAVV makes a diagnosis of VWD likely was based on the fact that 267/401 of this group had protein-altering variants which would be expected to cause autosomal dominant VWD, primarily through a dominant negative effect. The remainder, 134/401, had pLoF variants. It has been suggested that (some of these) variants are codominant, and hence (can) cause VWD if present on a single allele, which is the argument supporting their inclusion in the gaVWD group.

Before exploring the bleeding phenotype of each of the three genetically defined VWD groups, the overlap was assessed between participants in the gaVWD group and the 39 UKB participants in whom a diagnosis of VWD was recorded during a hospital episode. The latter group is referred to as 'clinically determined VWD' (cdVWD). Five cdVWD participants were also assigned the gaWVD label (Figure 4.5B, page 150), which represents a statistically significant overrepresentation (OR = 43.03, P = 2.49 x 10^{-7}), compared with 34 cdVWD cases out of 117,298 participants who were not in the gaVWD group. In contrast there was no enrichment (OR = 3.80, P = 0.10) of cdVWD participants in the grVWD group. One possible explanation for the lack of greater overlap between the cdVWD and gaVWD groups is that the former may carry *VWF* variants absent from the PAVV list, perhaps because they are not SNVs or indels. Another explanation is the effect of the ABO histo-group (Subsection 1.2.4, pages 51-52). Twenty three out of 39 of participants in the cdVWD group (58.0%) had an OO genotype, higher than the 43.1% in

the overall UKB BAC. However, this did not represent a significant enrichment of the O allele (OR = 1.37, $P = 0.27$). In contrast 155 out of 401 in the gaVWD group (38.7%) had an OO genotype, comparable to the overall population. Although not reaching statistical significance, these findings are compatible with the observation that individuals with a small quantitative reduction in VWF below the reference range (0.30 - 0.50 IU/ml) - referred to as 'low VWF' or type 1 VWD depending on the classification system used (James et al., 2021) - have a higher frequency of OO genotype than compared with the general population (Lavin et al., 2017).

Individuals in the UKB cohort had ICD-BAT scores of 0-5 (Figure 4.6, page 153). The distribution of the scores were positively skewed, with values (calculated using the Fisher-Pearson moment coefficient of skewness) of 2.43, 2.30, 2.32, and 2.34 for the no PAVV, gdVWD, guVWD, and grVWD groups, respectively. Consistent with this over 96% of individuals in all groups had scores of 0 or 1 (Table 4.1, page 154).

Considering the whole UKB BAC, 10,739 out of 63,855 (16.82%) females had an ICD-BAT score of 1 compared with 7941 out of 53,844 (14.75%) males; for a score of 2 this was 1608 out of 63,855 (2.52%) and 1040 out of 53,844 (1.93%) respectively. This suggested that females had a higher ICD-BAT score than males. To explore this further, a cumulative link model (Christensen, 2018), CLM, was fitted to the UKB BAC using the default settings. This approach was taken because the response variable, ICD-BAT score, was ordinal. Four predictor variables were chosen:

- ABO histo-group, which was dichotomised as group O or non-O. The former was defined as participants who were genotypically *OO*, the latter those who were *AA*, *AO*, *BO*, *BB*, and *AB*.
- Age. A continuous variable in years, defined as the age of the participant on 01 April 1997, the start of the period over which ICD-BAT defined hospital bleeding episodes were analysed (Figure 4.3, page 146).
- Genetically defined VWD status (control, gaVWD, guVWD, grVWD; Figure 4.5, page 150).
- Sex. A binary variable (Female, Male).

Figure 4.6

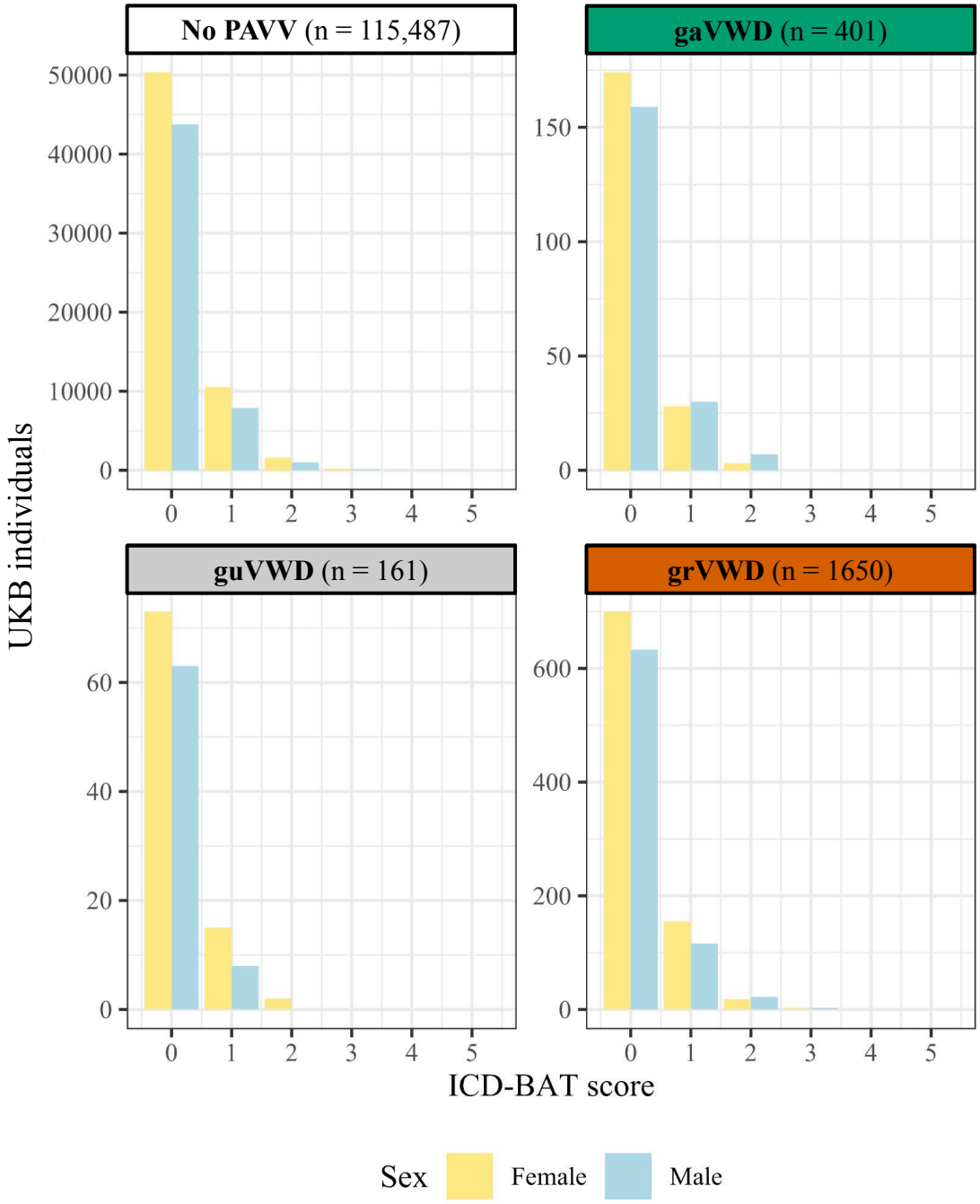


Figure 4.6 | ICD-BAT scores of groups within the UKB BAC. A bar graph showing the number of UKB BAC individuals which had an ICD-BAT score between zero and five, stratified by sex. No participants in any group had an ICD-BAT score greater than five. The four separate facets represent the four groups assessed: participants with no PAVV - the majority of the UKB BAC population - and participants in the genetically accepted [ga], undecided [gu], and rejected [gr] VWD groups respectively. The y-axis has a different scale on each panel based on the number of participants in each group.

ICD-BAT score	Controls | 115,487	Cases | 2212		
		gaVWD | 401	guVWD | 161	grVWD | 1650
0	50,334 (80.30%)	174 (84.88%)	73 (81.11%)	700 (79.91%)
	43,787 (82.93%)	159 (81.12%)	63 (88.73%)	633 (81.78%)
1	10,541 (16.82%)	28 (13.66%)	15 (16.67%)	155 (17.69%)
	7887 (14.94%)	30 (15.31%)	8 (11.27%)	116 (14.99%)
2	1585 (2.53%)	3 (1.46%)	2 (2.22%)	18 (2.05%)
	1011 (1.91%)	7 (3.57%)	0 (0%)	22 (2.84%)
3	200 (0.32%)	0 (0%)	0 (0%)	3 (0.34%)
	107 (0.20%)	0 (0%)	0 (0%)	3 (0.39%)
4	22 (0.04%)	0 (0%)	0 (0%)	0 (0%)
	11 (0.02%)	0 (0%)	0 (0%)	0 (0%)
5	2 (0.003%)	0 (0%)	0 (0%)	0 (0%)
	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Table 4.1 | ICD-BAT score by genetically defined VWD group. The numbers represent the number of UKB BAC participants. The light yellow and light blue rows represent females and males, respectively. In parentheses are the individuals with the corresponding ICD-BAT as a percentage of the total number of the given sex in the group.

Sex, age, and ABO histo-group all had statistically significant effects in the CLM. Female sex, increasing age, and histo-group O made a higher ICD-BAT score more likely (Table 4.2, page 155). In contrast, a participant's genetically defined VWD status had no significant effect on the ICD-BAT score. To explore the latter further, and control for co-

Predictor	Estimate	*P* value
Female sex	0.181	$< 2 \times 10^{-16}$
Age	0.008	$< 2 \times 10^{-16}$
Histo-group O	0.073	1.54×10^{-6}
guVWD	-0.231	0.29
gdVWD	-0.098	0.46
grVWD	0.045	0.47

Table 4.2 | The effect of predictor variables on ICD-BAT score. The output of the cumulative link model. The predictor variables are in order, top to bottom, of increasing *P* value.

variates by matching (Rubin, 1973), the MatchIt R package (Ho et al., 2007; Ho et al., 2011) was used to select pairs from the control group and each of the gaVWD, guVWD, and grVWD separately. Nearest-neighbour propensity score matching (Rosenbaum & Rubin, 1983) was used to balance the groups, 1:1, based on sex, age, and histo-group O, such that 401 controls were matched against gdVWD, and 161 and 1650 against guVWD and grVWD respectively. Subsequent to this the CLM was rerun for each subset. On this iteration, only the effect of being in a gaVWD, guVWD, and grVWD group (predictor variable) on ICD-BAT score (response variable) was analysed, but as for the initial analysis, there was no statistically significant effect on the ICD-BAT score: *P* values were 0.39, 0.92, and 0.67 for each group, respectively, despite close matching being achieved in all subsets (Figure 4.7, page 156).

4.3.3. Bleeding-free survival

Although the ICD-BAT score provided a means of aggregating total bleeding events across the whole UKB cohort and comparison within subgroups, use of the total ordinal score did not provide the means to establish whether certain ICD-BAT coded hospital bleeding episodes varied as a function of genetically defined VWD status. This was important to

Figure 4.7

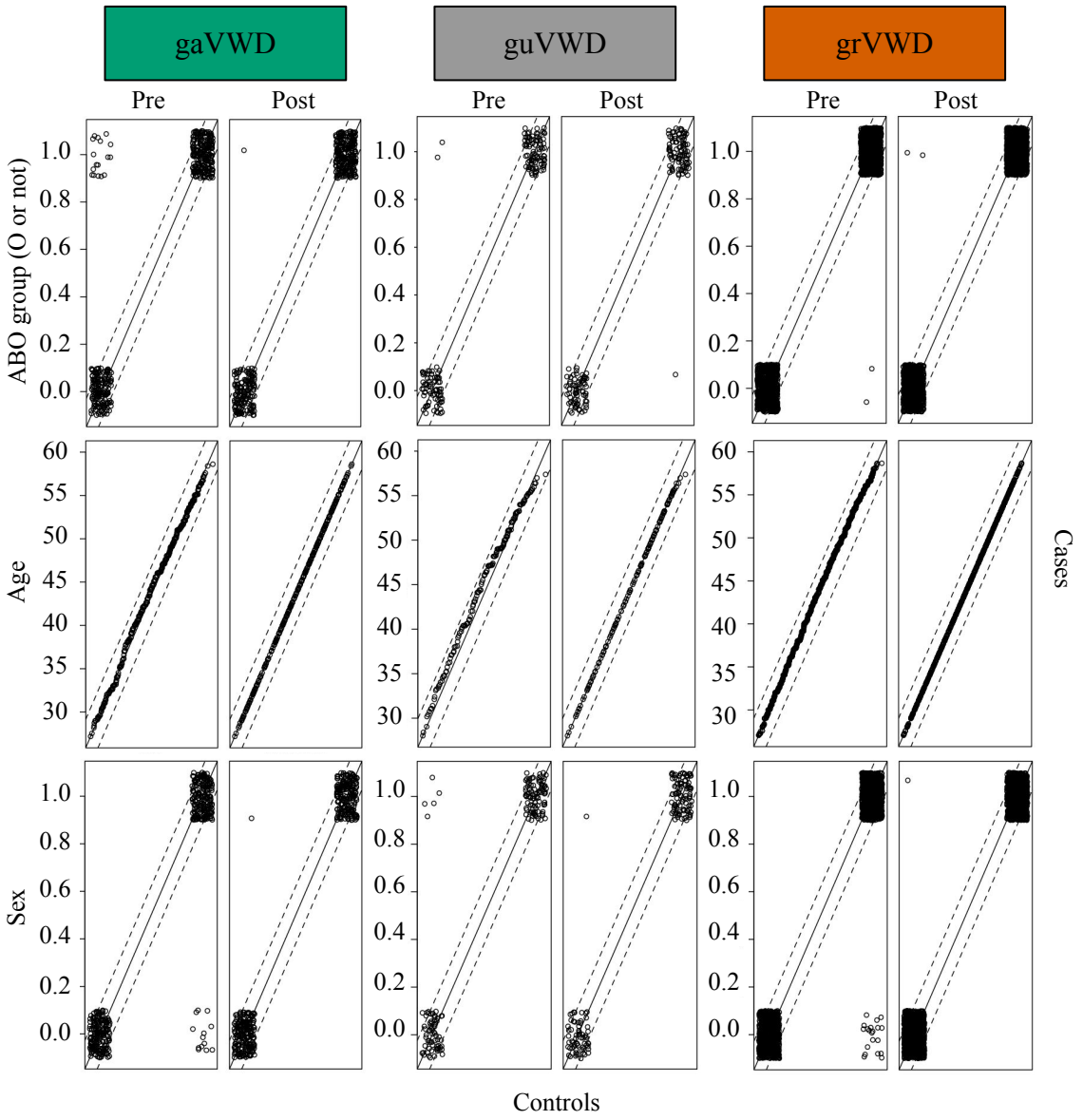


Figure 4.7 | Outcome of propensity score matching between cases and controls in the UKB BAC analysis of ICD-BAT scores. Quantile-quantile plots representing the probability distribution of the controls on the x-axis compared with the cases on the y-axis. Dots represent individuals, and those outside of the dashed lines represent those divergent from the distribution of the controls. The rows represent the three different covariates matched using propensity scoring. Columns one and two represent the gaVWD cases, three and four the guVWD cases, five and six the grVWD cases. Columns one, three, and five demonstrate the probability distributions before propensity score matching and two, four, and six after this was done. The plots demonstrate how the matching has balanced the groups according to the given covariate. For example, for ABO group, the standardised mean differences (SMD) were 0.091, 0.068, and 0.002 pre matching to 0, 0.013, and 0.001 post, for gaVWD, guVWD, and grVWD, respectively.

take into consideration as Italian investigators previously established that bleeding after tooth extraction and surgical bleeding (combined under the category 'procedural bleeding' in the ICD-BAT), menorrhagia, and epistaxis, all positively predicted for VWD1 (Tosetto et al., 2007). To explore this further, bleeding episodes that occurred in more than 1,000 UKB BAC participants - GI bleeding, haematuria, menorrhagia, procedural, CNS, and epistaxis (Figure 4.4C, page 148) - were selected for analysis by multivariable logistic regression (MLR). The six variables used in the MLR were the same as for the CLM as outlined in Table 4.2 (page 155). The MLR results for the categorical predictors (i.e. all except for age) are shown in Figure 4.8 (page 158). For four out of five of the episode types common to both sexes - i.e. bleeding from the central nervous or gastrointestinal systems, haematuria, and epistaxis - females had a significantly lower risk of these events occurring than males: the odds ratios calculated from the regression model were 0.70 (CI 0.63-0.79, $P = 1.64 \times 10^{-9}$), 0.89 (0.85-0.93, $P = 2.03 \times 10^{-7}$), 0.57 (0.53-0.60, $P = 6.62 \times 10^{-87}$), and 0.65 (0.57-0.73, $P = 2.64 \times 10^{-12}$), respectively. The odds ratios determined for histo-group O were consistently above 1 for all 6 types of bleeding episodes. These were statistically significant for epistaxis ($P = 0.014$) and menorrhagia ($P = 0.035$), such that histo-group O participants had a 16% and 7% increased chance of having a nosebleed and heavy menstrual bleeding, respectively, that required hospital attention. Of the genetically defined VWD groups, only one significant association was found. This was the increased risk of epistaxis in the gaVWD group (OR 2.19 [0.99-4.13, $P = 0.029$]). However, the result needs to be interpreted with caution as the 2.5% confidence interval was below one.

To understand how bleeding events accrued over time and if this was influenced by genetically defined VWD group, a Kaplan-Meier analysis was performed. The duration of follow-up of the UKB BAC was 23.5 years. Time point 0 corresponded to 01 April 1997, the first date on which HES data was available. The 23.5 year time point corresponded to 30 Sep 2020, which was the most recent date for which HES data were available when the data was downloaded from UKB (Figure 4.3, page 146). Each UKB BAC individual then was assigned one of two statuses, 0 or 1. Status 1 applied if the individual had an event during the follow-up period, and status 0 if there was no event. An event was defined as either death and/or the first occurrence of any one or more of the 16 ICD-BAT coded

Figure 4.8

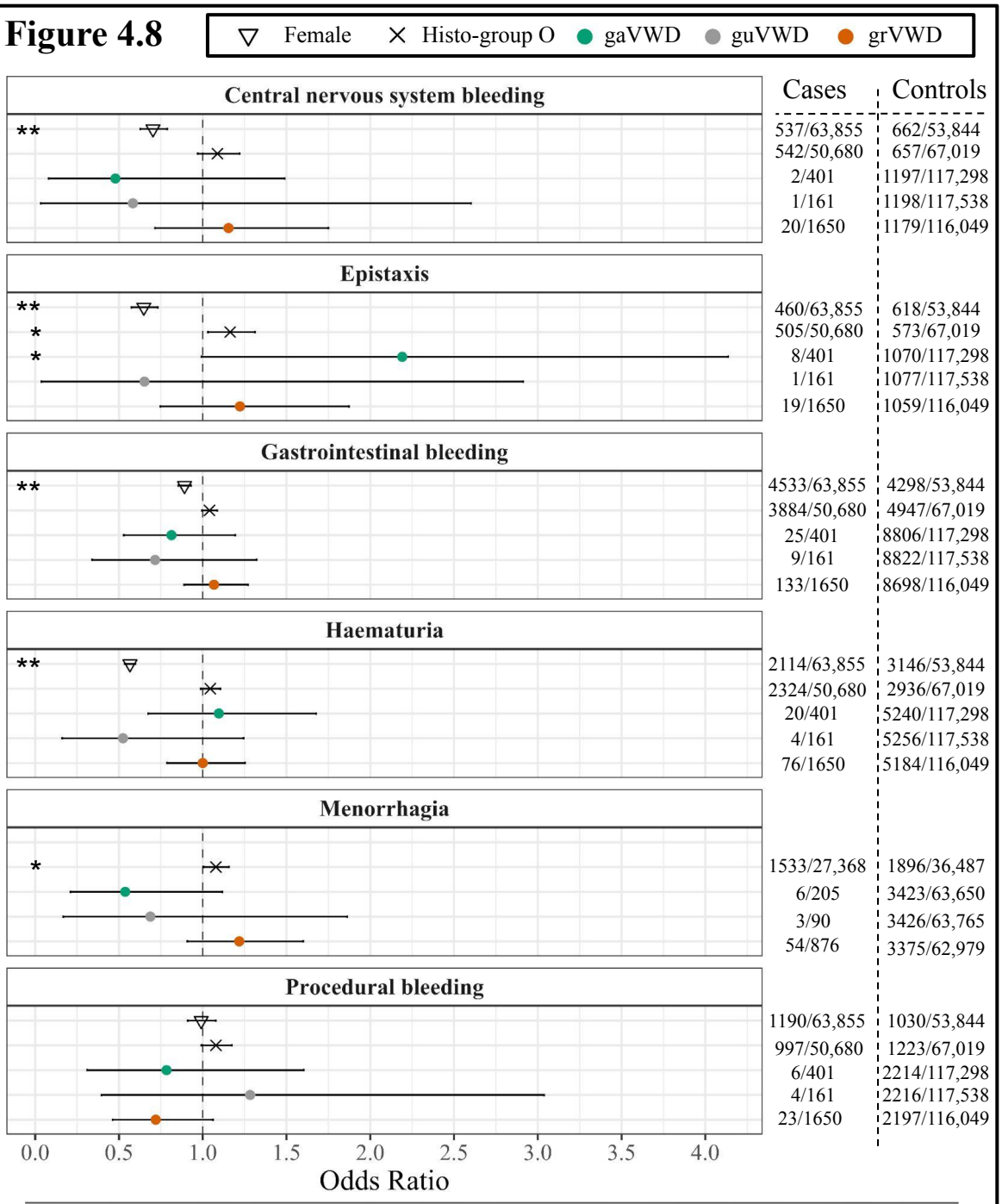


Figure 4.8 | Risk factors for the most common bleeding symptoms in the UKB BAC. A forest plot representing the odds ratio (OR) for the six ICD-BAT coded bleeding episodes (in separate facets) which occurred in the UKB BAC in more than 1,000 participants, determined using multivariable logistic regression. Each row of each facet represents one of five different predictor variables: female sex, histo-group O, genetically detetermined [ga], undecided [gu], and rejected [gr] VWD. The error bars represent the 95% confidence interval around the OR estimate. Statistically significant results are represented by * for $P < 0.05$ and ** for $P < 0.01$. The right hand columns represent the number of cases and controls depending on the parameter being evaluated e.g. for the top row, females are on the left and males on the right. The number before the '/' represents the individuals with the bleeding episode, and the number after represents the total individuals in the group, respectively.

hospital bleeding episodes (Figure 4.2, page 145). Taking death into consideration meant that the estimate of event probability took into account the number of participants remaining at any given time point. There were occasions when a single hospital episode fell under more than one ICD-BAT category and others where the bleeding episode and death occurred synchronously. The latter were classified as fatal bleeding episodes but causation could not be unequivocally proven. This was because not all of the ICD codes annotating the hospital admission were used in the analysis (Figure 4.3, page 146). Status 0 applied to individuals who were neither dead nor had a bleeding episode at the end of the follow-up period. The time allocated to an individual with status 1 was the time at which an event occurred. If the individual did not have an event (status 0), then time was recorded as 30 Sep 2020, i.e. they were "right censored" (Clark et al., 2003). Over the 23.5 year period, 26,008 events occurred in the whole UKB cohort, of which 4232 were deaths due to non-bleeding causes and 21,712 were non-fatal bleeding episodes. Only 64 were fatal bleeding episodes (Table 4.3, page 160), of which 37 were CNS, 22 GI, 2 procedural, 2 cardiac, and in one episode both CNS and GI bleeding co-occurred. CNS bleeding was significantly overrepresented in the fatal versus non-fatal bleeding episodes (OR 13.72, CI 8.89 - 20.94, $P = 2.2 \times 10^{-16}$), but this was not the case for the GI tract (OR 1.01, CI 0.60-1.64, $P = 1$).

The probability that at a given time point a participant was both alive and had not experienced a bleeding episode - hereafter referred to as the bleeding-free survival - is plotted against time for the whole UK BAC in Figure 4.9A (page 161) and for different subgroups in Figures 4.9B-D, 4.10A and 4.10B (pages 161-162). The vertical grey rectangle represents the time window during which participants were recruited at UKB assessment centres (13 March 2006 [time = 8.95 years] - 21 July 2010 [time = 13.31 years]). Therefore, the first 8.95 years provided a means to determine non-fatal bleeding in the UK BAC, which had a rate of 0.74% participants per year. The event rate in the time period from 13.31 years to 23.52 years (30 September 2020) was 1.37% per year which reflects the rate at which fatal and nonfatal bleeding events occur as well as death from other causes. The bleeding-free survival curves were significantly different between males and females, participants who were of the median age (46 years) or older and those

younger than this age, and histo-group O participants compared with those who were non-O (logrank test, $P < 0.0001$ for all comparisons).

(Sub)group	Total participants	Total events	Death - other cause	Fatal bleeding episode	Non-fatal bleeding episode	Figure
Overall UK BAC	117,699	26,008	4232 (16.27%)	64 (0.25%)	21,712 (83.48%)	4.9A
Female	63,855	14,285	1711 (11.98%)	24 (0.17%)	12,550 (87.85%)	4.9B
Male	53,844	11,723	2521 (21.50%)	40 (0.34%)	9162 (78.15%)	
≥ 46 years	59,055	14,640	3220 (22.00%)	50 (0.34%)	11,370 (77.66%)	4.9C
< 46 years	58,644	11,368	1012 (8.90%)	14 (0.12%)	10342 (90.97%)	
O	50,680	11,474	1775 (15.47%)	27 (0.23%)	9672 (84.29%)	4.9D
Non-O	67,019	14,534	2457 (16.91%)	37 (0.25%)	12040 (82.84%)	
gdVWD	401	85	17 (20.00%)	1 (1.18%)	67 (78.82%)	4.10A
guVWD	161	29	4 (13.79%)	0 (0.00%)	25 (86.21%)	
grVWD	1650	376	59 (15.69%)	0 (0.00%)	317 (84.31%)	
No PAVV	115,487	25,518	4152 (16.27%)	63 (0.25%)	21303 (83.48%)	
pLoF	134	28	4 (14.29%)	0 (0.00%)	24 (85.71%)	4.10B
Protein-altering	267	57	13 (22.81%)	1 (1.75%)	43 (75.44%)	
No accepted PAVV	117,298	25,923	4215 (16.26%)	63 (0.24%)	21645 (83.49%)	

Table 4.3 | Events in the UKB Bleeding Assessment Cohort. Deaths and first bleeding episodes (collectively aggregated as events) in the overall UK BAC (first row) and then in each of the subgroups, which were separately analysed. The second column represents the total number of participants in the (sub) group at time 0. The third column represents the total number of events occurring during the time period. The fourth - sixth columns break down the total number of events into those which were deaths from other causes, fatal bleeding episodes, and non-fatal bleeding episodes, respectively, with their percentage contribution (to the total events in the given [sub]group) shown in parentheses. The column on the far right hand side indicates the corresponding figure in which the (subgroup) Kaplan-Meier curves are displayed: Figure 4.9 on page 161 and Figure 4.10 on page 162.

Figure 4.9

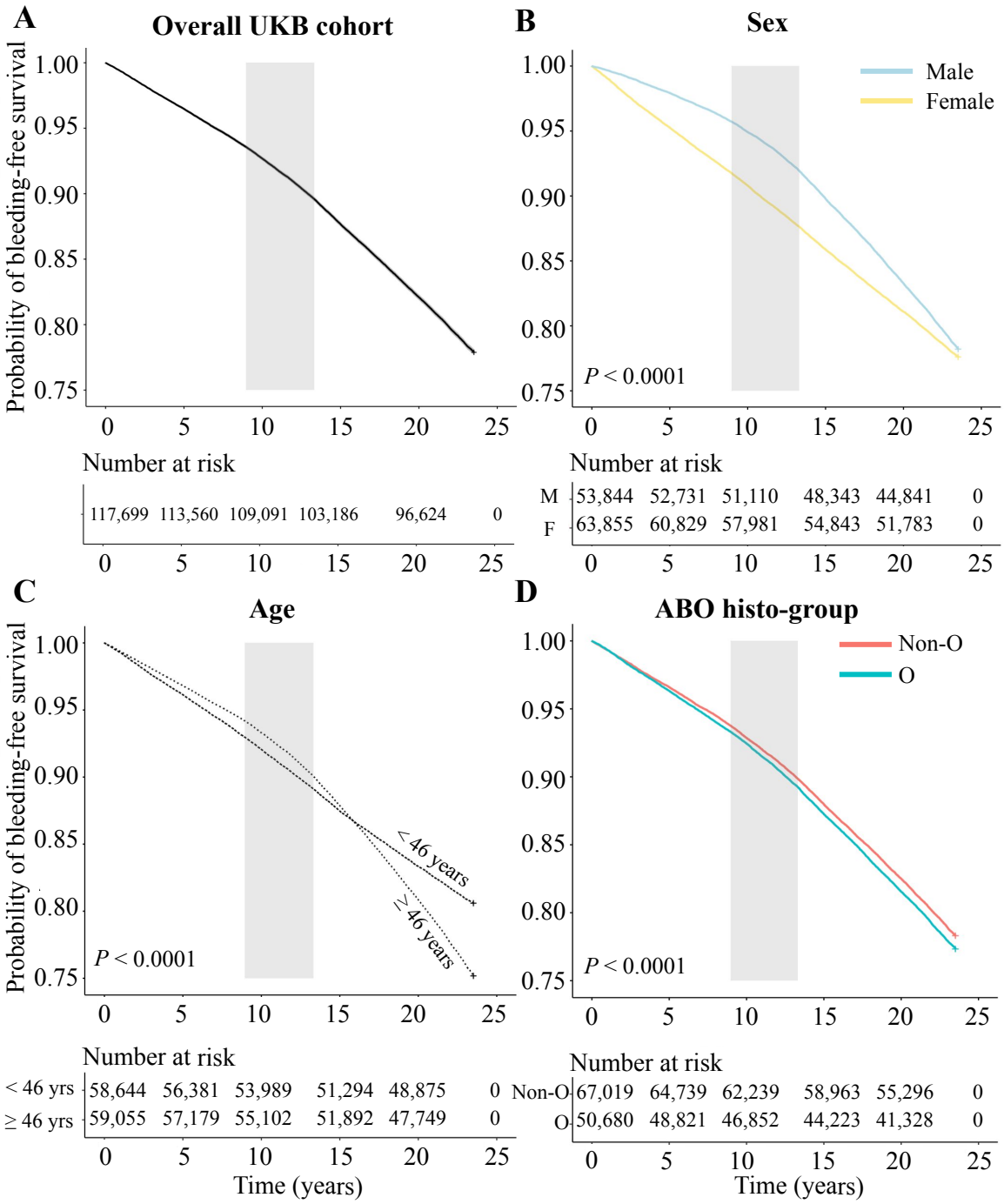


Figure 4.9 | Bleeding-free survival in the UKB BAC cohort. Kaplan-Meier curves representing, on the y-axis, the proportion of the population who remain alive and without a hospital bleeding episode as a function of time on the x-axis. Year 0 corresponds to 01 April 1997, the date from which hospital episode statistics were available. All individuals who had not died or had a bleeding episode were censored on 30 September 2020 corresponding to 23.5 years (marked by '+'). (A) shows the survival curve of all 117,699 participants, and (B) - (D) show, respectively, the bleeding-free survival of groups stratified by sex, age < or ≥ 46 years (the median), and genotypic O histo-group. The grey rectangle represents the time window in which participants were recruited. The *P* values were calculated from the log-rank test.

Figure 4.10

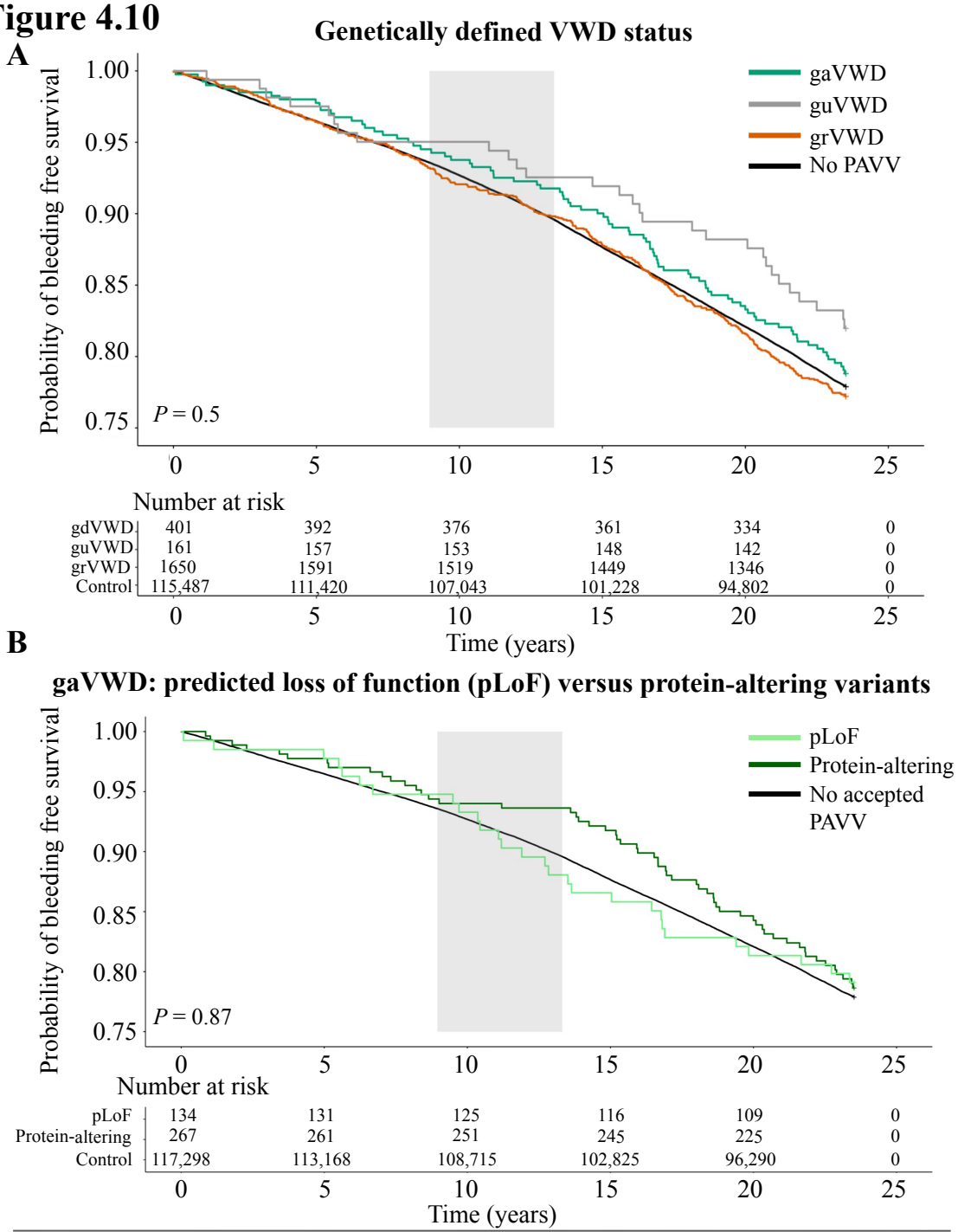


Figure 4.10 | Bleeding-free survival based on genetically defined VWD status and type of variant carried. Kaplan-Meier survival curves representing, on the y-axis, the proportion of the population who remain alive and without a hospital bleeding episode as a function of time on the x-axis. Year 0 corresponds to 01 April 1997, the date from which hospital episode statistics were available. All UK BAC participants who had not died or had a bleeding episode were censored on 30 September 2020 corresponding to 23.5 years (marked by '+'). (A) represents the survival of groups stratified by genetically defined VWD status to those with no PAVV. (B) represents gaVWD individuals with a pLoF or protein-altering PAVV compared to individuals with no accepted PAVV. The grey rectangle represents the time window in which individuals were recruited to UKB. The *P* values were calculated using the log-rank test.

The Cox proportional hazards model (Cox, 1972) was used to ascertain the relative effects of sex, age, histo-group O, and genetically defined VWD status on the likelihood of developing an event during the follow-up period, the results of which are in Table 4.4 (below). This shows that for a given individual, being female and histo-group O, increases the likelihood of an event by 6% and 5% respectively. For every year of increase in age, the event likelihood increases by 2%.

Variable	Hazard ratio	2.5% confidence interval	97.5% confidence interval	*P* value
Female	1.06	1.04	1.09	1.07×10^{-6}
Age	1.02	1.02	1.02	$< 2.00 \times 10^{-16}$
Histo-group O	1.05	1.03	1.07	6.18×10^{-5}
gdVWD	0.95	0.76	1.17	0.61
guVWD	0.77	0.54	1.11	0.16
grVWD	1.02	0.93	1.13	0.65

Table 4.4 | The output of the Cox proportional hazards model on bleeding-free survival in the UK BAC.

Bleeding-free survival was lower in females than in males despite fewer events being attributed to death in this group (Table 4.3, page 160) and less frequent occurrence of the most common non sex-specific bleeding episodes (Figure 4.8, page 158). The driver of this was female-specific bleeding episodes. Out of the 12,550 non-fatal bleeding episodes in females, 4812 (38.3%) were due to menorrhagia, postpartum haemorrhage, other obstetric or gynaecological bleeding. Out of these 4755 occurred alone, but 57 occurred in tandem with other bleeding episodes (both sex-specific and non sex-specific). There was no significant difference in the bleeding-free survival curves of the gdVWD, guVWD, grVWD groups when the comparison was restricted to these three groups (logrank test, $P = 0.3$), nor when a control group was also added for a four-way comparison (logrank test, $P = 0.5$; Figure 4.10A, page 162; Table 4.3, page 160). This was also supported by the outcome of the Cox proportional hazards model (Table 4.4, above).

As discussed in Subsection 1.2.4 (page 54) and Subsection 3.4.2 (pages 119-121), there is contention as to whether individuals who are heterozygous for *VWF* pLoFs, hereafter referred to as pLoF heterozygotes, express a phenotype of VWD. Of the 84 UKB PAVVs accepted by the MDT as being pathogenic (Figure 3.4E, page 110), 34 were pLoFs (Figure

3.5, page 117). Twenty two of these pLoFs were found in 45 individuals with VWF:Ag levels in the published literature, as were evaluated in Chapter 3 (Figure 3.7, pages 120-121). Of these 45 published pLoF heterozygotes, the median VWF:Ag was 0.39 IU/ml, below the lower end of the typical laboratory reference range and 31% were found to have a bleeding propensity. However, the small case series from which this data were extracted did not have control populations against which to compare bleeding phenotype. In the multicentre ISTH study (Castaman et al., 2006) - which did suggest an increase in haemorrhagic symptoms in pLoF heterozygotes - a control population of 215 individuals "matched by age and gender" was used. However, the deliberate selection of those who "had never been referred for evaluation because of hemorrhagic symptoms" potentially biases the control group in favour of those without a bleeding tendency.

In contrast, the UKB BAC provided a large background population within which to evaluate bleeding-free survival and to contextualise pLoF heterozygotes, of which there were 134 in the gaVWD group. In 132 participants these were present without other PAVVs. Two participants also carried another PAVV (p.Ala594Gly in one participant, p.Asn1231Thr in the other), both of which were PAVVs rejected by the MDT. Twenty nine different pLoFs were present in heterozygosity (Table 4.5, page 165), of which 28 were accepted by the MDT on the basis of published evidence that they cause VWD3 in homozygosity or compound heterozygosity with another pLoF. This didn't apply to c.6798+1G>T which was accepted on the basis of its attribution to a VWD1 phenotype in the Canadian cohort study (James et al., 2007).

The bleeding-free survival curves of the 134 gaVWD pLoF heterozygotes was no different from the 267 those who were heterozygous for accepted, protein-altering PAVVs nor from the 117,298 control participants who had no PAVV or were in the guVWD or grVWD groups (logrank $P = 0.98$ [two way comparison pLoF vs protein-altering], $P = 0.87$ [three way comparison including control group]; Figure 4.10B, page 162). Over the 23.5 year time period the pLoF heterozygotes experienced 28 events, of which 24 were non-fatal bleeding episodes (Table 4.3, page 160). There was no significant difference in the median ages, 48 and 46 years, of those with and without events respectively (Wilcoxon test, $P =$

0.09). Likewise there was no difference in the proportion of females (OR 0.78, CI 0.37-1.69, P = 0.47) or those with histo-group O (OR 0.78, CI 0.28-1.94, P = 0.67) between pLoF heterozygotes with or without events.

PAVV	Number of participants	Events (bleeding and/or death)	Event-free
p.Gly839GlufsTer4	17	3	14
p.Trp553LeufsTer97	14	2	12
p.Met814HisfsTer5	14	1	13
p.Gln2470Ter	13	5	8
p.Leu17PhefsTer25	10	2	8
c.3379+1G>A	10	3	7
p.Arg2535Ter	6	2	4
p.Arg365Ter	5	3	2
p.Arg1853Ter	4	0	4
p.Arg1779Ter	4	1	3
p.Cys331Ter	4	0	4
p.His2378AlafsTer13	3	0	3
p.Asp1283ProfsTer12	3	0	3
p.Arg34Ter	3	0	3
p.Asp93Ter	3	0	3
p.Gln1734Ter	3	2	1
p.Arg324Ter	2	2	0
p.Arg1659Ter	2	0	2
c.6798+1G>T	2	0	2
p.Ile1649SerfsTer44	2	0	2
c.3675-1G>A	2	1	1
c.5312-2_5312-1del	1	1	0
p.Arg1336Ter	1	0	1
p.Gln2543Ter	1	0	1
p.Pro2808LeufsTer24	1	0	1
p.Gln104ArgfsTer19	1	0	1
p.Glu644Ter	1	0	1
p.Gln1311Ter	1	0	1
p.Arg139AlafsTer32	1	0	1
Total	134	28	106

Table 4.5 | Bleeding events in gaVWD participants who were heterozygous for predicted loss-of-function variants (pLoFs). PAVVs which are underlined are those in which the published laboratory and bleeding phenotype was evaluated in Subsection 3.4.2 (pages 119-121). Variants are listed with HGVSp nomenclature, except for those positioned in introns which are named using HGVSc terms.

The individual level data available for participants helps to overcome the paucity of data available about the effects of pLoF heterozygotes in the literature. For example p.Trp553LeufsTer97 (c.1657dup) was only identified in one previous study in a patient with VWD1 (referred to as "nt1658 insT") who had a VWF:Act of 0.36 IU/ml and VWF:Ag of 0.40 IU/ml and an elevated ISTH-BAT score of 13 (Robertson et al., 2011). In contrast, of the 14 UKB individuals heterozygous for p.Trp553LeufsTer97, 12 experienced no bleeding episodes requiring hospital admission. For p.Gly839GlufsTer4, the most common *VWF* pLoF in the UKB BAC, there were no previous publications about its effect in heterozygosity. The only publication in which it could be found was a recent case series, in which p.Gly839GlufsTer4 was attributed to VWD3 in a European patient (Baronciani et al., 2021). The majority (14/17) of UKB participants with the variant for p.Gly839GlufsTer4 on one allele had no hospital bleeding episodes.

PAVVs were initially identified in VWDbase as a group in which at least one of the major repositories of these variants (ClinVar, EAHAD-CFDB, and HGMD) considered the variant to be potentially pathogenic (Figure 3.3C, page 104). My analysis demonstrated that individuals with genetically defined VWD did not differ to UK BAC controls with respect to ICD-BAT scores, risk of common types of hospital bleeding episodes, and bleeding-free survival.

4.4. Use of VWDbase in VWD patients missing a molecular diagnosis

One of the motivations for collating VWDbase (Figure 3.3, page 104) was to establish a centralised resource that could be used for the filtering and prioritisation of *VWF* variants identified using high throughput, short read sequencing in patients with a diagnosis of VWD. This section demonstrates how VWDbase can be harnessed for the analysis of WGS data generated from VWD patients.

4.4.1. Analysis of WGS data from the NIHR BioResource

The NIHR BioResource Rare Diseases (NBR-RD) study was a pilot of the 100,000 Genomes Project (100,000 Genomes Project Pilot Investigators, 2021) open to UK and international hospitals which recruited individuals from December 2012 to March 2017

(Turro et al., 2020). In order to organise recruitment within relevant clinical specialties, 15 different rare disease domains were established. One of these was the Bleeding, Thrombotic and Platelet Disorders (hereafter NBR-BPD) domain (Westbury et al., 2015), which recruited individuals from hospitals both in the UK and abroad. Patients were eligible if their clinician deemed that their BPD was thought to have a genetic basis but the cause had not yet been elucidated. Affected and unaffected relatives were also invited to participate. Detailed information about the individuals' clinical and laboratory phenotype, with a focus on bleeding, coagulation and platelet parameters, was entered by the recruiting physicians into a centralised electronic study database (Westbury et al., 2015). DNA from each study participant was generally extracted from blood and genotyped by WGS. The WGS results for the 13,037 participants in the NBR-RD project were all processed using the same analysis pipeline (Turro et al., 2020).

For this thesis the analysis was limited to 1176 NBR-BPD individuals for whom genotype and phenotype were available. Using the phenotype data, individuals enrolled with the diagnosis of VWD were identified, for a re-analysis using VWDbase. Quantitative VWF measurements were recorded for 643/1176 (54.7%) of NBR-BPD individuals. Consistent with the approach used for the MDT appraisal of PAVVs in UKB (Subsection 3.4.1, page 111-115), only NBR-BPD individuals with a VWF:Act and/or VWF:Ag level < 0.30 IU/ml irrespective of bleeding were considered to have a diagnosis of VWD, which is consistent with the latest international guidelines (James et al., 2021) (Table 1.2, page 40). Ten out of the 643 individuals (1.56%) met these criteria (Figure 4.11A, page 168), hereafter referred to as the NBR-VWD group. They had been recruited at seven hospitals in the UK and Belgium. Additional data including the VWD subtype assigned by the recruiting centre, FVIII activity (FVIII:C), platelet count, family history, self-declared ethnicity, additional haemostatic disorders, and the Bleeding Assessment Tool (BAT) score were captured from the NBR-BPD database. The BAT was calculated using the Molecular and Clinical Markers for the Diagnosis and Management of Type 1 von Willebrand disease (MCMDM-1 VWD) questionnaire (Tosetto et al., 2006). Furthermore, the bloodTyper algorithm (Lane et al., 2016; Gleadall et al., 2020) was applied to the WGS data in order to determine the participant's ABO histo-group (A , B, AB, or O). A summary of the

Figure 4.11

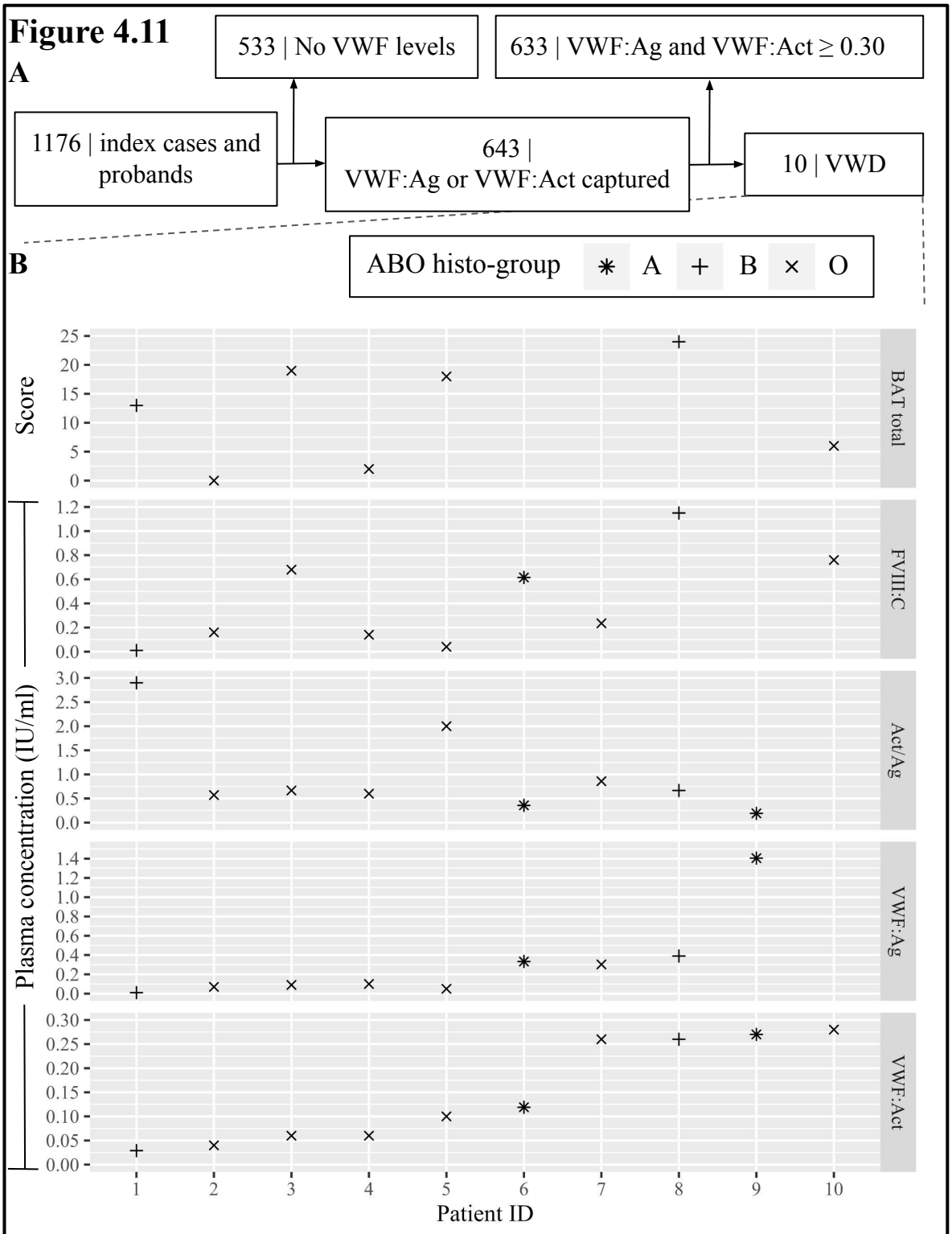


Figure 4.11 | Individuals diagnosed with VWD in the NIHR BioResource (NBR). (A) Flowchart representing the filtering steps taken to identify 10 VWD participants without a molecular diagnosis from the 1176 participants in the BPD domain who underwent WGS. (B) Dot plot representing, from bottom to top, the VWF activity (VWF:Act), VWF antigen (VWF:Ag), VWF activity/antigen ratio, FVIII activity (FVIII:C), and total BAT scores of the 10 VWD individuals. The x-axis represents the anonymised patient IDs which are in order, left to right, of ascending VWF:Act. The shape of the dot indicates the genetically determined ABO histo-group (A, B, or O).

phenotype of each of the 10 NBR-VWD individuals is presented in Figure 4.11B (page 168) and Table 4.6 (below).

ID	Age	Sex	Type	Level (IU/ml)			Plt (x 10^9/L)	ABO	BAT	Family history of VWD	Super-popn.	Additional confirmed haemostasis disorders
				VWF: Act	VWF: Ag	FVIII						
1	24	M	3	0.03	0.01	0.01	326	B	13	+ | Both parents	SAS	None known
2	36	M	2M	0.04	0.07	0.16	143	O	0	+ | 2 children	EUR	None known
3	69	F	NA	0.06	0.09	0.68	215	O	19	+ | 1 child	EUR	None known
4	70	M	2M	0.06	0.10	0.14	221	O	2	- | 2 children	EUR	None known
5	36	M	3	0.10	0.05	0.04	NA	O	18	+| 2 siblings	SAS	FXI 0.40 IU/ml
6	7	M	NA	0.12	0.33	0.62	342	A	NA	NA	EUR	None known
7	16	F	NA	0.26	0.303	0.24	235	O	NA	NA	EUR	FXI 0.26 IU/ml
8	35	F	1	0.26	0.39	1.15	341	B	24	+| Mother	EUR	PFD
9	10	F	NA	0.27	1.41	NA	262	A	NA	NA	EUR	None known
10	41	F	1	0.28	NA	0.76	166	O	6	+ | 2 children	EUR	None known

Table 4.6 | Phenotype of the 10 NBR-VWD individuals. The columns from left to right represent: anonymised ID assigned to patient; age at recruitment rounded to the nearest integer of year; sex based on determination of X and Y karyotype from the WGS data; plasma levels of platelet dependent VWF activity (VWF:Act), VWF antigen (VWF:Ag), and FVIII (FVIII:Act); platelet count (plt); ABO histo-group; total MCMDM-1 bleeding assessment tool (BAT) score; family history of VWD where '+' and '-' indicate 1st degree family members with and without VWD, respectively, which are specified to the right of the '|' sign; ancestry super-population as determined using the WGS data; and additional confirmed (inherited) haemostasis disorders. There were three individuals to whom the latter applied. Two had FXI deficiency (reference range for FXI is typically 0.60-1.50 IU/ml). One individual has a platelet function disorder, abbreviated as PFD. The participants with rows filled in a magenta colour already had a variant identified in the initial NBR-RD analysis (Turro et al., 2020). The participants whose rows are unfilled did not have a variant identified in this analysis.

Five out of the 10 of the individuals (NBR 2, 3, 6, 8, and 9) had variants in *VWF* - potentially causal of VWD - which were identified in the initial sequencing analysis reported by the NBR-RD consortium (Table 4.7, page 170; Turro et al., 2020). NBR 3 and 8 were heterozygous for different structural variants (SV) in *VWF*. No other NBR-VWD participants were found to have SVs deemed to be potentially causal of VWD. NBR 2, 6, and 9 had relevant SNVs in VWF. These variants are now considered in turn.

NBR 2 was listed by the recruiting physician as having VWD "Vicenza" (VWD1C), an autosomal dominant subtype of VWD (which was previously categorised as VWD2M, consistent with the diagnosis provided for the patient).

ID	Type	Effect	Variant	Zygosity	NBR MDT-adjudicated pathogenicity
2	SNV[1]	Protein-altering	c.3614G>A (p.Arg1205His)	Heterozygous	Pathogenic
3	SV[2]	pLoF[3]/ protein-altering	Exon 4-5 deletion (c.221-1681_c.533-7311del, p.Asp75_Gly178del)	Heterozygous	Likely pathogenic
6	SNV	pLoF	c.658-3C>A	Heterozygous	Pathogenic
8	SV	pLoF[3]/ protein-altering	Exon 4-5 deletion (c.221-977_c.532+7060del, p.Asp75_Gly178del)	Heterozygous	Likely pathogenic
9	SNV	Protein-altering	c.3569G>A (p.Cys1190Tyr)	Heterozygous	Likely pathogenic

Table 4.7 | Variants initially reported in the NBR-VWD participants. ID represents the anonymised NBR ID assigned to the patient. Abbreviations: [1]single nucleotide variant, [2]structural variant, [3]predicted loss-of-function variant. The NBR-RD convened its own MDT for BPD cases to review candidate variants against ACMG/AMP criteria and their conclusion on the pathogenicity of the variants is in the far right hand column (Turro et al., 2020).

VWD Vicenza is characterised by the accelerated clearance of VWF (Casonato et al., 2002) and occasionally requires the use of on-demand factor concentrates (Castaman et al., 2011), such as the Haemate P previously prescribed for this participant. The variant identified in this individual, p.Arg1205His, was the first to be attributed to VWD Vicenza (Schneppenheim et al., 2000), and is a UKB PAVV accepted as being pathogenic by the MDT, with an MAF in UKB of 5×10^{-6} (Appendix 7.4, page 277). Individuals with VWD Vicenza are reported to have laboratory values of FVIII:C < 0.20 IU/ml and VWF:Act < 0.10 IU/ml and the laboratory results of NBR2 are consistent with this (Castaman et al., 2011).

The other protein-altering variant, p.Cys1190Tyr, was found in NBR 9. It is a PAVV (Appendix 7.4, page 277), with a VWDbase summary assertion of 'pathogenic', because it was rated as an LPV in ClinVar and a DM variant in HGMD. This is supported by its rarity: it was not found in gnomAD v3.0, and NBR 9 was the only individual found to have this variant in the NBR-RD cohort (translating as an MAF of 3.84×10^{-5}). Furthermore, p.Cys1190Tyr was not found in the WES data of UKB participants and was therefore not appraised by the MDT which reviewed variants for VWDbase. The markedly discordant VWF:Act and VWF:Ag results found in this individual, 0.27 and 1.41 IU/ml respectively, correspond to a low Act/Ag ratio of 0.19 (Figure 4.11B, page 168) meaning

that she has VWD2. This fits with other patients heterozygous for p.Cys1190Tyr who are reported to have Act/Ag ratios of between 0.19 and 0.25 (de Jong et al., 2020; Schneppenheim et al., 2010). Therefore, it seems likely that NBR 2 and 9 seem to be fully explained by the variants identified by the NBR-RD analysis. This is in contrast to the variants identified in NBR 3, 6, and 8, as discussed below.

NBR 3 and 8 had heterozygous exon 4-5 deletions (ex4-5del), albeit with different breakpoints (Table 4.7, page 170). Inframe deletion of exon 4 and 5 is the most common SV reported in individuals with VWD (Christopherson et al., 2016; Sutherland et al., 2009b). In homozygosity or compound heterozygosity with a *VWF* pLoF variant, ex4-5del variants unequivocally result in VWD3 (Bowman et al., 2013; Christopherson et al., 2022; Sutherland et al., 2009a). However, the effect of ex4-5del in heterozygosity is much less clear. An in vitro experiment in endothelial colony forming cells obtained from a ex4-5del heterozygote showed that VWF mRNA levels were reduced, and that the VWF which was expressed was predominantly pro-VWF, retained in the ER with no significant increase in the amount released upon secretagogue stimulation (Starke et al., 2013). This suggests that ex4-5del both decreases transcriptional output but potentially also interferes with N-terminal multimerisation in a dominant negative manner, which would be consistent with the position of this variant in the propeptide (Cartwright et al., 2020). However, the degree to which these mechanisms result in an in vivo effect is highly variable: heterozygotes with ex4-5del have VWF:Act levels varying from 0.13-1.05 and VWF:Ag from <0.01 - 0.99 IU/ml (Christopherson et al., 2022; Sutherland et al., 2009b; Starke et al., 2013). Therefore, the degree to which the phenotypes of NBR 3 and 8 can be attributed to the heterozygous ex4-5del variants is uncertain.

NBR 6 was found to be heterozgyous for the splice donor variant, c.658-3C>A. This is a PAVV in VWDbase (Appendix 7.4, page 260) but not found in UKB so no further MDT assessment was made. NBR 6 was the only individual in the NBR-RD study found to have the variant (MAF of 3.84×10^{-5}), further supporting its rarity. However, the summary assertion of this variant in VWDbase is of 'conflicting interpretations of pathogenicity'. The VWD3 phenotype of a 15 year old male (Kumar et al., 2013) was attributed to

compound heterozygosity of c.658-3C>A with c.3379+1G>A (an accepted PAVV). Furthermore, c.658-3C>A was listed as a VWD1 variant in a French cohort study which included individuals with VWD1 if they had "VWF levels < 0.30 IU/ml [and] VWF:RCo/VWF:Ag > 0.6", but the levels of the affected individual was not provided (Veyradier et al., 2016). However, as previously illustrated there is variable expression of a laboratory phenotype in pLoF heterozygotes (Figure 3.7, pages 120-121). Therefore, the contribution of c.658-3C>A to the laboratory results of NBR 6 (VWF:Act 0.12 and VWF:Ag 0.33) is questionable. Furthermore, the VWF:Act/VWF:Ag ratio of 0.36 would be consistent with a VWD2 phenotype (Table 1.2, page 40), which would suggest the presence of an additional *VWF* variant that affects VWF function.

The NBR-RD analysis did not reveal a causal *VWF* variant for participants 1, 4, 5, 7, or 10, despite the severity of the phenotype of NBR 1, 4, and 5, all of whom have a high likelihood of having a variant within the *VWF* locus (Goodeve et al., 2007). Therefore, my primary objective was to use VWDbase to re-analyse the WGS data of these five individuals to establish whether (a) causal VWD variant(s) could be identified. This was combined with the analysis of the WGS data of the other NBR-VWD individuals for the following, secondary, objectives:

- Establish the utility of a CADD (C) score threshold for variant filtering and prioritisation.
- Search for additional *VWF* variants that could explain the laboratory phenotype of NBR 3, 6, and 8 given that the NBR-RD identified variants were unlikely to be fully contributory.

NBR 2 and 9 were included in the analysis as positive controls, on the premise that my reanalysis should also identify the p.Arg1205His and p.Cys1190Tyr variants, respectively.

The starting point of my analysis was the VCF created from each individual's WGS data. The VCFs were produced by the NBR-RD using Illumina software, and involved steps including read alignment to GRCh37 and the calling of SNVs and indels (Turro et al., 2020). The processing steps that I subsequently took are shown in Figure 4.12 (page 173). These are now briefly outlined. Firstly, BCFtools (Li et al., 2009) was used to create

Figure 4.12

A

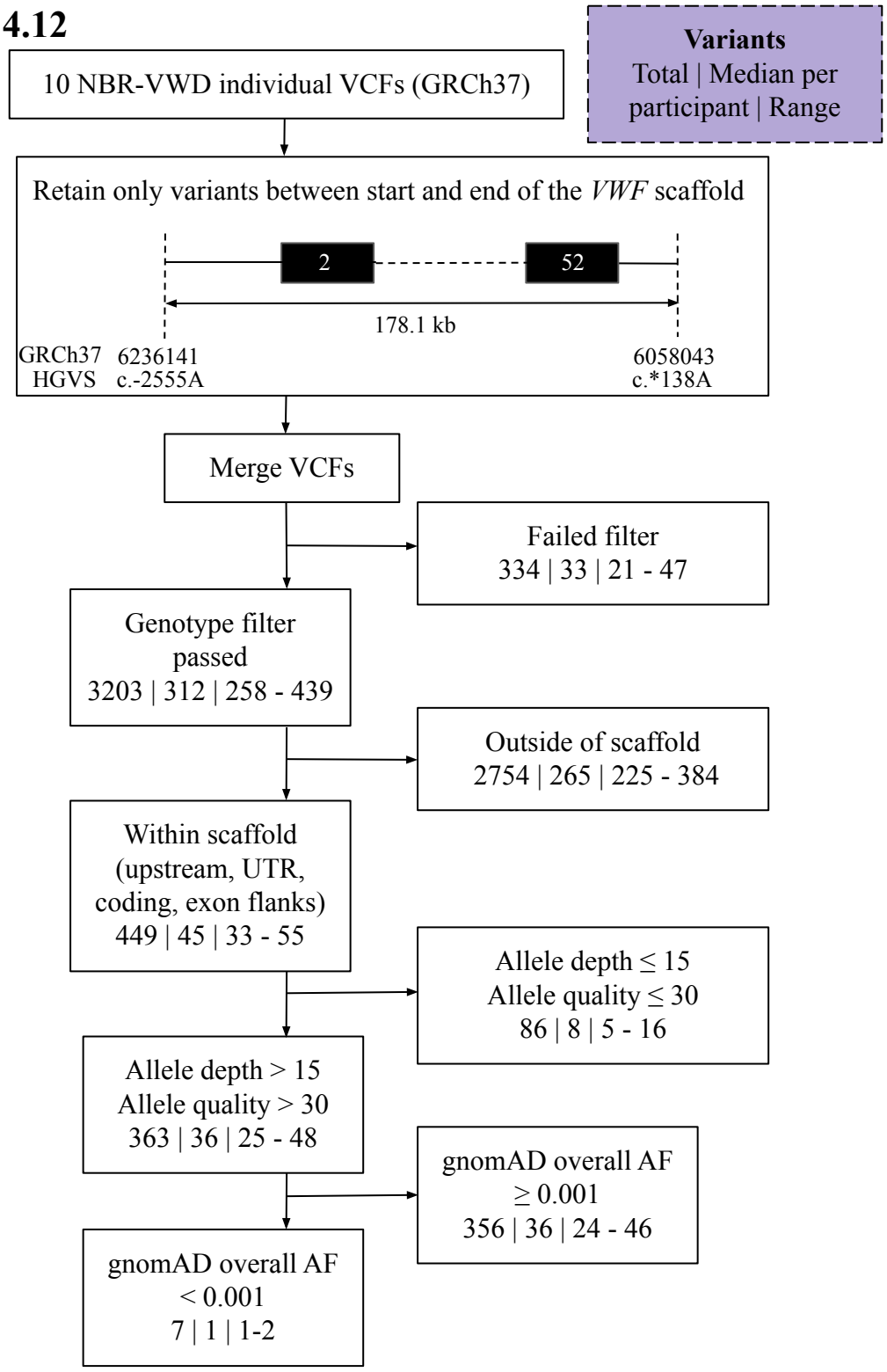


Figure 4.12 | Analysis of the WGS data from the 10 NBR-VWD individuals. VCFs from the 10 participants were first trimmed to only include variants within the start and end coordinates of the VWF scaffold. The VCFs were then merged, retaining the anonymised identifiers of individuals against each variant. The variants then went through a sequential series of filtering steps as shown by the flow chart, with the numbers indicated representing total variants, and median and range per participant.

trimmed VCFs which only contained variants between the start and end coordinates of the *VWF* scaffold (Subsection 3.3.1, pages 101-102). These were annotated with the NBR IDs (Table 4.6, page 169), prior to merging each of the 10 VCFs into a single file. After this initial merge, there were 3537 variants, with a median of 343 and range of 303 - 477 variants per participant. Out of these, 3203 variants passed the filter as defined by the Isaac variant caller software. Only these variants were retained.

The next step was to narrow the variant shortlist to those in regions of the *VWF* locus known to harbour potentially causal SNVs and indels and to liftover the variant positions to GRCh38 to facilitate annotation with gnomAD v.3.0 (see below). Therefore, only the 449 variants occupying positions in the 30,513 nucleotide *VWF* scaffold were kept (Figure 3.2, page 103). These were then further filtered to retain only those with a genotype quality (GQX) score > 30 consistent with the approach used for filtering PAVVs from the UKB WES data (Figure 3.4, page 110). A depth filter of greater than 15 was applied, because all samples in the NBR-RD pipeline were covered to at least this depth "in 95% of reference autosomes" (Turro et al., 2020).

Following quality and depth filtering, 363 variants remained. These were then annotated with their MAFs in gnomAD v3.0 as this version determined MAF using WGS data (in GRCh38 build) and was more ancestrally diverse than gnomAD v2.1 (Francioli & MacArthur, 2019). Consistent with the threshold used for the UKB WES analysis, and for the reasons outlined in Subsection 3.4.1 (page 109-111), only variants with an MAF < 0.001 in gnomAD were retained, thus constraining the final set of variants to just seven. These variants were then annotated with C scores (Subsection 2.1.4, page 74) and relevant VWDbase information (i.e. PAVV or not, summary pathogenicity assertion, and UKB MAF). Reassuringly, I replicated the identification of the three short *VWF* variants published by the NBR-RD (Turro et al., 2020) - p.Arg1205His, p.Cys1190Tyr, and c.658-3C>A - but also identified four further potentially relevant variants, including in NBR 1 and 5, whose VWD was hitherto unexplained. These seven variants are detailed in Table 4.8 (page 175). NBR 4 and 7 remained unexplained. Likewise no additional causal variants were found to further explain the phenotypes of NBR 3, 6, and 8. No novel

candidate VWD variants were identified.

ID	Variant	Zygosity	Location	PAVV | VWDbase assertion	UKB PAVV | UKB AF | MDT outcome	gnomAD v3.0 AF	C score (CADD v1.6)	Newly identified in NBR-VWD participants
1	c.8155+6T>A	Hom	Intron 50-51	Yes | Pathogenic	No | NA | N/A	NA	23.6	Yes
2	c.3614G>A (p.Arg1205His)	Het	Exon 27	Yes | Pathogenic	Yes | 5.0×10^{-6} | Accept	NA	22.7	No
2	c.8115+193A>G	Het	Intron 49-50	No | N/A	No | N/A | N/A	NA	0.378	Yes
5	c.5455+2T>C	Hom	Intron 31-32	Yes | Pathogenic	Yes | 2.0×10^{-6} | Accept	NA	33.0	Yes
6	c.658-3C>A	Het	Intron 6-7	Yes | Conflicting interpretation	No | NA | N/A	7.0×10^{-5}	23.0	No
9	c.3569G>A (p.Cys1190Tyr)	Het	Exon 27	Yes | Pathogenic	No | NA | N/A	NA	24.9	No
10	c.5312-19A>C	Het	Intron 30-31	Yes | Conflicting interpretation	No | NA | N/A	9.6×10^{-4}	2.2	Yes

Table 4.8 | Variants identified in the analysis of NBR-VWD individuals using VWDbase. ID represents the anonymised NBR ID assigned to the patient. In the zygosity column, Hom and Het stand for homozygous and heterozygous, respectively. In the UKB PAVV and gnomAD v3.0 AF columns, NA means that the variant was not observed in the UKB WES or gnomAD v3.0 WGS data, respectively, which either means that the variant is absent from these sources, or that it did not pass the genotype filters which were applied to the sequencing data before extracting the MAFs. N/A stands for 'not applicable'. This is used in two contexts in the UKB PAVV column. In the case of c.8115+193A>G, N/A indicates that MAF was not determined because the variant was not a PAVV. In all other instances, it reflects the fact that MDT appraisal of pathogenicity did not take place if the variant was not a UKB PAVV (Figure 3.4, page 110). C scores are reported to 3 significant figures. Variants are marked as being newly identified in NBR-VWD individuals if they were absent from those published in Turro et al., 2020 but were found using the analysis outlined in Figure 4.12, page 173. Rows filled in grey indicate variants which have an uncertain contribution to the VWD phenotype of the participant, and those in green represent variants considered to be causal of VWD in the given participant.

Out of the seven identified, two of the variants were located at deep intronic positions: c.8115+193A>G and c.5312-19A>C. The former was not found in VWDbase and occurred in NBR 2, in whom p.Arg1205His had already been identified as the causal variant. Furthermore, the C score of c.8115+193A>G was 0.37. As shown in the UKB PAVV analysis, a C score of below 10 was a strong predictor of a variant being rejected as pathogenic (Figure 3.8, page 123). Therefore, this would suggest that c.8115+193A>G is not pathogenic. c.5312-19A>C was identified in NBR 10, who had a VWF:Act of 0.28 IU/ml (Table 4.6, page 169). Functional work using platelets obtained from a patient with

type 1 VWD and heterozygous for c.5312-19A>C showed this variant does not alter splicing of *VWF* (O'Brien et al., 2003). This suggests, as does the C score of 2.2, that c.5312-19A>C is not the cause of NBR 10's phenotype. Of the remaining five variants, c.658-3C>A was found again in NBR 6, with an uncertain contribution to phenotype as discussed above (page 171-172). The remaining four variants, highlighted in green in Table 4.8 (page 175), are likely to be causal of the individual's phenotype. All four variants were found in VWDbase, highlighting the utility of this resource. None of these four variants had a C score of 35 or greater, which I had proposed, in Chapter 3 (Subsection 3.4.3, pages 122-124), as a threshold for high confidence pathogenic *VWF* variants. Instead the C scores of these four 'green' variants ranged between 22.7 and 33.0 (Table 4.8, page 175). Two of them (c.8155+6T>A and p.Cys1190Tyr) were absent from both gnomAD v3.0 and UKB. The MAFs of both p.Arg1205His and c.5455+2T>C were 5.0×10^{-6} and 2.0×10^{-6} in UKB, respectively, but but both variants were absent from gnomAD v3.0. Therefore, all 4 'green' variants had MAFs $< 10^{-5}$ and C scores > 20. As shown by Figure 3.8 (page 123), 44/65 (68%) of UK PAVVs meeting these thresholds were accepted by the MDT as being pathogenic for VWD, suggestive that these may be more workable thresholds for future *VWF* variant prioritisation from short read sequencing. Two of the four 'green' variants were not identified in the original NBR-RD analysis (Turro et al., 2020). These newly identified variants were c.5455+2T>C and c.8155+6T>A (Table 4.8, page 175). Both variants were present in homozygosity.

c.5455+2T>C was found in NBR 5. This variant is in VWDbase as a UKB PAVV and was accepted as pathogenic by the MDT (Appendix 7.4, page 291). The recruiting medical team had given him a prior diagnosis of VWD3, in keeping with his VWF:Ag of 0.05 IU/ml (Table 4.6, page 169). In homozygosity this variant has been previously reported to cause VWD3 (Bowman et al., 2013). This evidence, taken alongside the lack of other candidate variants revealed in the analysis, are consistent with homozygous c.5455+2T>C being the cause of VWD3 in NBR 5.

c.8155+6T>A was identified in NBR 1, a male who was 24 years of age at the time of recruitment into the NBR-RD study. He had the most severe laboratory phenotype of the

NBR-VWD group (Figure 4.11, page 168; Table 4.6, page 169), consistent with VWD3. c.8155+6T>A is a PAVV (Appendix 7.4, page 303), with a summary VWDbase assertion of 'Pathogenic'. It is rare as evidenced by its absence from the genotyping data from both gnomAD v3.0 and UKB. The position of this single nucleotide transversion at the sixth base pair 3' of the exon 50/intron 50-51 junction is shown in Figure 4.13 (page 178). The donor splice site (DSS) is defined as the final two nucleotides of an exon and the first six nucleotides of the following intron (Senapathy et al., 1990). Therefore, c.8155+6T>A is situated at the last nucleotide of the exon 50/intron 50-51 DSS. The WGS data for NBR 1 is shown in Figure 4.13B (page 178), confirming that he is homozygous for c.8155+6T>A. This variant was first reported in a pedigree investigated and treated at a London hospital (Harrington et al., 2018). Two out of the three propositi in this publication were homozygous for c.8155+6T>A. Both of these individuals had VWF:Ag levels of 0.01 and 0.02 IU/ml, consistent with VWD3 (Harrington et al., 2018).

The only other report of c.8155+6T>A is from a study investigating the genetic basis of VWD3 in individuals from Pakistan (Ahmed et al., 2019). The authors of this study identified the variant in homozygosity in an 18 year old male with a VWF:Ag level < 0.03 IU/ml. Using in silico tools (Desmet et al., 2009; Rogozin & Milanesi, 1997) they predicted that c.8155+6T>A either results in the generation of a new cryptic acceptor site or abolishes the existing donor site. The latter is more likely based on the assertion of the authors of Harrington et al. (2018) who stated that "the effect of [c.8155+6T>A] was elucidated by RNA analysis, which revealed that exon 50 is spliced out". However, they did not publish experimental details or results to support this conclusion.

The removal of exon 50 is also the proposed mechanism underpinning the deleterious effect of four other variants situated in the intron 50-51 DSS: c.8155+1G>T (Solimando et al., 2012), c.8155+3G>C (Corrales et al., 2011), c.8155+3G>T (Mertes et al., 1994), and c.8155+6T>C (Platè et al., 2010). Furthermore, the NetGene2 (Brunak et al., 1991), Genesplicer (Pertea et al., 2001), and NNSPLICE (Reese et al., 1997) tools were used by the authors of Corrales et al. (2011) to assess the effect of c.8155+3G>C. The tools universally predicted that this variant would result in DSS destruction.

Figure 4.13

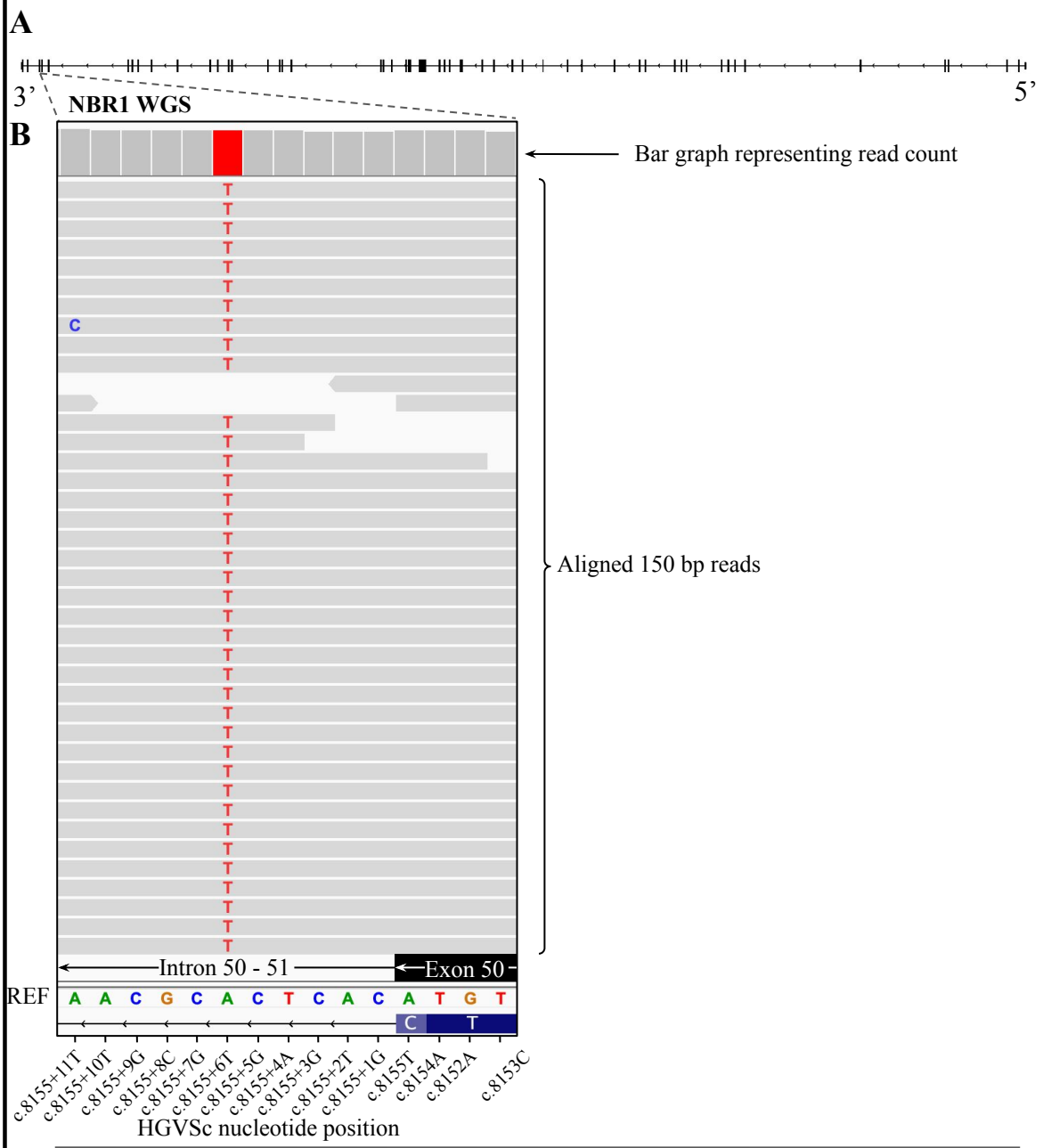


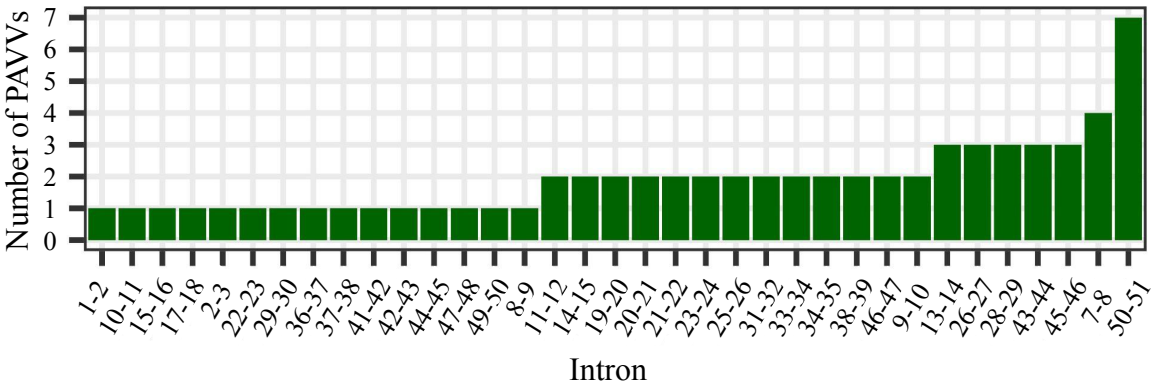
Figure 4.13 | The variant c.8155+6T>A is located in the donor splice site of intron 50-51. (A) A linear diagram, to scale, in which the 52 exons of *VWF* are represented by vertical black bars. In between the exons are the 51 introns. (B) A magnified representation of the 15 bp region at the junction of exon 50 and intron 50-51 annotated with the WGS data of NBR 1. Nucleotide position is shown using HGVSc nomenclature, with the corresponding forward strand reference sequence shown above (marked 'REF'). The HGVSc nucleotide is complementary to the forward strand nucleotide because *VWF* is positioned on the reverse strand. The blue rectangles represent the final two residues of exon 50 with the amino acids represented by one letter codes. The GRCh37 aligned 150 bp reads are represented by grey rectangles with points. The red T overlying the reads and the bar chart at the top of the figure indicate that at position GRCh37 12-5951838 (c.8155+6T) the reference nucleotide, A, has been replaced with the alternate, T, in all aligned reads.

Given the number of variants reported at the intron 50-51 DSS, a search was undertaken for all the PAVVs in VWDbase located in the 5' half of introns. Seven PAVVs, including c.8155+6T>A and the four variants mentioned above, are located in the 5' half of intron 50-51, more than in any other intron (Figure 4.14A, page 180). All seven PAVVs are in the DSS. The phastCons conservation score (Siepel et al., 2005) was downloaded for the 15 nucleotides at the exon 50/intron 50-51 junction shown in Figure 4.14B (page 180). The score is scaled from zero to one. Zero corresponds to nucleotides which are the least conserved, one indicates the most conserved. All seven of the intron 50-51 DSS PAVVs are positioned at nucleotides with a phastCons conservation score of 1 and all have been reported to be causal of a VWD3 phenotype either in homozygosity (Ahmed et al., 2019; Solimando et al., 2012; Harrington et al., 2018) or compound heterozygosity (Corrales et al., 2009; Liang et al., 2017; Mertes et al., 1994; Platè et al., 2010; Yadegari et al., 2012). This suggests that c.8155+6T>A and the other six intron 50-51 DSS PAVVs result in loss of function.

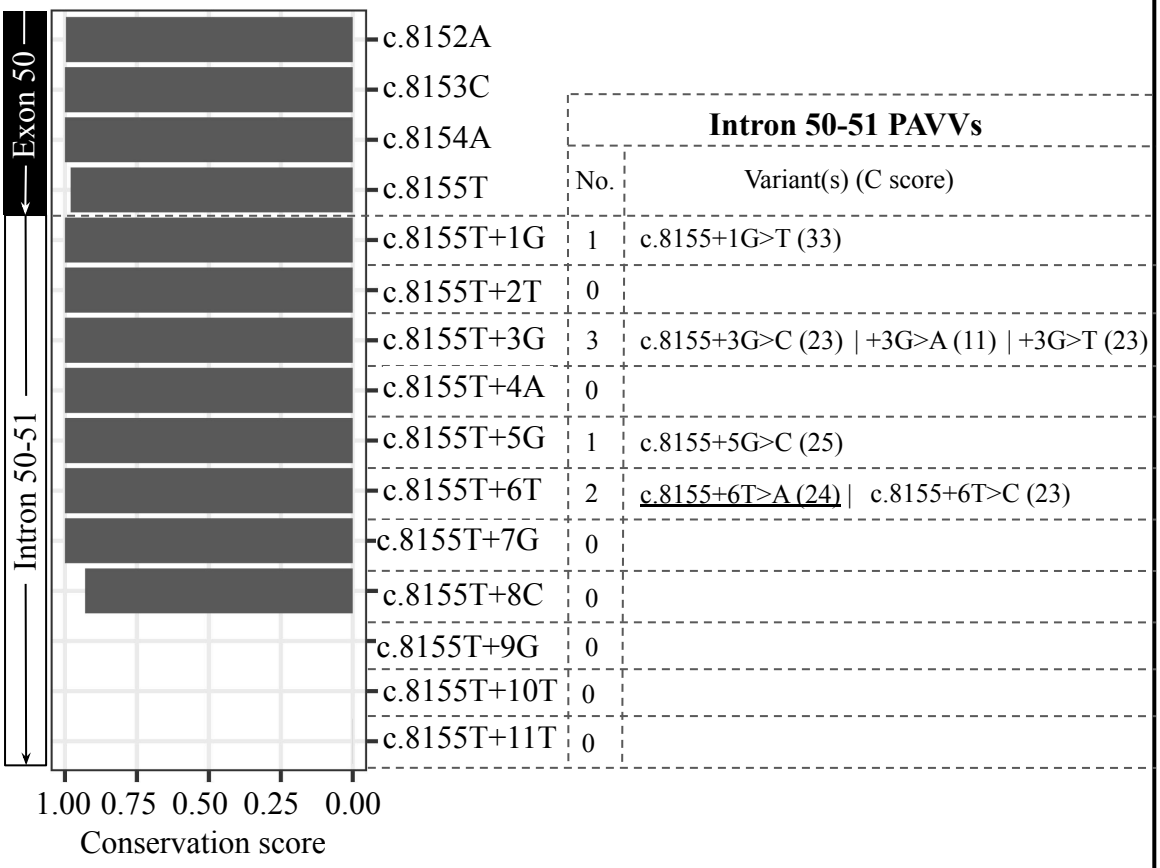
At 40 nucleotides in length (Mancuso et al., 1989), exon 50 is not a multiple of three. Therefore, if the exon skipping reported to occur in c.8155+6T>A (Harrington et al., 2018) has the same outcome as reported for c.8155+3G>C (Corrales et al., 2011) and c.8155+6T>C (Platè et al., 2010), it would be expected to result in a frameshift and premature termination codon (PTC). Nonsense mediated decay (NMD) is an evolutionarily conserved intracellular pathway for the rapid removal of some transcripts that harbour PTCs (Culbertson, 1999). Clearance of such transcripts is necessary as the translated truncated proteins can exert harmful, dominant negative effects (Chang et al., 2007). The only evidence about whether intron 50-51 DSS PAVVs result in transcripts susceptible to NMD comes from RNA sequencing of an individual with VWD3 who was compound heterozygous for both c.7082-2A>G and c.8155+3G>C (Borràs et al., 2019). The latter variant is situated in intron 50-51 (Figure 4.14B, page 180). In this individual, 95% of the reads came from transcripts resulting from c.8155+3G>C suggesting that these mRNAs did not (as readily) undergo NMD as compared to those transcribed from the c.7082-2A allele.

Figure 4.14

A



B



Intron 50-51 PAVVs	
No.	Variant(s) (C score)
1	c.8155+1G>T (33)
0	
3	c.8155+3G>C (23) | +3G>A (11) | +3G>T (23)
0	
1	c.8155+5G>C (25)
2	c.8155+6T>A (24) | c.8155+6T>C (23)
0	
0	
0	
0	
0	

Figure 4.14 | Intron 50-51 is a hot spot for donor splice site PAVVs. (A) Bar graph showing the number of PAVVs in the 5' half of an intron as a function of which intron they are located in (on the x-axis, in ascending order of the number of 5' PAVVs they contain). (B) A bar graph showing the phastCons conservation scores of 15 nucleotides at the exon 50/intron 50-51 junction of *VWF*. The table indicates the intron 50-51 PAVVs at the given nucleotide position and, in parentheses, their CADD (C) scores. The variant, c.8155+6T>A, identified in NBR1 is underlined.

Taking all this background information into consideration, I selected NBR 1 and the c.8155+6T>A variant for further phenotypic, genotypic, and functional characterisation. The reasons for this were as follows. Firstly, c.8155+6T>A is a PAVV in VWDbase that did not undergo MDT scrutiny because of its absence from UKB. Secondly, the DSS of intron 50-51 is a hotspot for PAVVs (Figure 4.13, page 178). Thirdly, the mechanism by which c.8155+6T>A results in VWD3 is not clear. As outlined above, there is limited evidence suggesting that other DSS PAVVs in intron 50-51 (Figure 4.14, page 180) result in transcripts which do not undergo NMD. But, on the flip side, in individuals homozygous for c.8155+6T>A, there is consistent evidence that no or minimal plasma VWF can be detected. So what happens in between? Is VWF expressed? And if so, why is it not released? These questions pertaining to the intracellular consequences of c.8155+6T>A and other intron 50-51 splice donor variants have not previously been addressed and are the focus of the remainder of this chapter and the next. The objectives for the rest of this chapter are:

1) To further explore and clarify the phenotype of NBR 1 beyond the information captured for the NBR-BPD study.
2) To confirm whether c.8155+6T>A causes exon 50 to be skipped in NBR 1.
3) If exon 50 is skipped, does this result in a PTC and where is this situated?
4) If the transcripts are shown to harbour PTCs, are they translated into VWF protein?

4.5. c.8155+6T>A is a donor splice site variant

4.5.1. c.8155+6T>A causes VWD3 in homozygosity

Consent was taken from NBR 1 to enrol in an additional study, called BRIDGE, that permitted further clinical information and blood samples to be obtained (Subsection 2.1.6, pages 75-76). This was four years after his initial enrolment in the NBR-RD study, and so he was 28 years of age at the time of recontact. An invitation to participate in BRIDGE was also sent to NBR'1 first-degree relatives, but they declined.

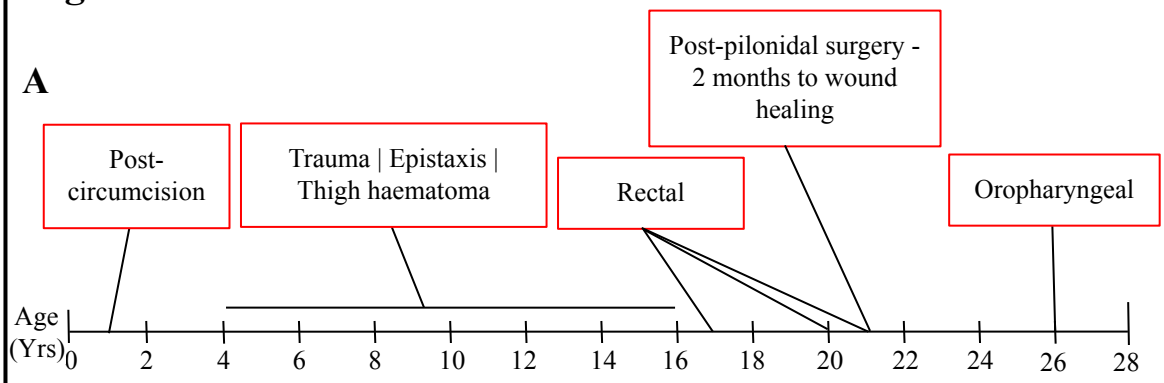
NBR 1 was first diagnosed with VWD3 when he was six months old. At that time he had been circumcised and the bleeding had failed to stop. This is a well-established mode of presentation of hitherto undiagnosed inherited bleeding disorders (Biss et al., 2010; Lak et

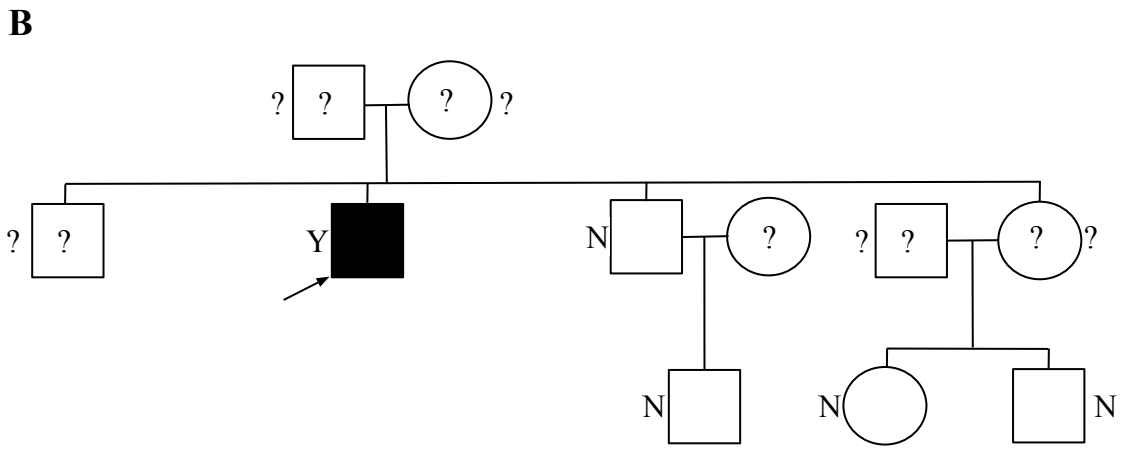
al., 2000; Rodriguez et al., 2010). A timeline of his bleeding symptoms is shown in Figure 4.15A (page 183). The rectal bleeding episodes were recurrent and culminated in excision of a pilonidal sinus. This took 2 months to heal despite regular administration of plasma derived VWF concentrate (Stadler et al., 2006).

The pedigree tree of NBR 1 is shown in Figure 4.15B (page 183). This reveals a number of key points that would support c.8155+6T>A behaving as a recessive allele in NBR 1's extended pedigree. First, NBR 1 is the only family member diagnosed with VWD. Second, aside from his paternal grandfather (not shown), who died of a brain haemorrhage, he was not aware of any other family members who had a bleeding diathesis. Third, his parents were first cousins, a known risk factor for autosomal recessive disorders (Woods et al., 2006). Because of NBR 1's parental consanguinity, regions of homozygosity were searched for in NBR 1's WGS data (Subsection 2.1.5, page 75). A 22 Mb region of homozygosity was identified on the short arm of chromosome 12, which included *VWF* (Figure 4.15C, page 183). In order to determine the effect of c.8155+6T>A on the VWF transcript, platelets were isolated from a blood sample provided by NBR 1 as well as two female controls (C1 and C2) who were 41 and 53 years of age, respectively (Subsection 2.1.6., pages 75-76). Neither C1 or C2 had a diagnosis of VWD.

From these platelet samples, RNA was extracted, which was then reverse transcribed to cDNA. The relevant regions of *VWF* were amplified using PCR, and the products analysed using agarose gel electrophoresis (Subsection 2.2.1, page 76-79). The images taken using UV transillumination are shown in Figure 4.16A (page 184). As a positive control for VWF transcription, exon 36-37 was amplified because it was positioned 5' of exon 50. A ~ 400 bp product in NBR 1 could be detected, and was the same size as the product identified in C1 and C2. In order to determine whether exon 50 was spliced out of all *VWF* transcripts in NBR 1, primers were designed to amplify a region spanning exons 49 through to 52 (Table 2.1, page 78). This yielded a ~ 370 bp product in C1 and C2 (consistent with a distance of 335 bp between the two primers, and the two primers each being 24 bp in length). In contrast, the product generated in NBR 1 was ~ 330 bp in length. Therefore, the difference between the two products, 40 bp, is the known length of exon 50

Figure 4.15





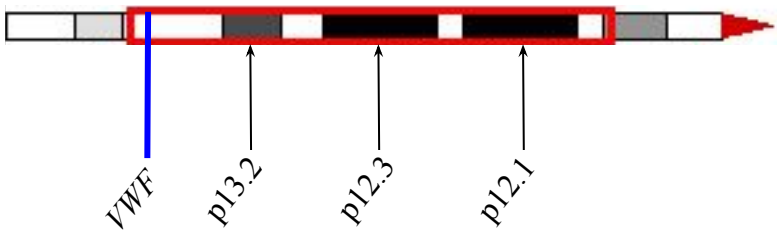


Figure 4.15 | The bleeding and family history of participant NBR 1. (A) The timeline of bleeding episodes in NBR 1: the red boxes provide details and the lines point to the approximate age at which these occurred. (B) The family tree of NBR 1. He is identified with an arrow. Males are represented by squares, females by circles. Filled shapes indicate individuals confirmed to have VWD. Unfilled shapes indicate individuals in whom VWD has not been diagnosed, unless there is a '?' inside the shape, in which case this is uncertain. On the left or right hand side of each shape there is a 'Y', 'N', or '?'. This indicates whether the individual has a bleeding history, no bleeding history, or this is not known. (C) The short arm of chromosome 12, showing in red one of the runs of homozygosity identified in NBR 1. The blue line is the position of *VWF*.

Figure 4.16

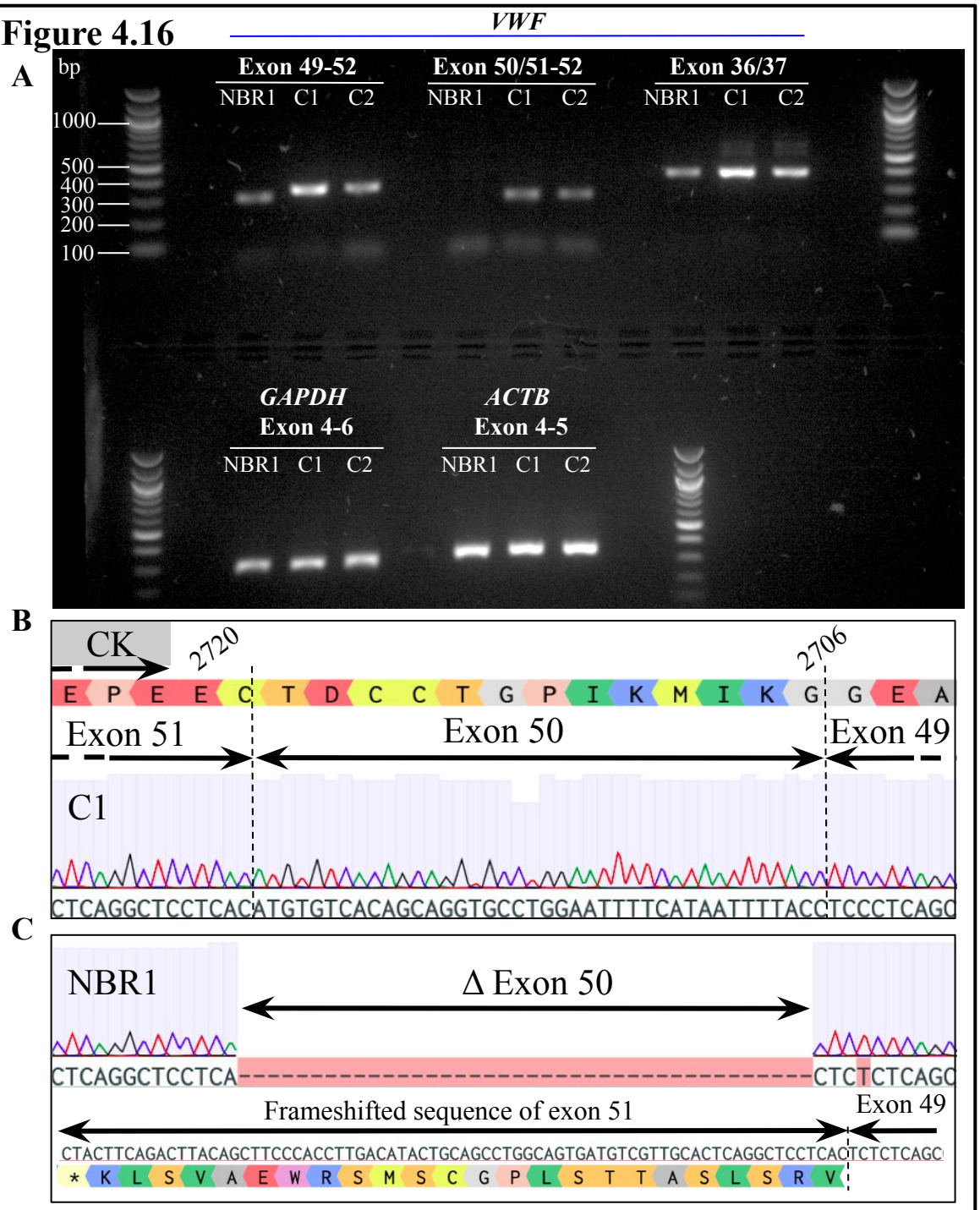


Figure 4.16 | Homozygosity for c.8155+6T>A results in the aberrant removal of exon 50. (A) The agarose gel used to resolve the presence and sizes of the PCR products generated using 5 different primer pairs on platelet cDNA obtained from NBR 1 and two control individuals (C1 and C2) (three biological samples; two independent experiments, the results of the second are shown). (B) and (C) show aligned Sanger sequences of a portion of the exon 49-52 product generated using the exon 52 primer, for C1 and NBR 1, respectively (two biological samples; one independent experiment; sequencing not repeated). NBR1 has a 40 nucleotide stretch which does not align to the reference. This corresponds to a deletion the length of exon 50. Consequently, after exon 49, the sequence is frameshifted. The predicted amino acid sequence (p.Gly2706ValfsTer25) is shown in (C) with the termination codon marked (*).

and would be consistent with it being skipped. Two further analyses provide orthogonal confirmation of this finding, as detailed below.

Firstly, PCR amplification was also carried out in which the same exon 52 reverse primer was used as for the aforementioned reaction. However, a different forward primer was used which was designed to span the exon 50/51 junction. In both C1 and C2, a 330 bp product was generated. In contrast, in NBR 1, no product was detected. This is consistent with the loss of the exon 50/51 junction due to exon 50 being aberrantly spliced out.

Secondly, the exon 49 - 52 product obtained from both NBR 1 and C1 were analysed by Sanger sequencing using the exon 52 primer. Figure 4.16B (page 184) shows the sequence spanning 63 base pairs across the end of exon 49, the whole of exon 50, and the start of exon 51. The sequencing results obtained from C1 were concordant with the reference sequence. In contrast, the sequence generated from NBR 1 shows the expected 40 bp deletion corresponding to exon 50. This results in a new junction being created between exons 49 and 51. As a result, the exon 51 sequence is out-of-frame and a PTC is created after 72 nucleotides (Figure 4.16C, page 184), corresponding to 24 amino acids not normally found in VWF. Therefore, the first AA alteration due to the frameshift is glycine to valine at position 2706, which would normally correspond to the first residue of exon 50. The corresponding HGVS term for the frameshift variant is p.Gly2706ValfsTer25. This is the same predicted consequence as reported for c.8155+3G>C (Corrales et al., 2011) and c.8155+6T>C (Platè et al., 2010), suggesting that p.Gly2706ValfsTer25 is the final common outcome of all seven of the known intron 50-51 DSS PAVVs (Figure 4.14, page 180). Therefore, the c.8155+6T>A transcripts generated from NBR 1 do not contain the sequence encoding the final 14 amino acids of the (74 amino acid) VWC6 domain and all of the 93 amino acids of the CK domain. As an aside, Figure 4.16C (page 184) shows that NBR 1 has a homozygous C>T transition in the first nucleotide of the last triplet codon of exon 49. This variant has an SAS MAF in gnomAD of 0.12 and is therefore not relevant to the phenotype observed in NBR 1.

4.5.2. Modelling the effect of c.8155+6T>A in HEK293T cells

To understand whether the transcripts arising from c.8155+6T>A (p.Gly2706ValfsTer25) are translated into VWF, an experimental approach using HEK293T cells was used. The pcDNA3.1-WT-VWF (WT) vector was used (Figure 4.17A, page 187; Subsection 2.2.2, pages 79-82), as it contains the cDNA sequence corresponding to the ORF of VWF (Zhang et al., 2019). Thirteen overlapping primers were designed (Table 2.4, page 82) to analyse the *VWF* sequence contained on pcDNA3.1-WT-VWF. Six SNVs (Gln852Arg, Thr1381Ala, Asp1472His, c.4641T>C, c.7239T>C, c.8241C>T) were identified. The last three are synonymous and therefore do not alter the protein sequence. The first five are all VWDbase variants which were classified as benign (Appendix 7.4, pages 256-304) and have gnomAD v3.0 MAFs between 0.08 and 0.21. Therefore, I deemed these variants not to be relevant for VWF function.

Site-directed mutagenesis was used to modify the pcDNA3.1-WT-VWF vector. In order to model p.Gly2706ValfsTer25 as closely as possible, mutagenic primers were used to substitute the first codon of exon 50 with a termination codon (Figure 4.17B, page 187; Subsection 2.2.2, pages 79-82), hereafter referred to as p.Gly2706Ter. This variant is not in VWDbase and I could not find it in a subsequent search of the literature. Furthermore, no known variants of any of the constituent nucleotides of the triplet codon encoding Gly2706 were found in gnomAD v3.0. Therefore, p.Gly2706Ter is not a naturally occurring variant. As site-directed mutagenesis can introduce nonspecific changes (Bachman, 2013), the whole of *VWF* ORF was re-sequenced in the mutant plasmid, and reassuringly the only difference with the WT plasmid was p.Gly2706Ter.

The WT and p.Gly2706Ter vectors were then transfected into HEK293T cells in order to compare intracellular expression of VWF (Subsection 2.2.3, pages 82-84). The efficiency of transfection was equivalent for both vectors (Figure 4.18A-C, page 188). Cell lysates harvested from the transfected cells were reduced and then analysed by immunoblotting with a polyclonal VWF antibody (Figure 4.18D, page 188). Some of the lysate material did not undergo any further processing steps before electrophoresis and blotting. These lysates are marked by ‘no digest’ above the corresponding wells on the blot.

Figure 4.17

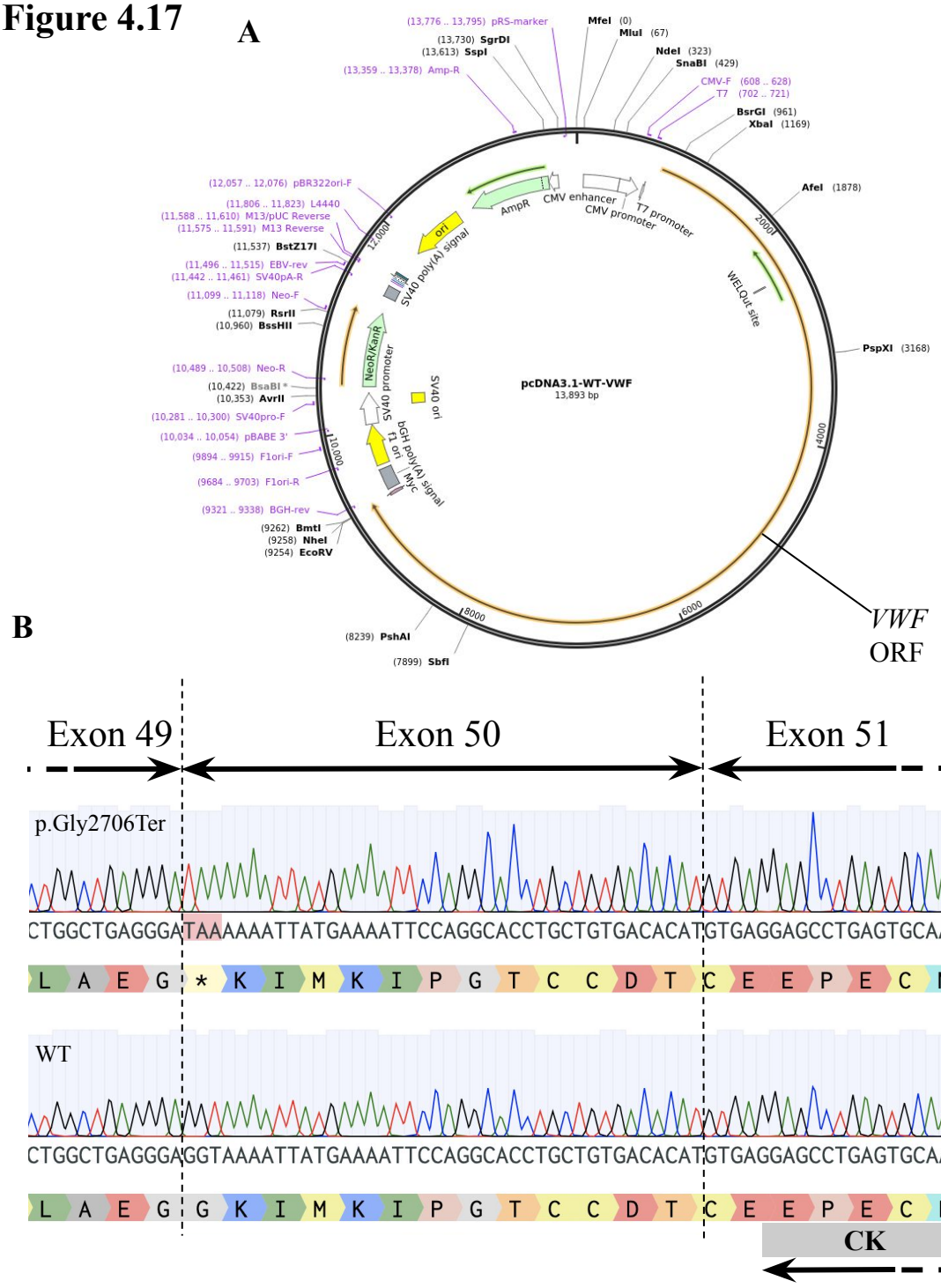


Figure 4.17 | Site-directed mutagenesis of the pcDNA3.1-WT-VWF plasmid. (A) The plasmid sequence map of pcDNA3.1-WT-VWF (reproduced from https://www.addgene.org/124794/). (B) Traces of Sanger sequencing of the pcDNA3.1-WT-VWF which underwent site-directed mutagenesis, p.Gly2706Ter (top) and the WT template (pcDNA3.1-WT-VWF) (two biological samples; one independent experiment; two aliquots of the same plasmid DNA of p.Gly2706Ter were sequenced, one of which is shown; one aliquot of WT plasmid DNA was sequenced). The nucleotides highlighted in pink are those which were mutated to a termination codon (*). CK = C-terminal cysteine knot.

Figure 4.18

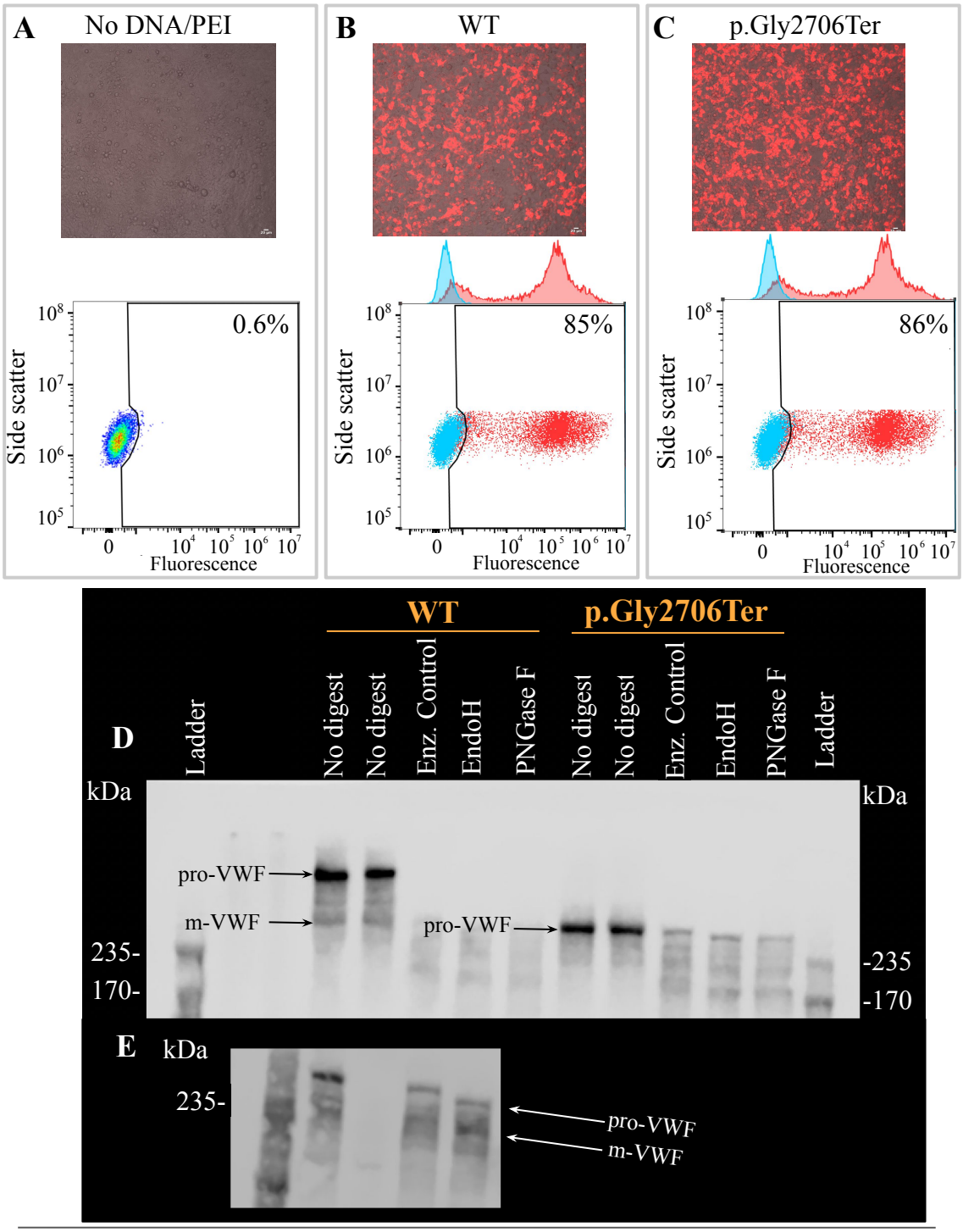


Figure 4.18 | Ascertaining the effect of p.Gly2706Ter in HEK293Ts. (A) - (C) show microscopy images and flow cytometry plots on the top and bottom rows respectively. Both WT (B) and p.Gly2706Ter (C) were co-transfected with the fluorescent vector ER-mcherry. "(D) & (E) show immunoblots of reduced protein extracted from lysates transfected with the WT and p.Gly2706Ter plasmids, first probed with rabbit polyclonal VWF antibody and then a fluorescent anti-rabbit IgG antibody for visualisation (two biological samples; one independent experiment [transfection, lysate extraction, enzyme digestion]; Western blot carried out twice for WT, once for p.Gly2706Ter - as explained in Subsection 4.5.2, page 190)."

The remaining lysate that was analysed was digested for 12 hours using the endoglycosidase enzymes (EndoH and PNGase lanes). The 'Control' lane represents a lysate treated in the same way but without the glycosidase. There are two main conclusions that can be drawn from the immunoblot.

First, the plasmid containing p.Gly2706Ter expressed a truncated form of VWF. The most fluorescent band visible in the two p.Gly2706Ter 'no digest' lanes has a molecular weight of ~ 250 kDa. This is in comparison to the principal band of the WT lysates which had a higher molecular weight.

Second, pro-VWF is the principal product of p.Gly2706Ter translation (where pro-VWF refers to full-length VWF comprising both the N-terminal propeptide and mature(m-) VWF [Figure 1.1A, page 17]). The reason for coming to this conclusion is that the ~250 kDa leading band in p.Gly2706Ter HEK293T cells migrated to the same position in the 'no digest' and 'control' lanes, but had increased electrophoretic mobility when treated with both EndoH and PNGase F (Figure 4.18D, page 188). This is consistent with sensitivity of the ~250 kDa band to the removal of glycan side chains by both EndoH and PNGase. As detailed in the introduction, VWF undergoes extensive post-translational N-glycosylation as it moves through the ER and Golgi (Subsection 1.1.4, page 23). In the medial Golgi the two outermost mannose saccharides are trimmed from pro-VWF prior to more complex forms being added (Wagner, 1990). Glycosylated proteins lacking these mannose saccharides cannot be cleaved by EndoH but can be by PNGase F (Freeze & Kranz, 2008). Subsequently, but prior to its release from the Golgi, pro-VWF is cleaved by furin into its constituent propeptide and m-VWF subunits (Rawley & Lillicrap, 2021). Hence the majority of pro-VWF is EndoH and PNGase sensitive but m-VWF is EndoH resistant but retains PNGase sensitivity (Carew et al., 1999; Lyons et al., 1992; Lentz & Sadler, 1993).

In contrast to p.Gly2706Ter, the 'no digest' WT VWF lanes contained both the principal band and a distinct, electrophoretically faster, band below it (Figure 4.18D, page 188).

This second band may represent m-VWF, as previous reports have demonstrated that reduced pro-VWF and m-VWF have molecular weights of 260 kDa and 220 kDa, respectively. This second band did not appear to be present in the 'no digest' conditions of p.Gly2706Ter. However, because the corresponding bands were unfortunately not visible on the WT glycosidase treated or enzyme control wells, this was repeated for the WT cells with EndoH. The results are shown in Figure 4.18E (page 188). Notwithstanding the tilt on the immunoblot, the largest and most fluorescent band was EndoH sensitive consistent with it representing pro-VWF. In addition, there did appear to be a less well-defined, Endo H-resistant band below it.

In summary, these results suggest that, in HEK293T cells, some WT VWF is able to move through the biosynthetic pathway and produce m-VWF, whereas in p.Gly2706Ter the majority of VWF is retained, as pro-VWF, prior to, or in the medial Golgi.

4.6. Conclusions

My analysis of the UK BAC addresses the important question of haemorrhagic risk in VWD from a different perspective than that used previously. This is because I defined VWD genetically, i.e. individuals heterozygous for a PAVV, rather than using the conventional definition based on phenotype (Table 1.2, page 40) employed in the development of BAT scores (Bowman et al., 2008; Bowman et al., 2009; Rodeghiero et al., 2005). This distinction is important because, in the future, increasing numbers of individuals will be identified with PAVVs. One reason for this is the increased use of DNA sequencing for perinatal diagnosis of VWD (Corrales, 2022; Swystun & James, 2017). Furthermore, WGS is increasingly being used for the diagnosis of rare disorders and the identification of driver mutations in cancer patients in the healthcare systems of developed nations (100,000 Genomes Project Pilot Investigators, 2021; Turro et al., 2021). This will inevitably result in an increased number of secondary findings with the consequent need for counselling of the individual tested regarding the significance of any unexpected findings (Gomez et al., 2019; Snape et al., 2019). The individual incidentally found to have

a PAVV may reasonably ask, “will this increase my chance of bleeding?” Based on the findings from the UK BAC, the short answer to this question is no.

Within the UK BAC, individuals heterozygous for a PAVV (genetically defined VWD) were categorised based on the MDT decision (Figure 3.5, page 117) regarding the PAVV that they carry. Those with accepted, undecided, or rejected PAVVs were placed in the gaVWD, guVWD, and grVWD groups, respectively (Figure 4.5, page 150). These groups were compared to the remaining 115,487 controls in the BAC who had no PAVV. The ICD-BAT score was devised as a means for assessing the presence or absence of 16 different types of bleeding episodes (Figure 4.2, page 145; Figure 4.3, page 146). The ICD-BAT score of the gaVWD, guVWD, and grVWD subgroups was no different to controls (Figure 4.6, page 153). Likewise, ~ 80% of participants in each of the genetically defined VWD subgroups were alive and free of bleeding at the end of the 23.5 year follow-up period over which hospital episode statistics were evaluated, no different to the control group (Figure 4.10A, page 162). What is surprising is that this still held true for the 234 participants with protein-altering PAVVs in the gaVWD group (Figure 4.10B, page 162), despite these variants being accepted by the MDT as autosomal dominant, pathogenic variants causal of VWD.

In summary, in the UKB BAC population, PAVVs did not have an effect on the total number of first bleeding episodes across 16 different ICD-BAT domains or on bleeding-free survival. But why is this the case? Although female sex, increasing age, and histo-group *OO* genotype all increased the ICD-BAT score (Table 4.2, page 155; Table 4.4, page 163), when these were controlled for by using propensity score matching (Figure 4.7, page 156) there was still no significant effect of PAVVs on the ICD-BAT score. This suggests that there are other potential explanations for the apparent phenotypic silence of PAVVs. Broadly, these explanations can be categorised into methodological, genetic, and environmental factors. These are now considered in turn.

By focussing on hospital-defined bleeding episodes, only the most severe types of bleeding were captured. Six categories of bleeding symptoms - bleeding after minor

wounds, surgical bleeding, cutaneous bleeding, menorrhagia, epistaxis, and bleeding after tooth extraction - are reported to be significantly associated with VWD (Tosetto et al., 2006). Aside from surgical bleeding, it would seem likely that the other five symptoms are principally managed on an outpatient basis. In support of this, only 18% of cases of epistaxis were managed as inpatients in a US case series (Purkey et al., 2014). Therefore, it is plausible that having genetically defined VWD does affect bleeding, but is being managed without the need of, or access to, medical inpatient care. An alternative explanation is that bleeding episodes are being treated in hospital outpatient clinics or in general practice, and therefore not captured by the UKB HES data (UKB, 2020b). The other limitation of the methodology I used to capture bleeding events was that only the first inpatient episode corresponding to a given bleeding domain was recorded (Figure 4.3, page 146). This means that if genetically defined VWD influences the cumulative occurrence of, for example, epistaxis, then this would not have been detected by the ICD-BAT or the Kaplan-Meier analyses.

There are 17 quantitative trait loci for VWF:Ag other than *VWF* and *ABO* (Table 1.4, page 52); cumulatively they may be exerting a stronger influence on plasma VWF levels than PAVVs. Furthermore, variants in other genes encoding proteins integral to haemostasis can exert an effect on their quantity and function (Goodeve et al., 2012). Medical comorbidities such as hypertension and diabetes increase VWF levels (Atiq et al., 2018; Conlan et al., 1993). It is estimated that 9% of the UK population over the age of 35 are taking an antiplatelet medication (Scholes & Mindell, 2018) and 2% of the US population are prescribed an anticoagulant (McBane, 2015). Aspirin and warfarin remain the most commonly prescribed antiplatelet and anticoagulant medications used in the UK (Afzal et al., 2021; Wilcock, 2015); individuals who take them have annualised bleeding rates of 1-4% (Hansen et al., 2010; Lamotte et al., 2006; Simes et al., 2014) and 2-18% (Connolly et al., 2009; Friberg et al., 2012; Patel et al., 2011), respectively. Therefore, it is apparent that the effect of PAVVs in heterozygosity, even those accepted as pathogenic variants by the MDT, do not exert an effect on inpatient bleeding episodes that can be detected above the compound effect of a multitude of other variables, both acquired and genetic, that are contributing to overall haemorrhagic risk.

Having looked at the collective effects of PAVVs in UKB I then shifted attention to the use of VWDbase in the analysis of WGS of individuals with hitherto molecularly unexplained VWD but a strong phenotype. These 10 individuals had been recruited to the NBR-RD study and prior analysis had identified causal VWD variants for two of them and possible candidates for another three (Turro et al., 2020). I identified homozygous donor splice site (DSS) variants, c.8155+6T>A and c.5455+2T>C, as the molecular explanation for two individuals, NBR 1 and NBR 5, who have a diagnosis of VWD3 (Table 4.6, page 169; Table 4.8, page 175). Both variants are PAVVs in VWDbase. Why these variants were not detected in the original NBR-RD analysis (Turro et al., 2020) of the WGS data is unclear. Both variants fit two of the key criteria for inclusion in MDT assessment: MAF < 1/1,000 and a VEP consequence of "splice_region_variant" [McLaren et al., 2016]). However, this discrepancy does illustrate the advantage of analysing *VWF* with a specific focus on VWD, taking into account, for example, the need to identify biallelic pLoF variants for individuals with VWD3. Out of the four causal VWD variants identified (highlighted in green in Table 4.8, page 175), all were PAVVs in VWDbase. This suggests that the majority of SNVs and indels potentially causal of VWD are now known - indeed only five novel discoveries of new variants linked to VWD have been published in the past year (Casonato et al., 2022; Fels et al., 2022; Okamoto et al., 2022; Pagliari et al., 2022; Yadegari et al., 2022; Zhang et al., 2021). Therefore, priority should be given to understanding the mechanism of PAVVs shown to be causal of VWD but for which the mechanism is not known, such as c.8155+6T>A which was identified in NBR 1.

The analysis of platelet cDNA from NBR 1 demonstrated that c.8155+6T>A results in the skipping of exon 50 and a frameshift variant that results in a premature termination codon, p.Gly2706ValfsTer25. It has been shown that, in general, PTCs occurring near the 3' end of a transcript do not undergo NMD (Brogna et al., 2016). The nearest coding nucleotide to the c.8155+6T>A variant, c.8155T, is only 287 nucleotides from the final coding nucleotide of the *VWF* transcript (c.8442A). Put another way, 97% of the transcript occurs before c.8155T. Therefore, it seemed likely that c.8155+6T>A would not trigger NMD supported by data from the RNA sequencing of another intron 50-51 variant,

c.8155+3G>C (Borràs et al., 2019). A plasmid containing the ORF of WT VWF was altered by site-directed mutagenesis to contain a termination codon at Gly2706 in order to model as closely as possible p.Gly2706ValfsTer25. HEK293Ts transfected with the p.Gly2706Ter plasmid expressed a truncated VWF protein, further suggesting that NMD is not activated. However, this possibility is not fully excluded, because transcripts of other genes bearing PTCs have been shown to undergo NMD escape in HEK293Ts (Sato & Singer, 2021). Although the protein itself was not sequenced, the truncated protein that resulted from p.Gly2706Ter would be expected to completely lack the C-terminal CK domain which would also be the case for p.Gly2706ValfsTer25. This domain contains three cysteine residues important for the dimerisation of VWF prior to its exit from ER (Katsumi et al., 2000) (Subsection 1.1.5, page 23). Chemical inhibition of VWF dimerisation at the CK in ECs and COS-1 cells transfected with a vector expressing p.Arg2663Ter both showed that reduced VWF was only present in its pro-VWF form (Voorberg et al., 1991; Wagner et al., 1986). This is in keeping with my findings that HEK293T cells transfected with the p.Gly2706Ter vector only contained pro-VWF whereas in those expressing the WT vector, some VWF did progress to the cleaved form, m-VWF (Figure 4.18, page 188), which is the constituent of WPBs. The relevance of these findings to p.Gly2706ValfsTer25 VWF expressed in endothelial cells is covered in the next chapter.

5. Results: the effect of c.8155+6T>A in endothelial cells

5.1. Background

The HEK293T experiments suggested that that there was a maturation block in the VWF biosynthetic pathway as a result of creating a VWF protein truncated before the C-terminal cysteine knot (CK) through the replacement of the first codon of exon 50 with a termination codon (Figure 4.17B, page 187; Figure 4.18 D&E, page 188). However, as previously outlined (Subsection 1.2.6, page 62) there are limitations of HEK293T models of VWD, two of which are relevant to ascertaining the effect of c.8155+6T>A. First, because HEK293T cells do not normally transcribe VWF, delivery of the open reading frame (ORF) of VWF relies on a plasmid vector (Michaux et al., 2003). In this system the effect of non-coding variants cannot be modelled. Second, in the HEK293T plasmid constructs I used, the ORFs of both WT and p.Gly2706Ter VWF are controlled by a strong CMV promoter (Figure 4.17A, page 187). This is likely to result in VWF overexpression (de Boer & Eikenboom, 2019), which is fine for the assessment of post-translational differences, but not suitable for determining whether a variant affects whether VWF is expressed or not in its endogenous context. Therefore to overcome these limitations I used NBR 1-derived endothelial colony forming cells (ECFCs). As outlined in the introduction (Subsection 1.2.6, pages 63-64) these cells are derivatives of a progenitor population that circulates in the bloodstream which can be expanded ex vivo (Melero-Martin, 2022; Paschalaki & Randi, 2018). When derived from patients with VWD they provide a useful cellular endophenotype model for analysing the effect of *VWF* variants on the intracellular handling and release of VWF in ECs (Starke et al., 2013; Wang et al., 2013b). As detailed in Section 5.3 of this chapter, the ECFC approach confirmed that VWF produced in NBR 1 ECFCs (predicted to be p.Gly2706ValfsTer25[Figure 4.16, page 184]) did not progress through the biosynthetic pathway into Weibel-Palade Bodies, and compared to WT, had an increased number of cells in which no VWF was detected at all. Given that p.Gly2706ValfsTer25 is the anticipated consequence of all eight intron 50-51 donor splice site variants (Figure 4.14, page 180), these findings have wider pathomechanistic implications beyond c.8155+6T>A, especially as the intracellular phenotype of these

variants has not previously been evaluated (Ahmad et al., 2019; Borras et al., 2019; Corrales et al., 2009; Corrales et al., 2011; James et al., 2007; Liang et al., 2017; Harrington et al., Mertes et al., 1994; Plate et al., 2010; Solimando et al. 2012; Yadegari et al., 2012). As has previously been suggested (Harrington et al., 2018), it is likely that the effect of this variant is mediated by the absence of the CK because of the role of this domain in coupling VWF monomers together as dimers through disulfide bonding in the ER (Marti et al., 1987). Therefore in order to develop insight into this process I wanted to derive genetically engineered endothelial cells in which VWF was truncated just before CK (the start of this domain is the third amino acid encoded by exon 51 [Figure 4.16B, page 184]).

Although CRISPR/Cas9 has been used to successfully knockout (KO) VWF in cord blood-derived ECFCs (Schillemans et al., 2019), this approach is limited by the loss of proliferative ability of ECFCs, as I also corroborated in my experiments (Subsection 5.4, page 209). There are no published studies attempting to genetically modify VWF in other primary ECs. Likely reasons for this include the difficulty in transfecting these cells (Abrahimi et al., 2015) and the reduction of VWF producing-capacity with successive passages (Howell et al., 2004). Passaging also erodes the expression of other markers of endothelial identity in a process termed endothelial-to-mesenchymal transition (Fleenor et al., 2012; Piera-Velazquez & Jimenez, 2019). This may explain why investigators wanting to abrogate the function of VWF have relied instead on knockdown using short interference RNAs (Dushpanova et al., 2016), but this is not a suitable method for editing VWF to synthesise a truncated product. Therefore, I was motivated to develop a different approach for creating ECs truncated prior to CK (hereafter referred to as ΔCK) and turned to human induced pluripotent stem cells (hiPSCs).

In late 2006, two groups simultaneously demonstrated that human dermal fibroblasts (HDFs) could be reprogrammed into a new cellular state that was able to differentiate into the three essential germ cell layers (endoderm, mesoderm, ectoderm). These cells were therefore given the name, human *induced* pluripotent stem cells because of the use of lentiviral transduction of the ORFs of four transcription factors that forced a new,

pluripotent identity upon the HDFs (Takahashi et al., 2007; Yu et al., 2007). Two transcription factors, *POU5F1* (better known as *OCT-4*) and *SOX2* were common to both protocols but *KLF4* and *MYC* were the additional factors used by Takahashi et al. and *NANOG* and *LIN28A* employed by Yu and colleagues. Between 20 and 30 days after viral transduction, cells with a high nuclear:cytoplasmic ratio and the ability to form tight colonies emerged, akin to human embryonic stem cells (hESCs). These cells had an RNA expression profile more similar to hESCs than their somatic cell derivatives and, like hESCs, also demonstrated the ability to form a teratoma when the cells were injected into mice with severe combined immunodeficiency (SCID), thereby confirming their pluripotency. It has been subsequently demonstrated that a wide variety of somatic cells can be used as the starting material for hiPSC-generation, including peripheral blood CD34+ cells (Loh et al., 2009). Some of the limitations of hiPSCs include the low efficiency of reprogramming (Raab et al., 2014), the consequences of retained epigenetic memory from the parental cell type (Bar-Nur et al., 2011), and the acquisition of somatic mutations during transduction and subsequent cell culture (Pera, 2011). Notwithstanding these challenges, the capacity of hiPSCs to be genome-edited, derived from individuals with inherited disorders (Soldner and Jaenisch, 2012), indefinitely divide (Freiermuth et al., 2018), and differentiate to functional cells (Grandy et al., 2019), means that they are now at the forefront of in vitro approaches to model human inherited diseases (Pamies et al., 2017). Methods to differentiate hiPSC to ECs (iECs) were described shortly after hiPSCs were first described. The techniques used to make iECs can be categorised into three groups as summarised in Table 5.1 (page 198).

The derivation of iECs was first reported by Choi et al., 2009 and based on methods they previously used to differentiate hESCs. Using fibroblast-derived hiPSC lines, they employed a feeder co-culture system to induce differentiation to haematopoietic and endothelial cell subsets in eight days in the absence of cytokines. However methods relying on feeder-cells are limited by poor differentiation efficiency to iECs of approximately 3% (Choi et al., 2009; Taura et al., 2009).

Culture method	First described by	Advantages	Disadvantages
Co-culture	Choi et al., 2009	No cytokines needed	Non-human cells; low differentiation efficiency
Embryoid body	Lee et al., 2009	Common haemato-endothelial precursors generated which can be used to produce multiple different cell types	Difficult manual dissection technique; low differentiation efficiency; heterogeneous definitions of embryoid body; lengthy process
Monolayer	Azhdari et al., 2013	Easier standardisation of approach	Less useful if multiple mature cell types required

Table 5.1 | Methods for generating iECs. Categorisation based on the schema from Williams & Wu, 2019.

The embryoid body method involves the initial generation of a spheroid mass in culture that contains all three germ layers. The general approach is to place the hiPSCs in suspension culture with either serum (Li et al., 2011) and/or a differentiation media (Adams et al., 2013). The differentiating hiPSCs pass through one or two intermediate stages after which embryoid bodies emerge, which are then manually dissected and re-cultured in EC-specifying conditions (Lee et al., 2009; Li et al., 2011; Adams et al., 2013). Embryoid body-based protocols take two weeks to obtain mature ECs, and, like feeder-cell methods, require positive selection of differentiated cells expressing a surface EC antigen. Major downsides include the lack of a standardised definition of an embryoid body (Bratt-Leal et al., 2009), the technical challenges of micro-dissection, and low differentiation efficiency of 1-5% (Lin et al., 2017; Patsch et al., 2015). This limits reproducibility and scalability, which are prerequisites for the generation of larger numbers of iECs for detailed functional analysis.

Monolayer-based methods for deriving iECs have been recently developed. This technique involves culturing hiPSCs in media on a basement membrane in the presence of cytokines to induce mesoderm and then EC specification. Drawbacks of the early versions of these protocols include the use of serum (Orlova et al., 2014) and “small clusters” of hiPSCs as the starting material (Azhdari et al., 2013) making the process difficult to standardise. The Cowan group was the first to develop a monolayer-based protocol to derive mature iECs in serum-free, chemically defined conditions (Patsch et al, 2015). The purified cells which are derived using this approach have a transcriptomic and metabolomic signature that strongly correlates with primary ECs. Subsequently, other groups have published

serum-free, monolayer-based methods with similar efficiency using different hiPSC cell lines, reagents and timescales (Harding et al., 2017; Liu et al., 2016) confirming the general reproducibility of this approach.

Inherited vascular and bleeding disorders including pulmonary artery hypertension (Sa et al., 2017) and haemophilia A (Son et al., 2022) have been modelled using iECs. In this chapter I demonstrate a new model for VWD using hiPSC-derived iECs. First I developed and validated a robust protocol for iECs with a focus on VWF synthesis and release. Second I then applied CRISPR/Cas9 (as introduced in Subsection 1.2.6, pages 63-64) to create both ΔCK and KO iECs. Finally I show the utility of this model by elucidating the pathomechanism of c.8155 + 6T>A, but also more broadly on the impact of *VWF* variants that lead to the removal or disruption of CK.

5.2. Aims

1) Evaluate the effect that c.8155+6T>A has on intracellular VWF localisation in patient-derived ECFCs.

2) Develop a new in vitro approach for modelling VWD variants using hiPSC-derived endothelial cells, or iECs in short.

3) Use the approach developed in 2) in order to examine the effect of the removal of the CK from VWF using CRISPR/Cas9 on iECs.

5.3. NBR 1-derived endothelial colony forming cells

5.3.1. Homozygous c.8155+6T>A results in the perinuclear retention of VWF

Blood samples were collected from NBR 1 and three control volunteers (C3, C4, and C5) and mononuclear cells were isolated by density gradient centrifugation. These were then seeded onto flasks containing a culture medium and substrate shown to promote the proliferation of ECFCs (Ormiston et al., 2015) (Subsection 2.2.4, pages 84-88). I used

more than one control to safeguard against the potential failure to derive ECFCs, as it has been estimated that this occurs in about one quarter of individuals (Toupance et al., 2021). The controls were all deliberately selected to be matched for age with NBR 1, as there is uncertainty about whether the blood concentration of ECFCs declines with advancing age (Melero-Martin, 2022). None of the controls reported a history of VWD or any other bleeding disorder. Their demographics are shown in Table 5.1 (page 198). Plasma VWF:Ag measurements were made on NBR 1 and all three controls at the same laboratory using an automated analyser (Subsection 2.1.6, pages 75-76). This confirmed that NBR 1 had no detectable VWF:Ag (< 0.02 IU/ml). All three controls had normal VWF:Ag levels (Table 5.2, below).

ID	Sex	Age	Medical history	Medication	VWF:Ag (IU/ml)
C3	Male	29	None	None	1.35
C4	Male	24	Psoriasis	None	1.41
C5	Female	34	None	Progestogen-only pill	1.22

Table 5.2 | Control participants from which ECFCs were derived. Plasma VWF:Ag measurements were made on plasma derived from the blood samples taken at the same time as those used for ECFC derivation. Displayed are the results of three biological samples; blood was taken once from each individual; VWF:Ag measurements were made once.

Colonies of ECFCs were visible using a light microscope from D20 onwards (Figure 5.1, page 201), and, when confluent they had a cobblestone appearance consistent with previous reports (de Jong et al., 2019; Yadegari et al., 2022). The timing of colony emergence compared favourably with other groups who report that this normally occurs between D15 (Ingram et al., 2004) and D21 (Smadja et al., 2019). ECFCs from C3, C4, and two colonies from NBR 1 were then analysed by flow cytometry and immunofluorescence confocal microscopy at D36 and 40 respectively. Two colonies from NBR 1 were selected because of the known differences in VWF expression that can occur between colonies from the same donor (de Jong et al., 2019). Colonies from C5 were not used for these assays as an insufficient number of cells were available at these time points.

Figure 5.1

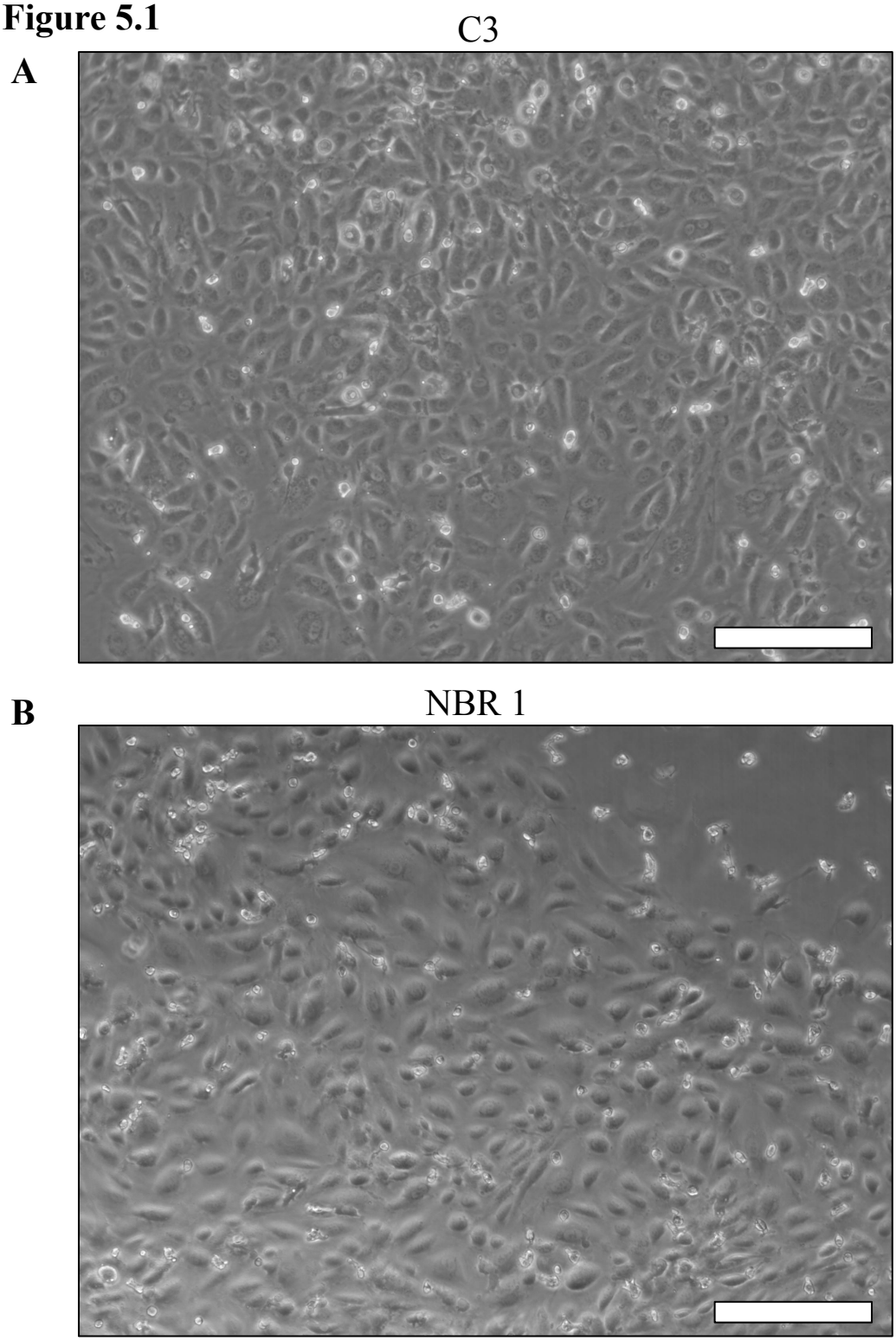


Figure 5.1 | Endothelial cell colony forming cells. Light microscopy images taken with a x 10 objective of monolayers of ECFCs grown on T25 flasks, 20 days after initial seeding of peripheral blood mononuclear cells. (A) and (B) represent colonies from C3 and NBR 1, respectively. The scale bar in both images represents 200µm. Three biological samples were analysed (C3, C4, NBR 1); the experiment to derive ECFCs was carried out once; photographs on D20 post seeding were taken once and only images of C3 and NBR 1 are shown.

Figure 5.2

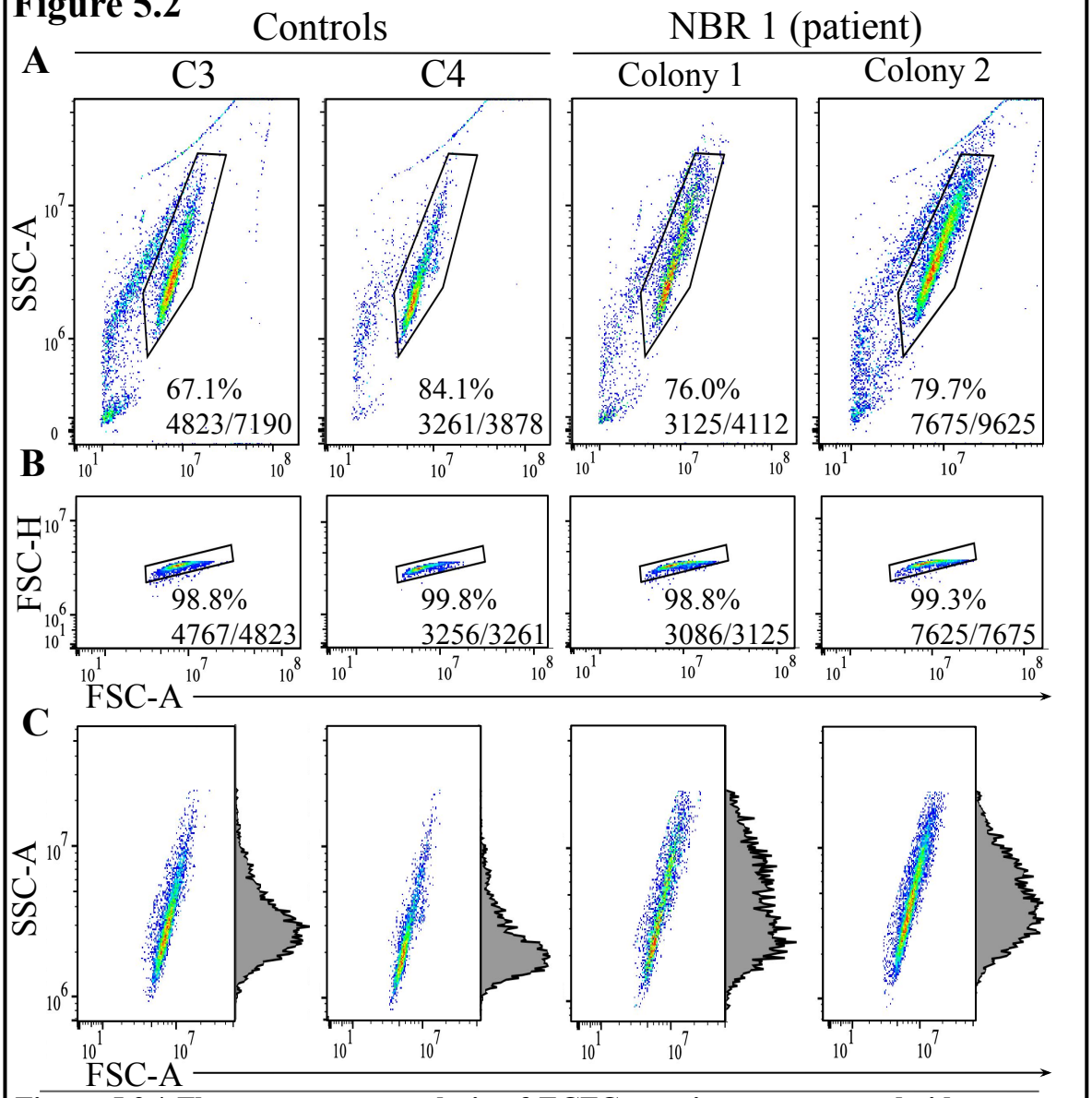


Figure 5.2 | Flow cytometry analysis of ECFCs: gating strategy and side scatter light comparison. The first two columns left to right are C3, C4 (control participants), and last two columns are two different colonies from NBR 1. Pseudo colour dot plots of events detected on the flow cytometer, such that blue represents the lowest event density, red the highest, and green is intermediate. Both x- and y-axes are in biexponential format. (A) Side scatter area (SSC-A) as a function of forward scatter area (FSC-A). The cells gate is represented by the polygon with black outline. (B) Forward scatter height (FSC-H) as a function of FSC-A of the events in the cells gate. The polygon here represents the singlets gate. In both (A) and (B), the number of events in each gate is shown as the numerator over the denominator (total in events shown), with the percentage indicated above. (C) SSC-A as function of FSC-A for events in the singlets gate with marginal plots showing density distributions. Three biological samples were analysed (C3, C4, NBR 1); colony 1 and 2 represent technical replicates from NBR 1 (colonies selected from different flasks); the experiment was carried out once. Flow cytometry measurements on each sample were made once. This applies to all other figures and tables displaying flow cytometry data from this experiment (Figure 5.3, page 204; Figure 5.4, page 205; Table 5.3, page 203; Table 5.4, page 209).

ECFCs were evaluated by multicolour flow cytometry using a panel comprising fluorophore-conjugated antibodies to CDH5, PECAM1, CD45, and CD14 (Subsection 2.2.4, pages 86-87). When compared to ECFCs incubated with isotype control antibodies, the majority of ECFCs from both the control participants (C3 and C4) and NBR 1 co-expressed the EC surface proteins PECAM1 and CDH5 (Figure 5.3, page 204; Table 5.3, below). Additionally ≥ 96% of all the ECFCs were double negative for the haematopoietic cell surface proteins, CD14 and CD45 (Figure 5.4, page 205; Table 5.3, below).

Antibody	Controls			
	C3	C4	NBR 1 Colony	NBR 1 Colony 2
PECAM1-APC	126318	52328	140009	129520
CDH5-FITC	76410	63679	71127	48883
CD45-AF405	7832	5818	7825	11830
CD14-AF700	-11690	-5340	-10475	-11357

Table 5.3 | Median fluorescence intensities (MFI) of the antibodies used to evaluate ECFCs. The values shown are those based on each of the four channels used to detect the fluorescence signal from one of four fluorophore-conjugated antibodies. The MFIs were calculated after correcting for fluorescent overspill using compensation beads.

The cell surface marker expression was consistent with that expected for ECFCs based on an international position paper (Smadja et al., 2019). The absence of CD14 and CD45 meant, reassuringly, that the ECFCs I derived were not myeloid angiogenic cells, which are considered to be of myelomonocytic rather than endothelial origin (Medina et al., 2010) but are also known to result from the ex vivo expansion of PBMCs in culture.

To explore intracellular VWF localisation, fixed ECFCs were imaged on a confocal microscope (Subsection 2.2.4, pages 87-88). The majority of ECFCs from both C3 and C4 control participants contained WPBs, most of which were round with occasional rod-shaped ones. The WPBs extended from a position close to the cell nucleus to the periphery of the cell (Figure 5.5, page 206). The pattern seen in WT ECFCs resembles that of other investigators (Berber et al., 2009; Selvam et al., 2017). In addition there were

Figure 5.3

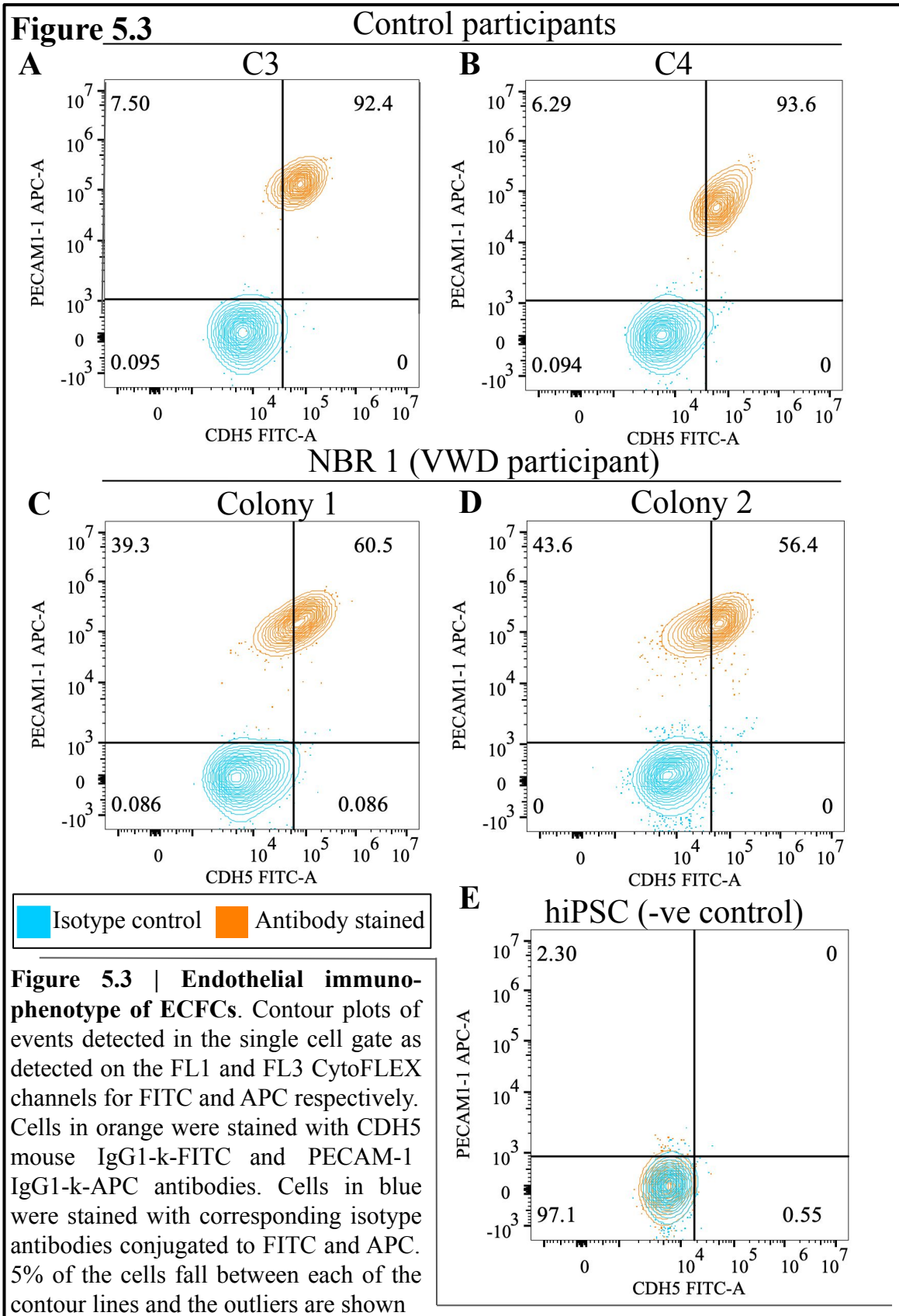


Figure 5.3 | Endothelial immuno-phenotype of ECFCs. Contour plots of events detected in the single cell gate as detected on the FL1 and FL3 CytoFLEX channels for FITC and APC respectively. Cells in orange were stained with CDH5 mouse IgG1-k-FITC and PECAM-1 IgG1-k-APC antibodies. Cells in blue were stained with corresponding isotype antibodies conjugated to FITC and APC. 5% of the cells fall between each of the contour lines and the outliers are shown as dots outside of these. The quadrant gate was set so that the horizontal and vertical lines touch the second outermost ring of the isotype control. The numbers in each quadrant represent the the percentage of antibody stained cells that fall within the gate. Human induced pluripotent stem cells (hiPSCs) were used as a negative control (E).

Figure 5.4

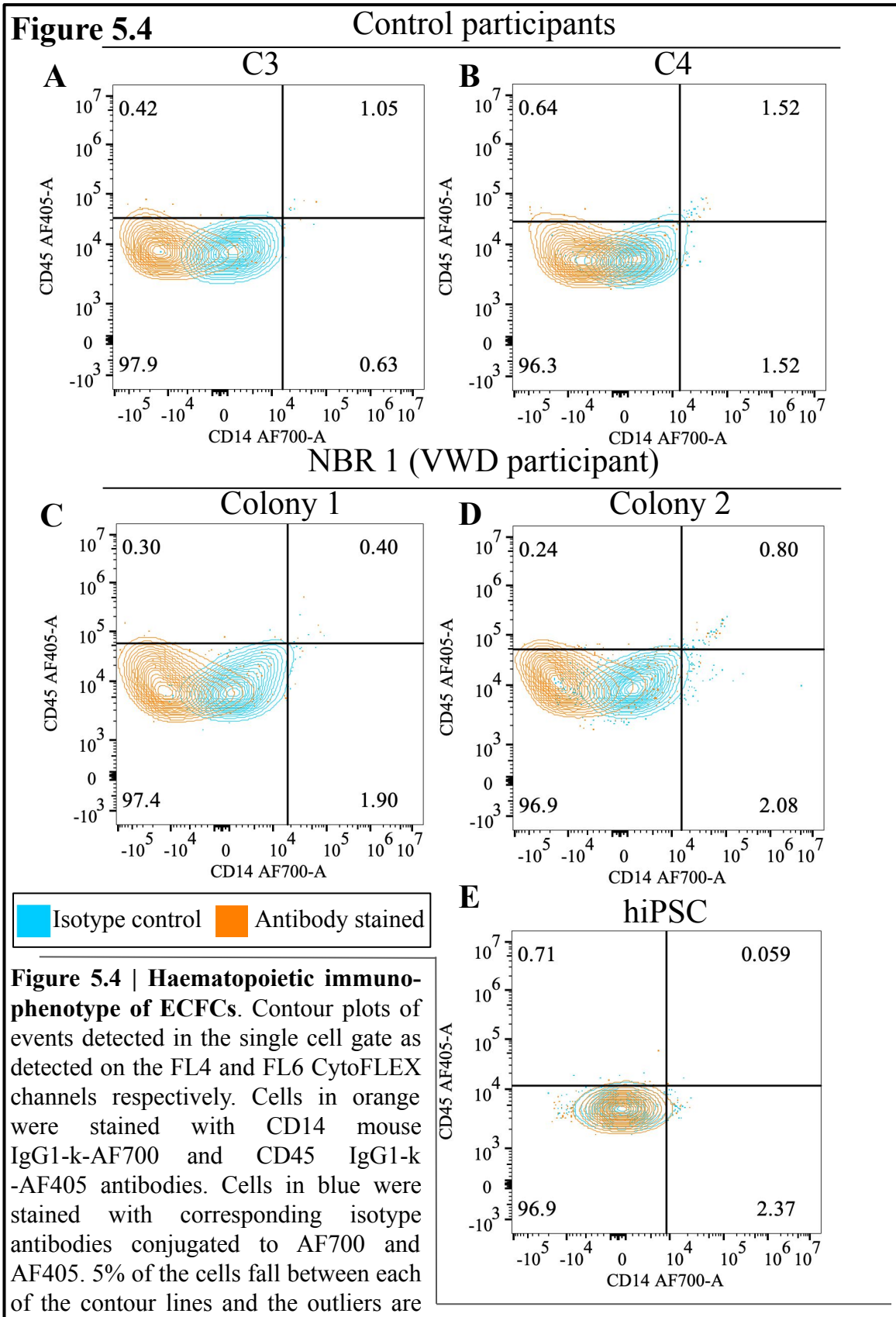


Figure 5.4 | Haematopoietic immunophenotype of ECFCs. Contour plots of events detected in the single cell gate as detected on the FL4 and FL6 CytoFLEX channels respectively. Cells in orange were stained with CD14 mouse IgG1-k-AF700 and CD45 IgG1-k-AF405 antibodies. Cells in blue were stained with corresponding isotype antibodies conjugated to AF700 and AF405. 5% of the cells fall between each of the contour lines and the outliers are the dots outside of these. The quadrant gate was set so that the horizontal and vertical lines touch the second outermost ring of the isotype control. The numbers in each quadrant represent the the percentage of antibody stained cells that fall within the gate. Human induced pluripotent stem cells (hiPSCs) were used as a negative control (E).

Figure 5.5

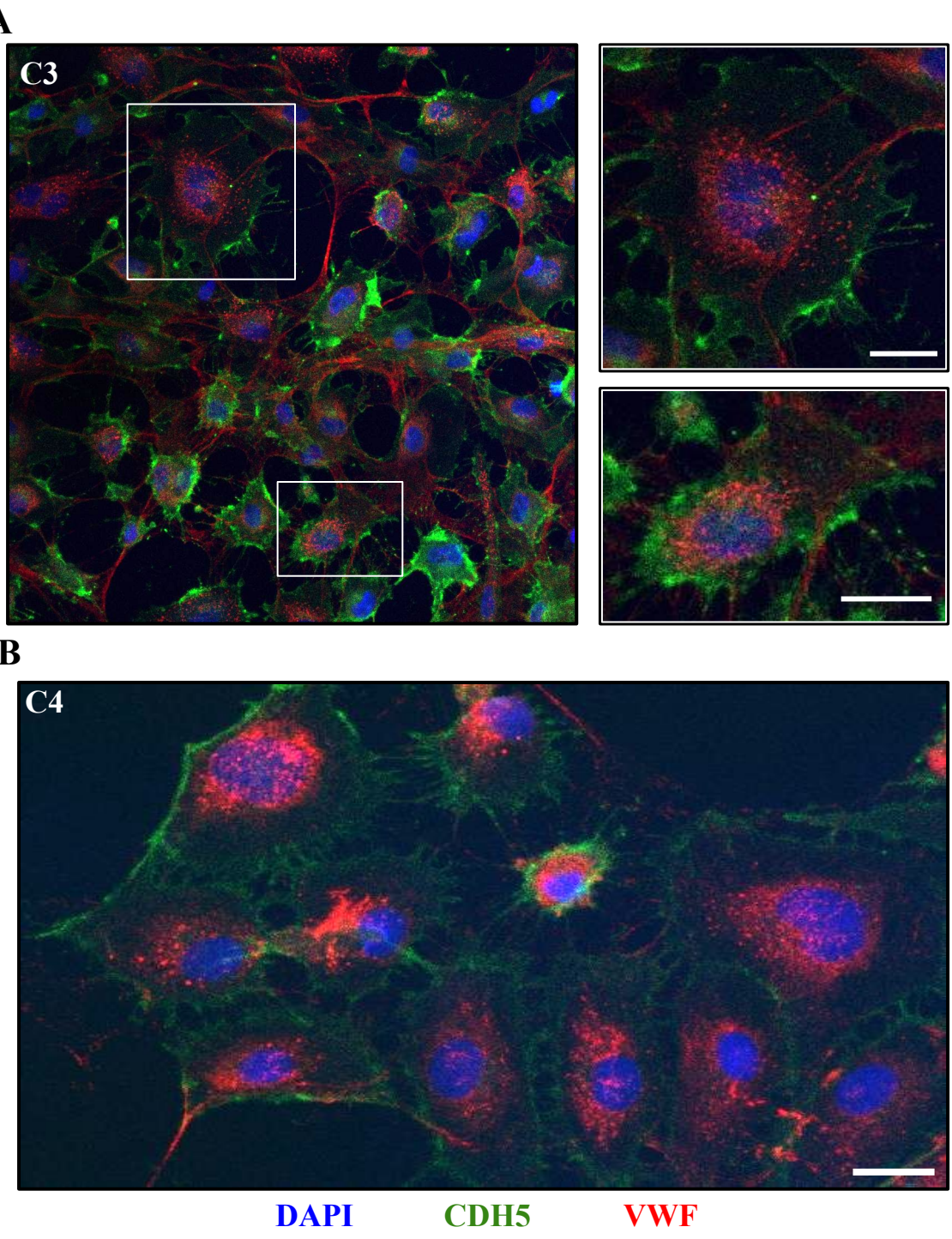


Figure 5.5 | ECFCs from control volunteers. Composite confocal images of ECFCs derived from the PBMCs from C3 (top panels) and C4 (bottom panel), both of whom had normal VWF:Ag levels. For C3 the right hand panels represent zoomed in projections of the insets. The nucleus was stained with DAPI (blue) and antibodies were used to probe for VWF (red) and CDH5 (green). The scale bar in all images represents 20μm. Both this figure and Figure 5.6 represent the analysis of three biological samples (C3, C4, NBR 1 colony 1); the experiment was carried out once; three confocal images were taken from non-overlapping positions for C3 (of which one is shown), one from C4 (as shown), and three from NBR 1 (two of which are shown).

long, extracellular strands of VWF consistent with strings of multimers of VWF following release from the ECFCs, consistent with the observations of other investigators under basal conditions (de Jong et al., 2019). In contrast, there were minimal WPBs and no VWF strings visible in ECFCs derived from NBR 1 (Figure 5.6, page 208). Instead, these ECFCs contained either no VWF, or in the cells in which it was detected, VWF was located as a circumferential halo around the nucleus. The confocal images suggest that in NBR 1 ECFCs, VWF is retained in an intracellular position consistent with the ER, there is a severe reduction of packaging of VWF into WPBs, and there is no evidence that it is secreted, consistent with the VWF:Ag measurements made on the patient's plasma. However, the lengthy set up and scan time on the confocal microscope and the effect of photobleaching with repeated laser scanning limited the number of ECFCs analysed to 54 from the control participants and 100 from NBR 1.

Therefore in order to gauge the effect of biallelic c.8155+6T>A mutation on VWF distribution in a greater number of ECFCs I went back to the flow cytometry data. I focussed on the side scatter light area (SSC-A) parameter. This is the area of the light pulse signal detected by the flow cytometer photomultiplier tube at right angles to the cell being illuminated (Givan, 2011). SSC-A is a measure of the internal complexity of a cell and is affected by the size and composition of organelles (Salzman, 1999; Marina et al., 2012). Therefore I hypothesised that the perinuclear retention of VWF in ECFCs and the apparent lack of WPBs may affect SSC-A. Indeed, HUVECs treated with monosodium urate release VWF from WPBs and have reduced side scatter compared with untreated cells (Kuo et al., 2008). The effect of VWF retention within the ER on SSC-A has not previously been reported. However, a different inherited disorder, alpha-1-antitrypsin (A1AT) deficiency, is characterised by ER accumulation of the 'Z' mutant of A1AT (Belorgey et al., 2007). Hepatocytes from mice homozygous for the Z mutant have an increased side scatter compared to WT counterparts, thought to be secondary to the birefringent properties of ER-retained Z A1AT (Lindblad et al., 2007). The dot plots in Figure 5.2C (page 202) display SSC-A as a function of forward scatter area (FSC-A) for the events collected in the single cell gate (Figure 5.2B). The marginal histograms show that there was a broader distribution of SSC-A measurements for NBR 1 colonies 1 and 2 than for the control

Figure 5.6

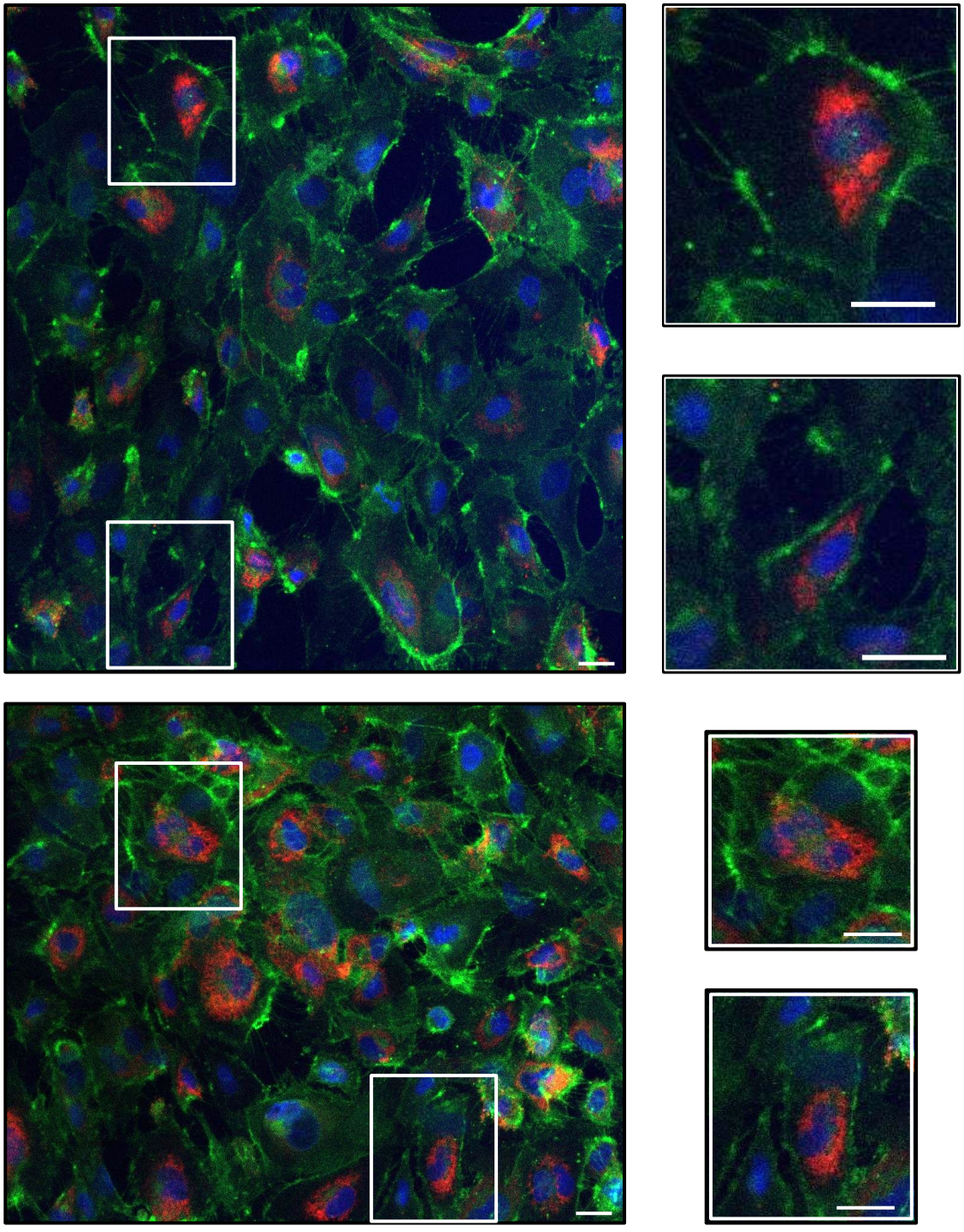


Figure 5.6 | ECFCs from NBR 1 colony 1. Composite confocal images of ECFCs derived from the PBMCs from NBR 1. The right hand panels represent zoomed in projections of the insets. The nucleus was stained with DAPI (blue) and antibodies were used to probe for VWF (red) and CDH5 (green). The scale bar in all images represents 20μm. The brightness settings used to display the images are the same as for Figure 5.5.

participants. The median SSC-A measurements were higher in the NBR 1 ECFCs than controls (Table 5.4, below). These results suggest that perinuclear retention of VWF in NBR 1 ECFCs increases SSC-A and that this effect counterbalances any effect that may result from the depletion of WPBs.

	Controls		NBR 1	
	C3	C4	Colony (Col) 1	Colony (Col) 2
Number of single cells	4767	3256	3086	7625
Median SSC-A	2971080	2147535	4129715	4437220

Table 5.4 | Side scatter assessment of ECFCs.

5.4. hiPSC-derived ECs (iECs): an alternative in vitro model for VWD

Only a finite number of ECFCs were derived from both control participants and NBR 1. After 3-4 passages a total of 1.7 million ECFCs were obtained from C3, 0.8 million from C4, and 2 million from NBR 1. It was also impractical to ask NBR 1 for repeated blood sample donations. Therefore I was motivated to develop a different approach for generating ECs that would enable me to further characterise the cellular endophenotype resulting from the c.8155+6T>A variant. In addition I was also keen to explore whether this variant still had the same EC effect when modelled in a different genetic context from NBR 1. Human induced pluripotent stem cells (hiPSCs) have unlimited capacity for self-renewal (Maherali & Hochedlinger, 2008), have been shown to both be capable of differentiation to ECs (Choi et al., 2009) and amenable to genome-editing (Yusa et al., 2011). Therefore, as outlined in the remainder of this section, I developed an approach for the production of hiPSC-derived ECs (iECs) and then used a number of assays to ascertain whether the resulting cells had the potential for modelling VWD variants.

5.4.1. QOLG_3 hiPSCs can be differentiated to iECs

Three hiPSC cell lines were in established use by our research group (Table 5.5, page 210) as they were able to generate megakaryocytes (MKs) using a lentiviral forward programming (FoP) approach (Moreau et al., 2016). An intermediary step in the FoP

protocol is the chemical induction of mesoderm. This is one of the three germ cell layers containing not only haematopoietic progenitors (relevant to MK generation) but also those of the vasculature including ECs (Kimelman, 2006). Therefore I reasoned that if these hiPSC lines could make mesoderm this increased the chance that they could be

hiPSC line	Source	Method of reprogramming	Transcription factors	References
A1ATDc	Adult dermal fibroblasts	Murine leukaemia virus vector	*POU5F1, SOX2, KLF4, MYC*	Rashid et al., 2010; Yusa et al., 2011
FFDK		Episomal DNA vector		Dalby et al., 2018; Kilpinen et al., 2017
QOLG_3		Non-integrative Sendai vector		

Table 5.5 | hiPSCs used for the initial development of the iEC protocol.

differentiated to endothelium and so all three were used to develop my approach for deriving ECs (hereafter the 'iEC protocol'). Before I acquired them, the cells had been acclimitised to feeder-free culture on vitronectin (Subsection 2.2.5, page 88-89) by other group members (Acknowledgements, page 8). On acquisition, I confirmed that the cells were round, had a high nuclear-to-cytoplasmic ratio, and grew as colonies in culture (Figure 5.7A, page 211) consistent with the original description and images of these cells (Takahashi et al., 2007).

I used a monolayer based approach to iEC differentiation (Table 5.1, page 198) as this most closely mirrored the conditions the hiPSCs were already being propagated in. The protocol I developed was a hybrid of that published by the Cowan, Mummery, and Sinha research groups (Cheung et al., 2014; Challet Meylan et al., 2015; Orlova et al., 2014). The final method is detailed in Subsection 2.2.5 (page 89-90) and an overview is displayed in Figure 5.7B (page 211). In brief, hiPSCs were dissociated and seeded onto matrigel-coated plates as single cells at a low seeding density in their regular maintenance (SF) media. Y-27632 was added to SF as it is an inhibitor of apoptosis known to increase cell survival during dissociation (Watanabe et al., 2007). Then 24 hours later the media was replaced with 'FCL' (Table 7.9, pages 254-255), named after the three key compounds which were added to it (FGF2, CHIR99021, and LY294002) in order to induce the small clumps of hiPSCs (Figure 5.8A ii., page 212) into mesoderm. FGF2 is a growth factor important for

Figure 5.7

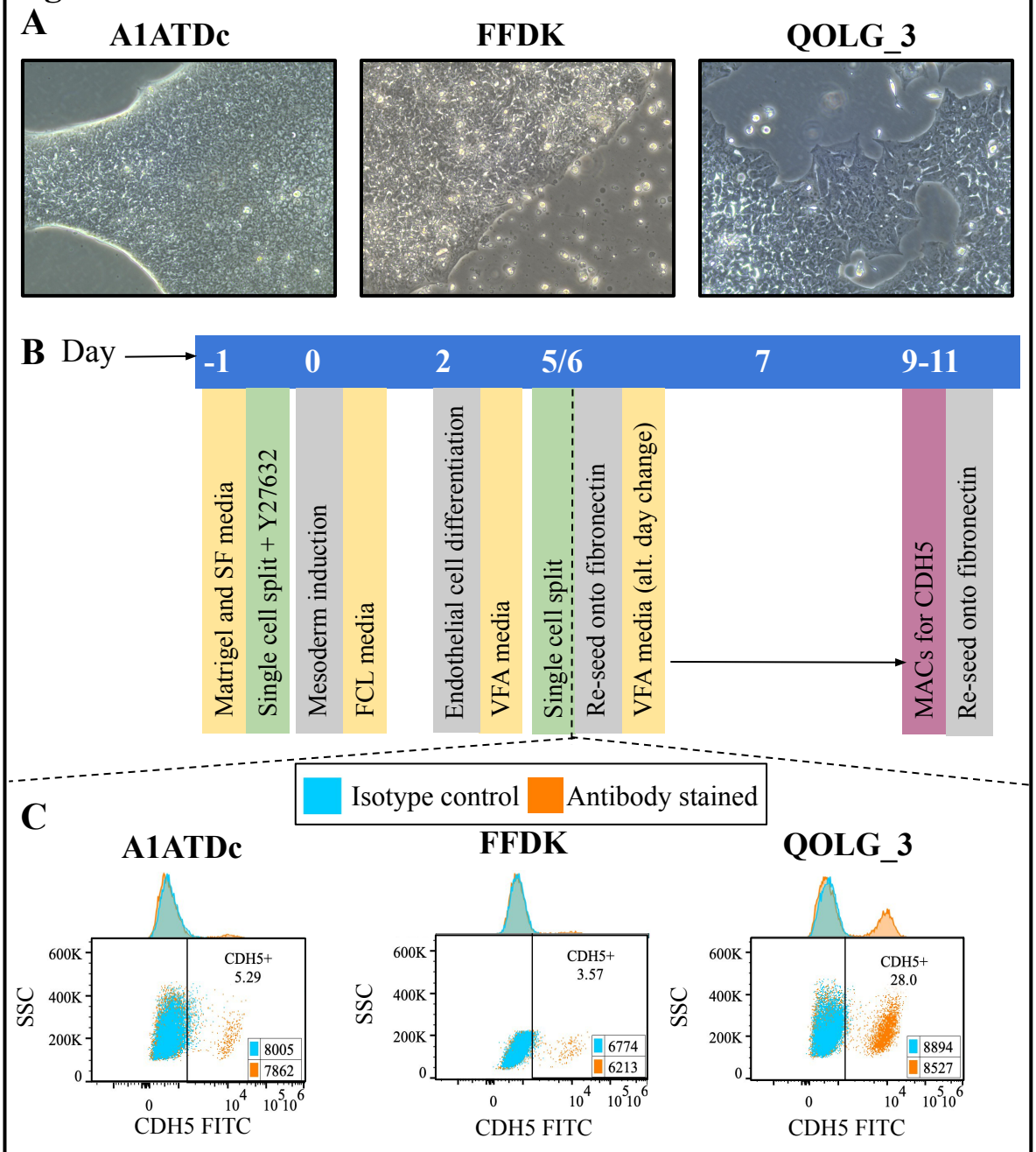


Figure 5.7 | Setting up the iEC protocol. (A) Brightfield images of each of the three hiPSC lines grown in StemFlex medium using a x 20 objective lens. (B) Outline of the key steps in the iEC protocol. (C) . Abbreviations: SF = StemFlex; FCL = FGF-2, CHIR 99021, LY294002; VFA = VEGF-A, Forskolin, Ascorbic acid; MACS = Magnetic cell separation. (C) Dot plots showing side scatter as a function of CDH5-FITC fluorescence at D5 of the iEC protocol. The marginal histogram shows the density distribution of fluorescence. The numbers in the top right hand corner represent the percentage of positive cells, in the bottom right hand corner, the total number of cells evaluated (in the cells gate) for isotype and antibody stained conditions. Three biological samples were analysed (A1ATDc, FFDK, QOLG_3); two independent iEC differentiation and flow cytometry experiments were carried out on each of A1ATDc and FFDK and four on QOLG_3, with the results of one of these displayed for each cell line.

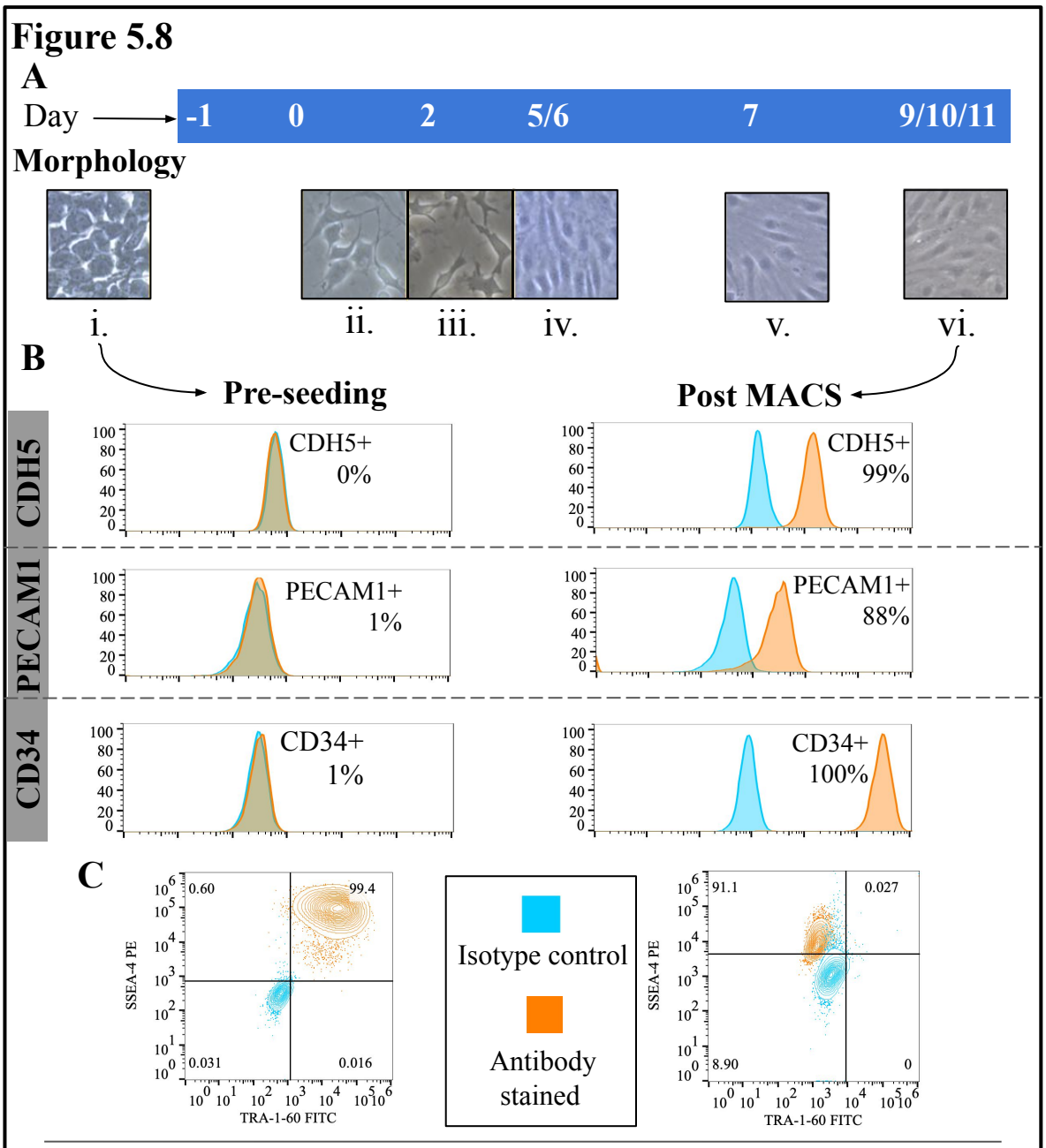


Figure 5.8 | The iEC differentiation protocol in QOLG_3. (A) Brightfield images taken at six different time intervals. HiPSCs prior to seeding are rounded and clumped (i) whereas larger, flattened cells emerged by D9. (B) Histograms of hiPSCs at D-1 (left) and post-MACS iECs at D9 (right) stained with CDH5 (top), PECAM1 (middle), and CD34 (bottom) antibodies. The y-axis is a linear scale representing the event count normalised to the mode, the x-axis represents, on a base 10 logarithmic scale, the compensated fluorescence on the channel used to detect the antibody with the intervals 10^0 to 10^6 marked by the bold vertical lines. The percentages are rounded to the nearest integer. (C) Contour plots of hiPSCs at D-1 (left) and post-MACS iECs at D9 (right) stained with TRA-1-60 and SSEA-4 antibodies with the percentage of events in each gate marked in the corner. The results above represent the analysis of one biological sample (QOLG_3). Eight independent experiments were carried out to differentiate QOLG_3 iPSCs to iECs and to magnetically separate them. The panels above represent the results of one of these replicates.

multiple steps in mesoderm induction including embryonic patterning (Poole et al., 2001). CHIR 99021 inhibits glycogen synthase kinase-3 beta at nanomolar concentrations (Ring et al., 2003), resulting in intracellular accumulation of catenin beta-1 (beta-catenin). The latter is the transcriptional activator downstream of the canonical Wnt signalling cascade (Moon et al., 2002), an essential pathway for mesoderm development during embryogenesis (Nusse and Varmus, 1992). LY294002 facilitates stem cell differentiation by indirectly increasing expression of T-box transcription factor T, also known as brachyury, which regulates mesodermal gene expression (Bernardo et al., 2011). After 48 hours the media was changed to one called VFA, named after the compounds added to it to specify endothelial cells (vascular endothelial growth factor A [VEGFA], forskolin, and ascorbic acid). VEGFA stimulates early embryonic vasculogenesis by triggering the migration of haemangioblasts which express its surface ligand, VEGFR-2 (Hiratsuka et al., 2005). Furthermore VEGFA maintains endothelial cell identity and survival by binding to VEGFR-2 which remains expressed on adult ECs (Hirashima, 2009). Forskolin activates protein kinase A (PKA) via a cyclic AMP (cAMP) dependent pathway (Misra & Pizzo, 2005). The cAMP/PKA pathway has been shown to promote sensitivity to VEGFA and improve EC differentiation in stem cells (Yamamizu et al., 2009). Ascorbic acid is reported to improve haematoendothelial differentiation of stem cells (Vodyanik & Slukvin, 2007).

Previous studies using a monolayer-based approach to differentiate hiPSC to ECs reported that VEGFR-2 transcripts rose three-fold by day 3 (Orlova et al., 2014) and that ~ 25% of cells expressed it on their surface at day 5 (Azhdari et al., 2013) post seeding. Therefore as a means of triaging which of the three hiPSC lines could successfully be differentiated to ECs, it seemed reasonable to assess them at D5. This was done by assessing the percentage of cells expressing CDH5 using flow cytometry (Figure 5.7C, page 211). The dot plots represent the first time this was carried out for each cell line, but was replicated once more for A1ATD and FFDK and three further times for QOLG_3, with a mean of 3.5%, 8.2%, and 25.2% CDH5-positive cells respectively. Therefore QOLG_3 were taken forward for further optimisation of the iEC protocol. The main aims were to determine whether a further 4-5 days in culture would yield an increase in the proportion of CDH5+ cells

obtained, decide on whether a method for iEC selection was required, and evaluate a wider panel of pluripotent and EC markers.

Fibronectin has been shown to be important for EC proliferation and survival (Wang & Milner, 2006) as well as providing a scaffold on which other extracellular matrix proteins, important for EC attachment, can bind (Hielscher et al., 2016). Therefore a step in the iEC protocol was introduced to dissociate the differentiating cells at D5 and seed them onto fibronectin-coated vessels (Figure 5.7B, page 211) and evaluate their CDH5 expression again at D9-11 (the exact day depending on availability of tissue culture hoods and scheduling of other experiments). Unfortunately although a further 6 days in culture enriched the proportion of CDH5+ cells up to 82%, the distribution remained bimodal, consistent with a proportion of the QOLG_3 hiPSCs being recalcitrant to differentiation to ECs as has been reported with other hiPSC lines and protocols (Kumar et al., 2018). Furthermore, in order to utilise the protocol for modelling VWD a method for obtaining a homogeneous population of ECs was required. Therefore magnetic cell separation (MACS) was used between D9 and D11 to positively select for CDH5 expressing iECs using CDH5-coated microbeads as this technique has previously been used for the purification of both hESC- (Tatsumi et al., 2011) and hiPSC- derived ECs (Challet Meylan et al., 2015; Patsch et al., 2016). This iEC protocol was then replicated eight times with QOLG_3 hiPSCs at passages between 28 and 31 subsequent to becoming feeder free. For every hiPSC seeded using the protocol, a mean of 3.6 iECs were derived (Table 5.6, page 216).

There was an increase in the expression of surface markers characteristic for ECs (CDH5, PECAM1, and hematopoietic progenitor cell antigen CD34 [CD34]) and concomitant decrease in the pluripotent ones, TRA-1-60 and SSEA-4, in the interval between when the hiPSCs were first seeded at D-1 and post-MACS at D9-11 (Figure 5.8B & C, page 212; Figure 5.9, page 215). This difference was statistically significant for CDH5 (paired t-test, $P = 0.049$), which had the highest number (five) of technical replicates.

Figure 5.9

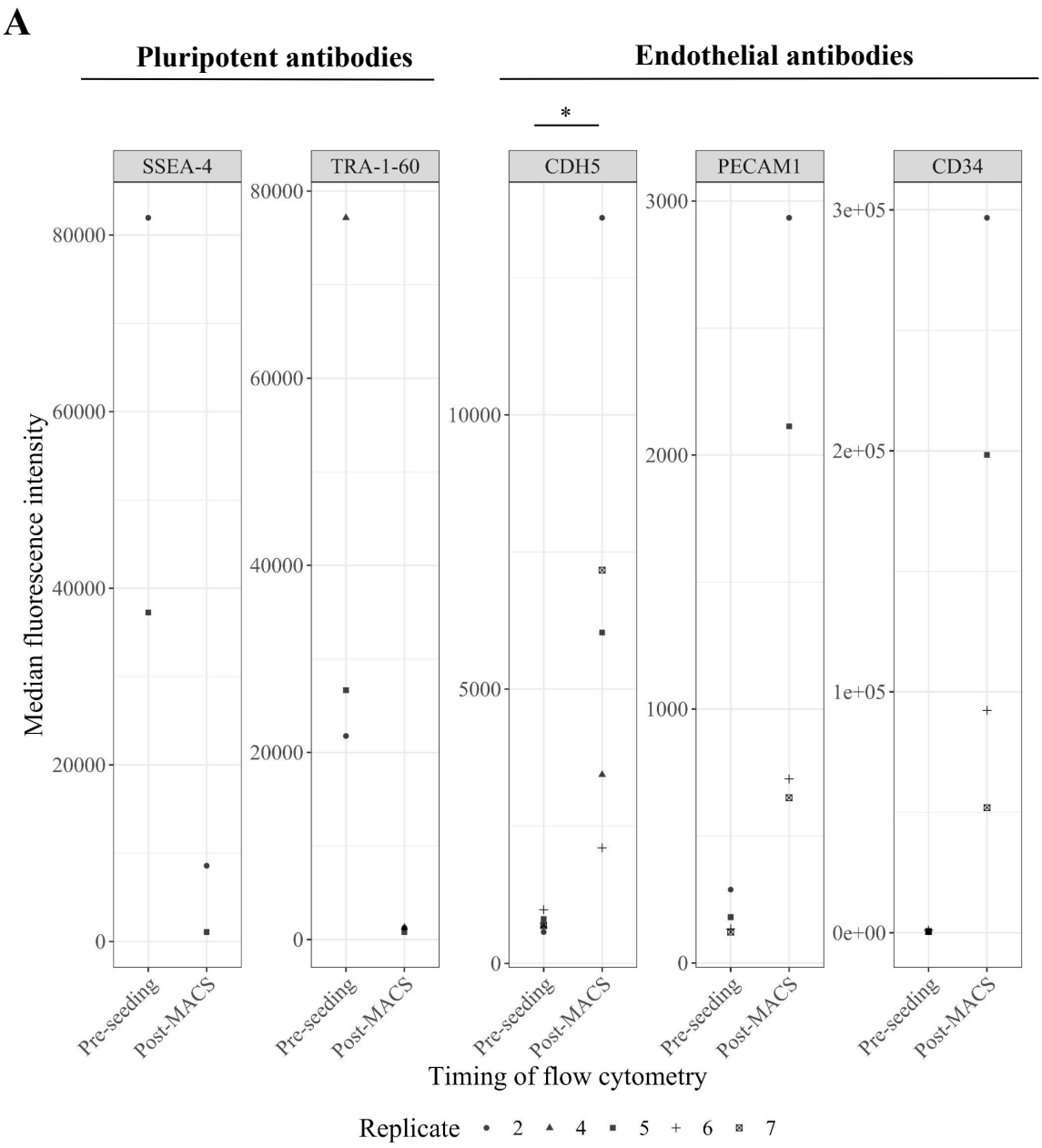


Figure 5.9 | The pluripotent and endothelial immunophenotype of QOLG_3 before and after differentiation to iECs. (A) Scatterplot showing median fluorescence intensity (MFI) as a function of the timing of flow cytometry. Pre-seeding corresponds to hiPSCs at D-1, post-MACS to D9-11 after selection of CDH5 positive cells using magnetic beads. Only replicates in at least one of five antibodies (SSEA-4, TRA-1-60, CDH5, PECAM1, and CD34) were used, pre-seeding and post-MACS, are shown. Furthermore only replicates in which the voltage and gain were the same at both time points were included. The shape of the dot corresponds to the replicate with the same numbering as Table 5.5. A two-sided, paired t-test was used to compare mean MFI at the pre- and post- time points using a 95% confidence interval. The *P* values were as follows: 0.208 (SSEA-4), 0.147 (TRA-1-60), 0.049 (CDH-5) marked by an asterisk, 0.071 (PECAM1), 0.063 (CD34).

Replicate	hiPSCs seeded (D-1)	iECs selected by CDH5 MACS (D9-11)	Yield (iECs/hiPSCs seeded)
1	960,000	4,783,333	5.0
2	1,200,000	3,317,000	2.8
3	1,200,000	3,290,000	2.7
4	2,720,000	9,473,333	3.5
5	4,927,500	13,160,000	2.7
6	3,340,000	11,512,000	3.4
7	7,539,333	57,400,000	7.6
8	7,360,000	5,383,333	0.7

Table 5.6 | Yield of cells from iEC protocol using QOLG_3. The ratios in the yield column are rounded to the nearest 1 decimal place. The results above represent the analysis of one biological sample (QOLG_3) that was differentiated to iECs and magnetically separated in eight independent experiments.

5.4.2. QOLG_3 hiPSCs are wild type for *VWF*

Having established that QOLG_3 could consistently be differentiated to cells with an endothelial cell surface immunophenotype, I wanted to determine if these produced VWF. However, first I needed to verify that there were no PAVVs present that could alter the expression or function of VWF. Based on information captured at the point of dermal skin cell donation, QOLG_3 was male, aged 35-39, of European ancestry and he did not report any diseases (HipSci, 2014). However as shown in Chapter 4, the absence of a phenotype of VWD does not rule out the presence of a PAVV. As part of the HipSci consortium project, feeder-free QOLG_3 hiPSCs were analysed by WES (Streeter et al., 2017), prior to when I acquired the cell line. The VCF of this WES data was processed using the same pipeline as that used for the NBR-VWD participants (Figure 4.12, page 173). Seventeen variants were identified within the *VWF* scaffold that had passed the genotype filter and that had had sufficient depth and quality. Reassuringly, all were common variants (gnomAD MAF 0.01 - 0.15). In summary the deposited QOLG_3 VWF sequence contained no rare PAVVs and was therefore considered WT.

5.4.3. The endothelial phenotype of WT QOLG_3 iECs

In order to determine whether QOLG_3 iECs transcribed the *VWF* gene, RNA was extracted from iECs obtained immediately after MACS selection for replicates 2, 4, 5, and 6 (Table 5.6, page 216). For comparison purposes, RNA was also extracted from human

microvascular endothelial cells (HMVECs) (Subsection 2.2.6, pages 90-91) and iECs differentiated from another hiPSC line, called C2 (Acknowledgments, page 8). C2 hiPSCs were reprogrammed from ECFCs derived from a 45 year old Caucasian female (Geti et al., 2012). This donor had no known diseases and the iECs were differentiated using an alternative protocol (Kiskin et al., 2018) to the one I developed. All three lines had *VWF* transcript levels > 2 $\log_2$ fragments per kilobase million ($\log_2$ FPKM) (Figure 5.10A, page 218), a level well above the threshold for consistent detectability of intracellular protein (Wang et al., 2019). Reassuringly, QOLG_3 iECs contained a significantly higher number of *VWF* transcripts than C2 iECs ($\log_2$ fold change[FC] 3.27, adjusted $P = 3.46 \times 10^{-133}$; Figure 5.10B, page 218). This could be due to a variety of reasons including inter-individual variation between hiPSC donors and/or differences in iEC differentiation protocols. In contrast, QOLG_3 iECs had significantly fewer *VWF* transcripts than HMVECs ($\log_2$ fold change[FC] -4.03, adjusted $P = 1.38 \times 10^{-211}$; Figure 5.10C, page 218).

One of the critical properties of ECs is their ability to synthesise a pool of VWF that can be released upon stimulation (Levine et al., 1982; Loesberg et al., 1983). To determine if QOLG_3 iECs were capable of this, I cultured the post-MACS iECs from replicate 5 (Table 5.6, page 216) for one day in VFA media (Subsection 2.2.7, pages 91-92). The supernatant was then aspirated and compared, by western blotting for VWF, to media which had not been used to culture any cells. The results are shown in the immunoblot in Figure 5.11B (page 219). This confirmed that the serum-free VFA media by itself did not contain VWF, but this was detected in the media used to grow the iECs, consistent with basal release of VWF by these cells. For the assessment of intracellular VWF content, hiPSCs were used as a negative control as immunoblotting of cell lysates confirmed, as expected, that they did not contain VWF (Figure 5.11A, page 219). Additionally, in three separate wells, iECs were either treated with different agonists, thrombin (2.5 U/ml) or adrenaline (100 μM), or (as a negative control) D-PBS. After 30 minutes of treatment, the iECs were then harvested. The iECs treated with D-PBS had a more visible band on immunoblotting than either the thrombin or adrenaline treated cells (Figure 5.11A, page 219). The signal intensity of each band was normalised against GAPDH and was lower for the agonist treated samples compared to the D-PBS control. These results suggest that the two major pathways for evoking VWF release, the calcium- and the cAMP- dependent

Figure 5.10

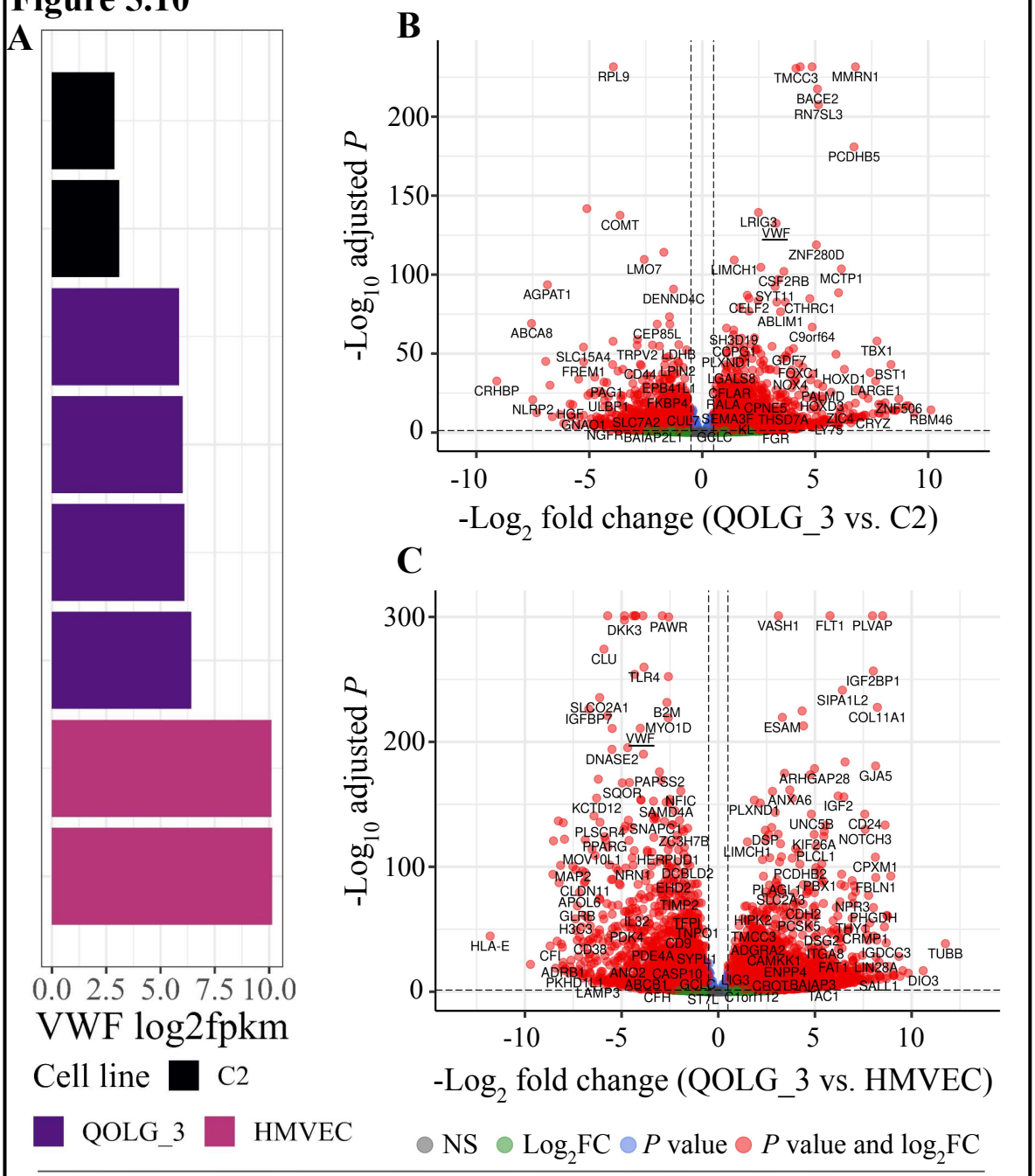


Figure 5.10 | WT QOLG_3 iECs express detectable VWF transcripts. (A) A bar chart showing the level of transcript expression of *VWF* in QOLG_3 iECs as compared with C2 iECs and HMVECs. The expression of *VWF* transcripts on the x-axis is the binary logarithm of fragments per kilobase million (log_2fpkm). The y-axis represents technical replicates of the different cell types as indicated by the colours used to fill the bars. (B) and (C) are volcano plots of differential gene expression, in which QOLG_3 is compared to C2 (B), or to HMVEC (C). Each dot represents a gene and those coloured in red are both statistically significant at the adjusted *P* value threshold of 1 x 10^{-4} and have log_2 fold change (FC) > 0.5. Blue dots represent those with adjusted *P* value < 1 x 10^{-4} only. Green dots represent those with log_2 fold change (FC) > 0.5 only. Grey dots are not significantly different (NS). VWF is underlined. Plots (B) and (C) were made by Dr Dinan (Acknowledgements, page 8).

Figure 5.11

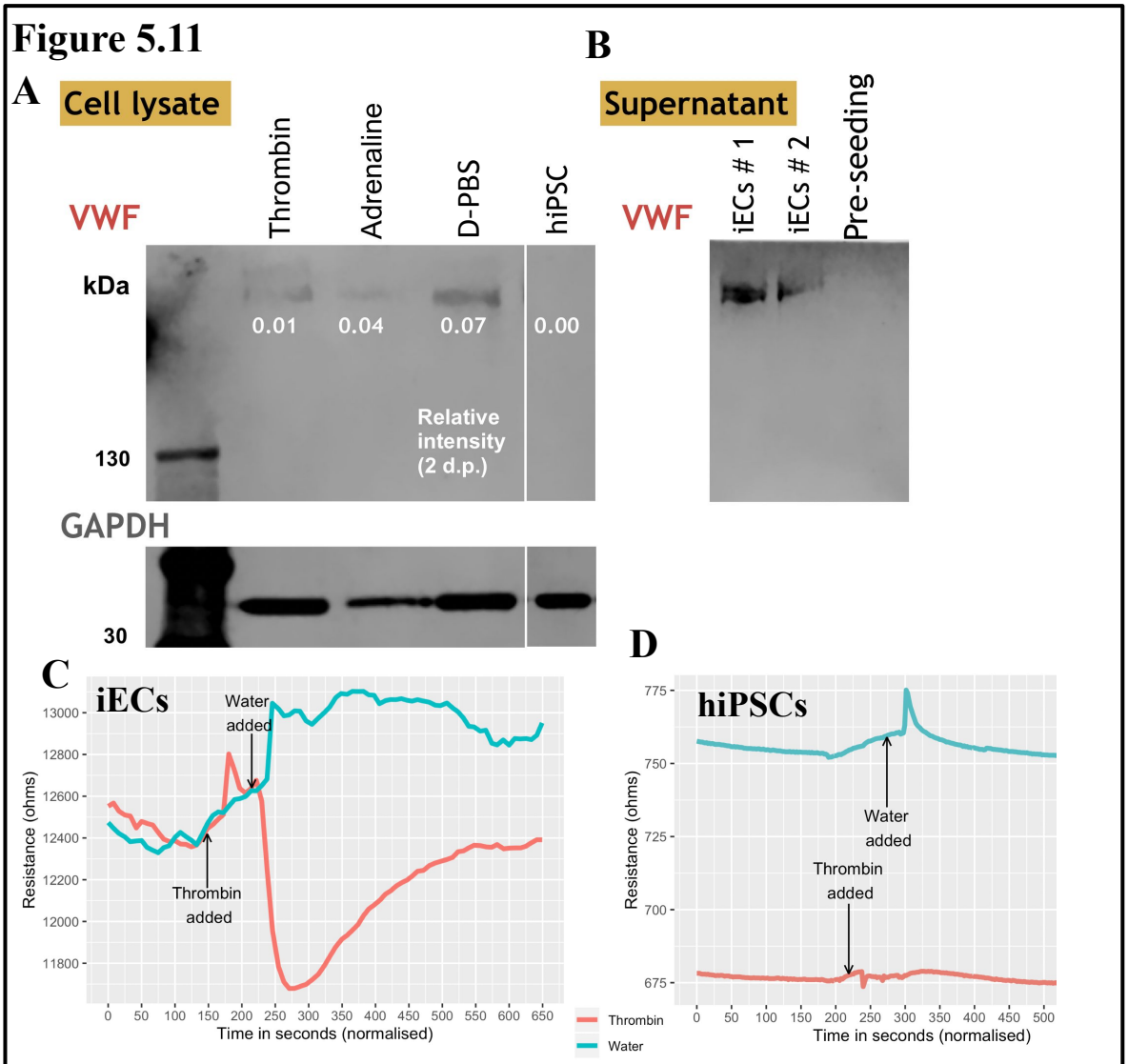


Figure 5.11 | WT QOLG_3 iECs produce VWF and are agonist responsive. (A) and (B) show the immunoblot of reduced protein extracted from iECs lysates (A) and media supernatant collected after being used to culture iECs (left two lanes) and a control sample which was not used to culture iECs (B). The blots were first probed with rabbit polyclonal VWF antibody and then a fluorescent anti-rabbit IgG antibody for visualisation. The ladder is shown on the left hand side of the blot with the corresponding size of the band marked. The relative signal intensity of the VWF bands as normalised to GAPDH are indicated in the lanes marked 'Thrombin', 'Adrenaline', and 'D-PBS'. (C) and (D) are line graphs of electrical resistance (measured at 4,000 Hz) as a function of time in electric cell-substrate impedance sensing (ECIS) experiments. The results above represent the analysis of one biological sample (QOLG_3). Eight independent experiments were carried out to differentiate QOLG_3 hiPSCs to iECs and to magnetically separate them. The iECs from one experiment (replicate 5; Table 5.6, page 216) were used for the subsequent experiment shown in panels A and B and the Western blot was performed once. The supernatant from two separate wells seeded with these iECs (iECs # 1 and # 2) was used for panel B (two technical replicates). The iECs from one experiment (replicate 4; Table 5.6, page 216) were used for the subsequent experiment shown in panels C and D and the ECIS measurements were made once.

(Rondaij et al., 2006), triggered by thrombin and adrenaline, respectively, are functional in WT QOLG_3 iECs.

An electric cell-substrate impedance sensing (ECIS) test was used to gather further evidence of the agonist response in QOLG_3 iEC. Using this technique, an alternating electrical current is passed through an adherent layer of cells between two electrodes. The resistance to this current is measured by a machine linked to the electrodes which provides information about the biophysical properties of the cells (Giaever & Keese, 1991). Post-MACS iECs differentiated during replicate four (Table 5.6, page 216) were seeded onto the ECIS arrays in VFA media and cultured overnight whilst measuring electrical resistance. Although the range of alternating current frequencies that can be used on ECIS varies between 62.5 and 64,000 Hz, measurements were made using 4,000 Hz because measurements at this frequency take into account the net resistance from cell-cell and cell-substrate connections and the cell membrane (Robilliard et al., 2018). The baseline resistance of iECs determined 24 hours following seeding on to the ECIS arrays was ~ 12,500 ohms (Figure 5.11C, page 219). This compares to ~ 2,500 ohms reported for iECs in two publications from the same group (Halaidych et al., 2018; van IJzendoorn et al., 2020). The resistance in hiPSCs was 18-fold lower than iECs (Figure 5.11D, page 219). One of the hallmarks of the endothelium is its ability to alter permeability in response to physiological stimuli (Claesson-Welsh et al., 2021). As shown in Figure 5.11C, when 5nM thrombin was added to the iECs (as 10µl added into a volume of 200ul media in the ECIS array), there was a sharp drop in resistance by 700 ohms which then recovered more slowly over 400 seconds. This is in comparison to the vehicle control (water) in which there was a small uptick in resistance (400 ohms) and then a flattening off of the resistance profile. These results are in contrast to those obtained in hiPSCs which showed no change in resistance in response to thrombin. Therefore, QOLG_3 iECs are able to transiently increase permeability in response to thrombin.

In summary, QOLG_3 hiPSCs, which neither produce VWF nor form a tight monolayer, can be differentiated, using the protocol I developed, to iECs which have the expected

characteristics, including being agonist responsive to adrenaline and thrombin. Therefore I felt that they had the potential as an in vitro cellular endophenotype model for VWD.

5.5. The use of CRISPR/Cas9 to model the EC effect of c.8155+6T>A

5.5.1. The CRISPR/Cas9 clones: ΔCK and KO

When considering how to model c.8155+6T>A in QOLG_3, I chose not to design a repair template in order to try and knock in the variant precisely using homology directed repair (HDR). Instead I relied on non-homologous end joining (NHEJ). The rationale behind this was twofold. Firstly, the HDR approach is technically more challenging than NHEJ and less efficient (Devkota, 2018; Liu et al., 2019). Secondly, as outlined in Chapter 4, the consequence of c.8155+6T>A (and other intron 50-51 donor splice site variants) is a frameshift and termination codon (p.Gly2706ValfsTer25) which removes the normal sequence encoding the CK domain and the distal 15 amino acids of the VWC6 domain (Figure 4.16, page 184). Therefore my reasoning was that as long as a termination codon was placed close to the end of exon 50 this would recapitulate c.8155+6T>A, i.e. truncation of VWF prior to the CK, and serve the dual purpose of ascertaining whether the abnormal distribution of VWF seen in NBR 1 ECFCs is a more generalisable consequence of the absence of this domain (hereafter ΔCK). In order to maximise the chances of achieving this goal, three different single guide (sg) RNAs were designed (sgRNA 6, 7, and 8) (Figure 5.12A, page 222; Section 2.2.8, pages 92-93; Table 2.5, page 93) to target exon 50. The analysis of the deposited WGS data of QOLG_3 hiPSCs from the HipSci consortium (Subsection 5.4.2, page 216) showed that there were no genetic variants in exon 50. However the cells I had acquired had been passaged since this baseline sequencing. Passaging can introduce new variants (Ji et al., 2012). Therefore, I aligned the RNA-seq data from replicate 2 iECs (Table 5.6, page 216) to the reference sequence which confirmed that there were no exon 50 variants (Figure 5.12A page 222). For the subsequent iEC differentiation and VWF localisation experiments, I needed to create an additional clone in which VWF was knocked out as a negative control. Therefore three sgRNAs were designed (named sgRNA 2, 4, and 5) targeting the start of exon 2 (Table 2.5, page 93) as this one contains the ATG codon and has previously been knocked

Figure 5.12

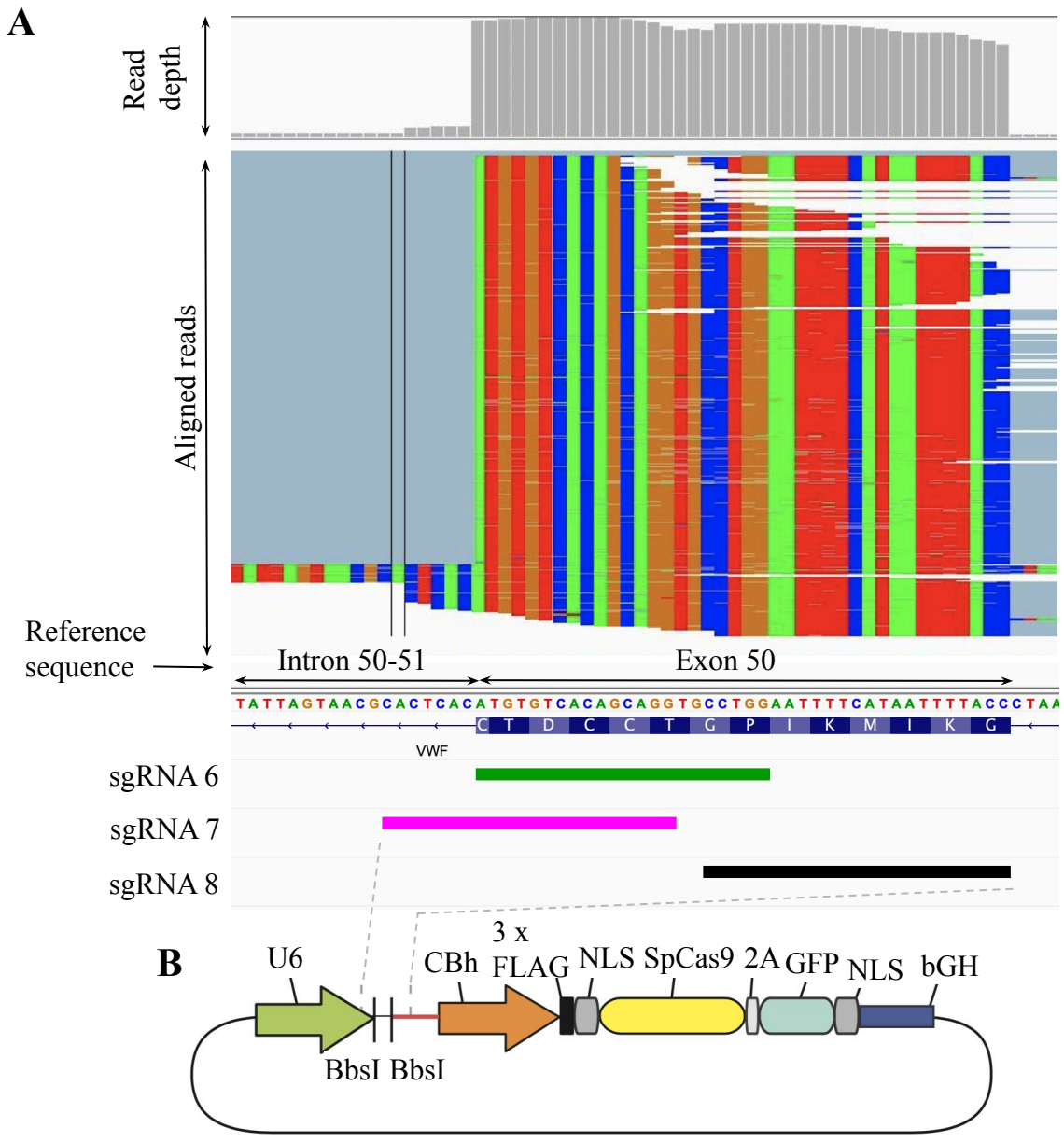


Figure 5.12 | Overview of CRISPR/Cas9 editing of QOLG_3 hiPSCs. (A) Aligned RNA-sequencing from QOLG_3 positioned above the reference sequence of exon 50 and the start of intron 50-51 as annotated. The positions of the single guide (sg) RNAs are shown below. (B) Schematic outline of the PX458 plasmid, modified from Ran et al., 2013. Abbreviations: U6 = U6 promoter sequence; BbsI = BbsI restriction site; CBh = chicken beta-actin promoter hybrid; FLAG = N-terminal FLAG-tag; NLS = nuclear localisation signal; SpCas9 = streptococcus pyogenes Cas9; 2A = 2A peptide sequence; GFP = green fluorescent protein; bGH = bovine growth hormone terminator sequence. Each of the sgRNAs were separately inserted into the vector between the BbsI restriction sites.

out using a similar design in ECFCs (Schillemans et al., 2019).

The methods for the steps carried out to obtain the ΔCK and KO clones is in Subsection 2.2.8 (page 92-95). In brief, all sgRNAs were inserted into a plasmid which contains the Cas9 endonuclease, in order to create the DSB, and a GFP tag which facilitates cell sorting (Ran et al., 2013) (Figure 5.12B, page 222). Therefore six plasmids were created, each containing one of the sgRNAs. These were then nucleofected into QOLG_3, with the same batch (passage number 30) being used as the starting point. Following nucleofection, single GFP-positive cells were sorted into 96 well plates and genomic DNA extracted from colonies three weeks later. PCR amplification of exon 50 was carried out for ΔCK clones and exon 2 for KO clones. There were four potential outcomes of the CRISPR experiment: WT/WT, variant/WT, variant/variant, or multi-allelic. To resolve this, A-tailed PCR amplicons from each clone were ligated into the vector, pGEM-T-easy. Using this approach two clones with the desired result were obtained. The successful ΔCK clone had a single nucleotide insertion of A (c.8139_8140insA) which resulted in a frameshift and termination codon (p.Thr2714AsnfsTer4). As shown, this PTC occurs 10 nucleotides 5' of the CK domain (Figure 5.13, page 224). There was also one successful clone that would be predicted to be a KO based on compound heterozygosity for an allele that was a 11 nucleotide deletion (c.3_13del) and the other that was a one nucleotide insertion (c.12_13insA) (Figure 5.14 A & C; page 225). Both variants would be predicted to cause a frameshift and termination codon 37 (p.Pro3CysfsTer35) and 41 (p.Arg5LysfsTer37) AAs following the ATG codon, respectively (Figures 5.14 B & D; page 225). I searched for both the c.8139_8140insA, c.3_13del, and c.12_13insA variants in VWDbase and gnomAD v.3.0 and none of them were found. Therefore there is no evidence that they exist as naturally occurring variants.

5.5.2. ΔCK recapitulates the phenotype seen in NBR 1 ECFCs

The selected ΔCK and KO hiPSC clones were then differentiated using the iEC protocol (Figure 5.7, page 211). Additionally, two other controls were set up. The first was WT QOLG_3 from the same batch of cells that had been used for nucleofection, referred to as WT hereafter. The second control was a clone that had been nucleofected with sgRNA 6

Figure 5.13

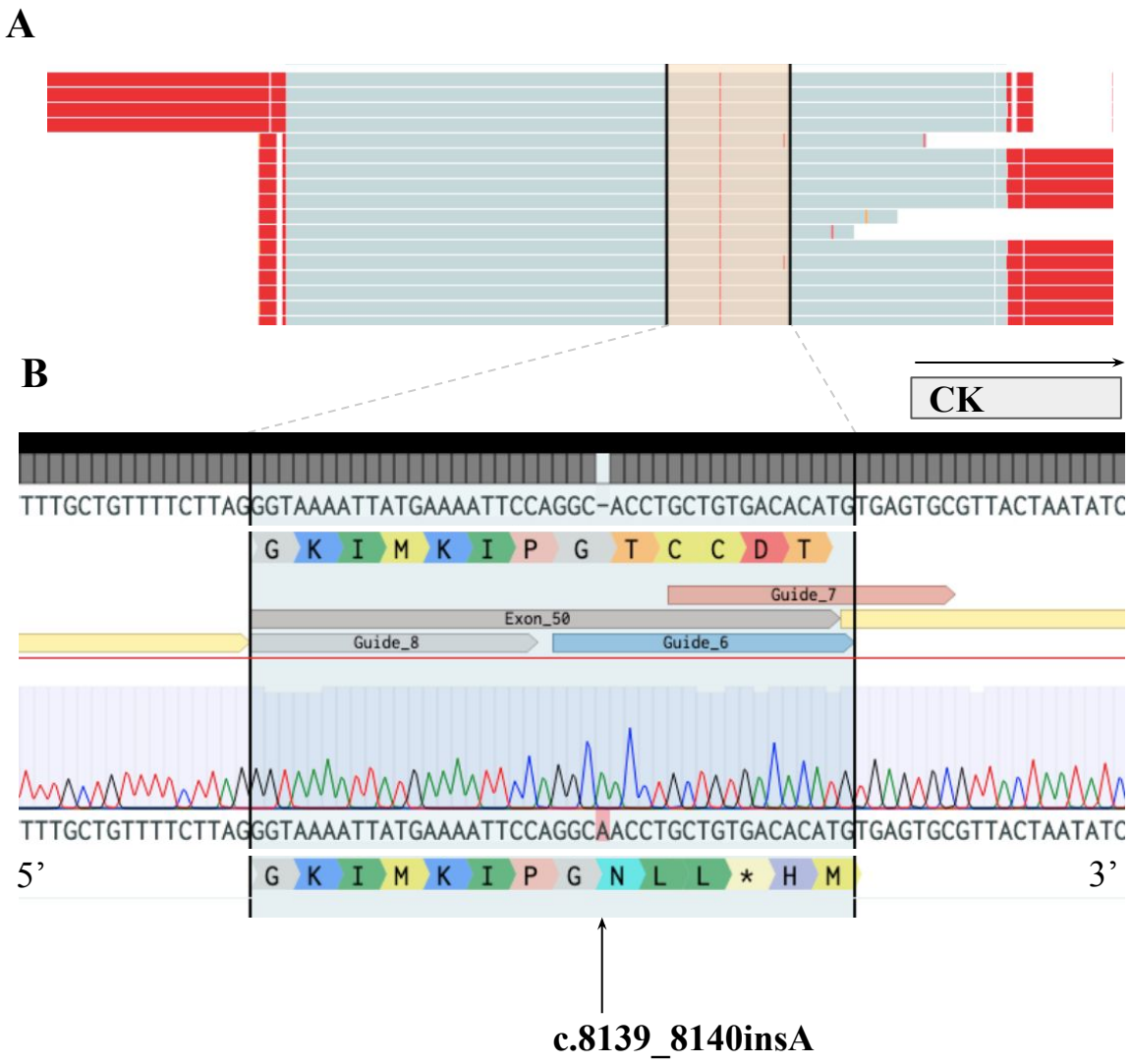


Figure 5.13 | Sequence of the selected ΔCK clone. (A) A-tailed amplicons were ligated into pGEM®-T-easy and then following bacterial transformation, single colonies were picked and analysed by Sanger sequencing. Of the 25 colonies which were successfully sequenced, all contained the same insertion of A (c.8139_8140insA) as marked in pink in (B). 17 of these sequences are represented. Where the rectangles are grey in colour they aligned to the reference sequence, where coloured in red they did not align. The red vertical line inside of the black bold vertical lines represents the position of c.8139_8140insA. (B) Represents zoomed in projection of the areas marked, focussing on exon 50. The reference nucleotide and amino acid sequence is at the top with the position of the three sgRNAs (guides) indicated. The Sanger sequence of one of the colonies is shown below with the position of c.8139_8140insA marked. Below is the resulting frameshifted amino acid sequence, which results in a termination codon (marked by *) after three new amino acids as a result of the frameshift (p.Thr2714AsnfsTer4). The position of the CK domain is marked by a grey box with a black outline above the reference sequence.

Figure 5.14

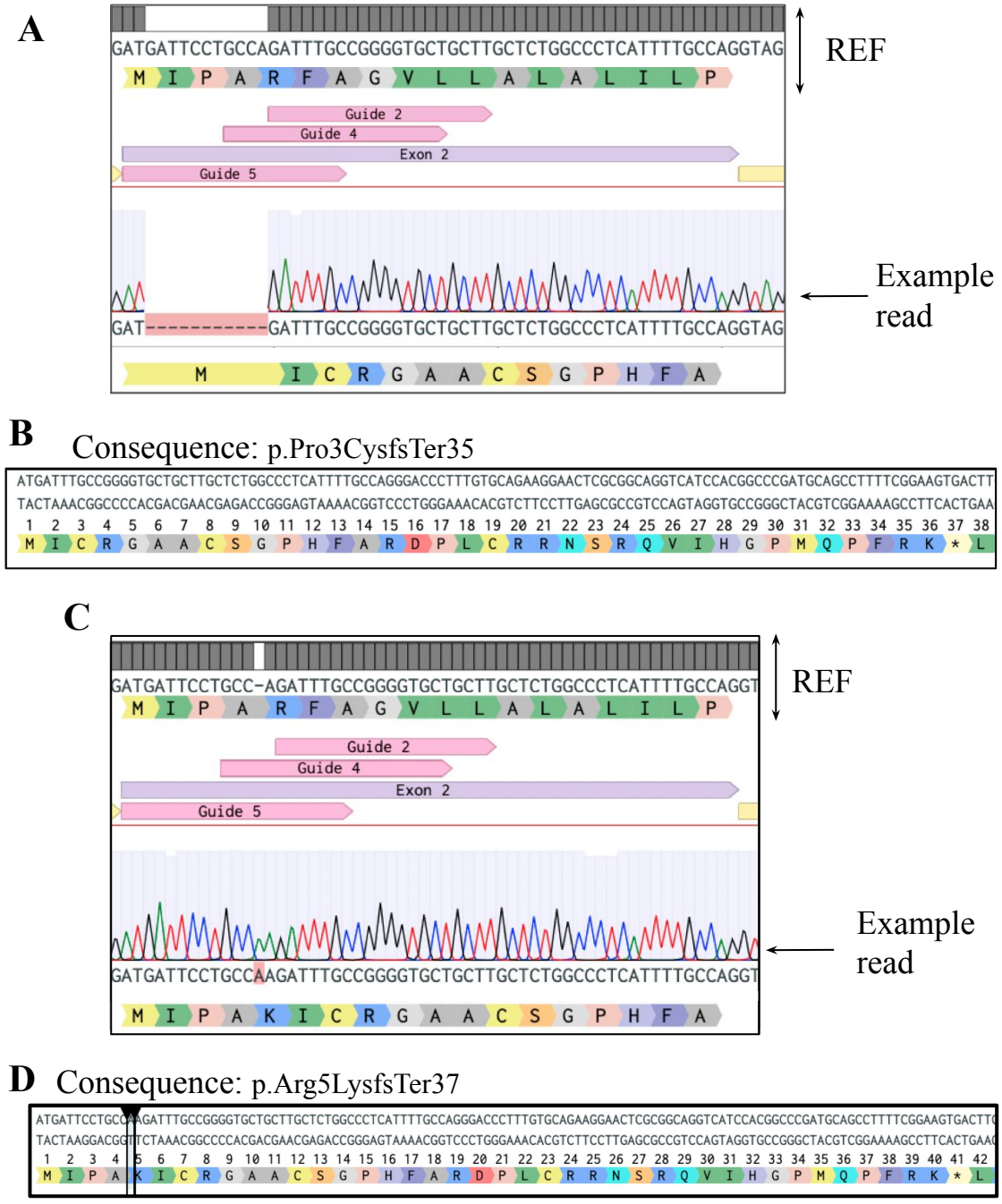


Figure 5.14 | Sequences of the selected KO clone. A-tailed amplicons were ligated into pGEM®-T-easy and then following bacterial transformation, single colonies were picked and analysed by Sanger sequencing. (A) and (C) represent example Sanger sequencing traces following single amplicon sequencing of 30 colonies from a potential KO clone. Seventeen reads had an 11 nucleotide deletion shown in (A), corresponding to c.3_13del. Thirteen reads had a 1 nucleotide insertion shown in (C), corresponding to c.12_13insA. The reference sequence is marked (REF) and the positions of the sg (guide) RNAs are shown. (B) and (D) show the predicted consequences of c.3_13del and c.12_13insA, respectively, which are resultant frameshifts and premature termination codons (marked with *) occurring at 37 and 41 amino acids after the initiation codon, respectively.

(as for the selected ΔCK line), but for which the sequencing confirmed that no changes occurred to the sequence of exon 50 - this is referred to as nucleofection control (nucl. cont.). The rationale for this latter control was to try and take into account CRISPR off-target effects (Kimberland et al., 2018).

There was one log_{10}-fold increase in the MFI of CDH5-FITC when iECs are compared to hiPSCs, and a corresponding decrease in the MFI for SSEA-4 (Table 5.7, below). Furthermore, the profiles of the contour plots of the cells evaluated in each of the four conditions are similar (Figure 5.15, page 227), except the distribution of SSEA-4 PE fluorescence for the KO cells where there is a small population of cells with reduced expression. One potential explanation for this is that there may have been some spontaneous differentiation of KO cells as these underwent more passages prior to differentiation to iECs than the other three conditions because of the need to subclone them as outlined above.

Antibody	**Clone**							
	ΔCK		**Nucl. cont.**		**KO**		**WT**	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
SSEA-4 PE	3.0×10^5	9.3×10^3	6.3×10^5	1.3×10^4	5.5×10^5	1.4×10^4	4.3×10^5	1.2×10^4
CDH5 FITC	3.8×10^3	5.6×10^4	3.3×10^3	7.4×10^4	3.4×10^3	8.6×10^4	3.9×10^3	4.6×10^4

Table 5.7 | Median fluorescence intensities (MFI) of the antibodies used to evaluate iECs pre and post differentiation. The values shown are those based on each of the two channels used to detect the fluorescence signal from one of the two fluorophore-conjugated antibodies. The MFIs were calculated after correcting for fluorescent overspill using compensation beads. The same instrument settings were used for the D-1 and D9 acquisition. In both this table and Figure 5.15, the results above represent the analysis of four biological samples (clones) following the CRISPR/Cas9 editing of QOLG_3 (ΔCK, nucleofection control [nucl. cont.], KO, WT) which were differentiated to iECs in a single experiment. Flow cytometry was carried out once at D-1 on hiPSCs pre differentiation (left hand column) and once on iECs at D9 post MACS (right hand column) for each clone.

To assess the effect ΔCK had on the intracellular distribution of VWF, frozen vials of these cells, obtained post-MACS, were thawed and expanded on fibronectin using a media normally used to culture HUVEC cells (EGM-2) with 10% fetal bovine serum added in order to encourage proliferation (Maciag et al., 1981). One of the limitations of the ECFC image analysis was that the cells were not fully confluent when evaluated by confocal

Figure 5.15

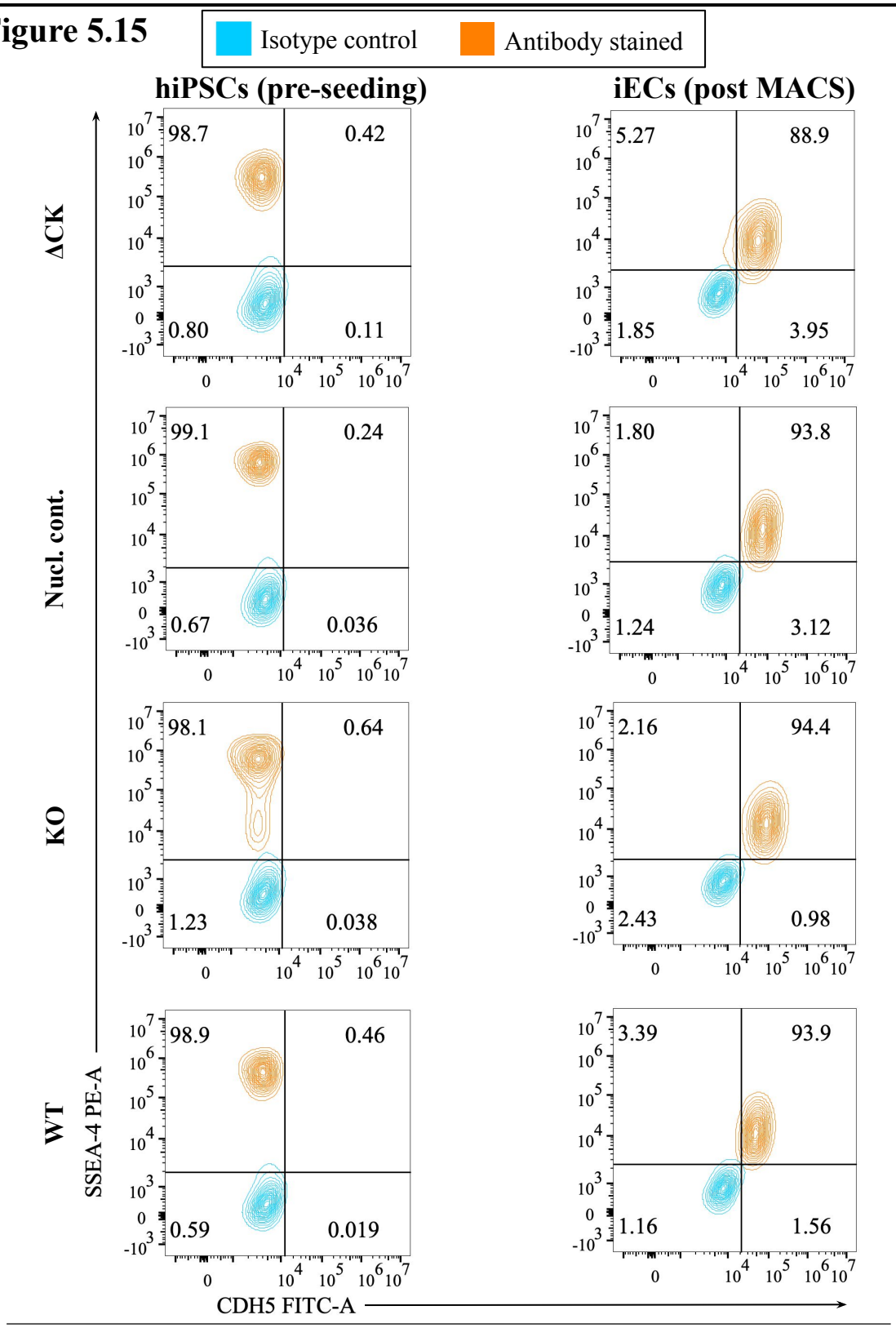


Figure 5.15 | Immunophenotype of the QOLG_3 iEC clones. Contour plots of events detected in the single cell gate of cells double stained with CD5 FITC and SSEA-PE (in orange) compared with respective isotype controls (in blue) and assessed before and after the iEC differentiation protocol. 5% of the cells fall between each of the contour lines. The quadrant gate was set so that the horizontal and vertical lines touch the second outermost ring of the isotype control. N.B. Nucl. cont. = nucleofection control.

microscopy (Figure 5.5, page 206; Figure 5.6, page 208). This is important because confluency itself has been reported to be a determinant of VWF expression in cultured HUVECs (Popa et al., 2022). Likewise, HUVECs at full confluence produce more and longer WPBs than those which are subconfluent (Howell et al., 2004). Following expansion, the iECs were then seeded onto fibronectin-coated glass slides. After 4 days the cells had been confluent for at least 48 hours and they were formaldehyde fixed. The same process was followed, in parallel, for both WT and KO iECs. Following staining with DAPI, VWF and CDH5 primary antibodies, and fluorescent secondaries, the cells were imaged using confocal microscopy (Subsection 2.2.9, pages 95-97). In total, 37 composite images were obtained: 6 WT, 9 KO, and 22 ΔCK. DAPI, CDH5, and VWF were detected following excitation with lasers of wavelength 405 nm (blue), 488 nm (green), and 633 nm (red), respectively. CDH5 was universally expressed on the plasma membrane of all iECs tested, and showed that the iECs were fully confluent. The expression of CDH5 is consistent with the maintenance of an endothelial cell identity after 8 days in culture post MACS (488 nm channel images of Figure 5.16, page 229). Reassuringly, the iECs from the unstained control, in which no DAPI, primary or secondary antibodies were added during the staining process, did not display any fluorescence from the cell membrane. However in this unstained control there was obvious fluorescence detected around the nucleus in the 488 nm channel (Figure 5.16A, page 229). This is likely to represent autofluorescence as an artefact of formaldehyde fixation (Yang et al., 2017a). There was no autofluorescence detected in the 405 nm (blue) or 633 nm (red) channels.

On initial review of the composite images with the clearest resolution, WT iECs expressed VWF both in a circumferential ring around the nucleus (perinuclear distribution), or in puncta in the cell cytoplasm (consistent with the formation of WPBs), or a combination of both patterns (Figures 5.16B, page 229 and Figure 5.17A & D, page 230). As expected, KO iECs showed a marked reduction in red fluorescence consistent with an absence of VWF in most cells, but some dimmer, more diffuse fluorescence could also be observed (Figures 5.16C, page 229; Figure 5.17B & E, page 230) which was of questionable relevance and is discussed below.

Figure 5.16

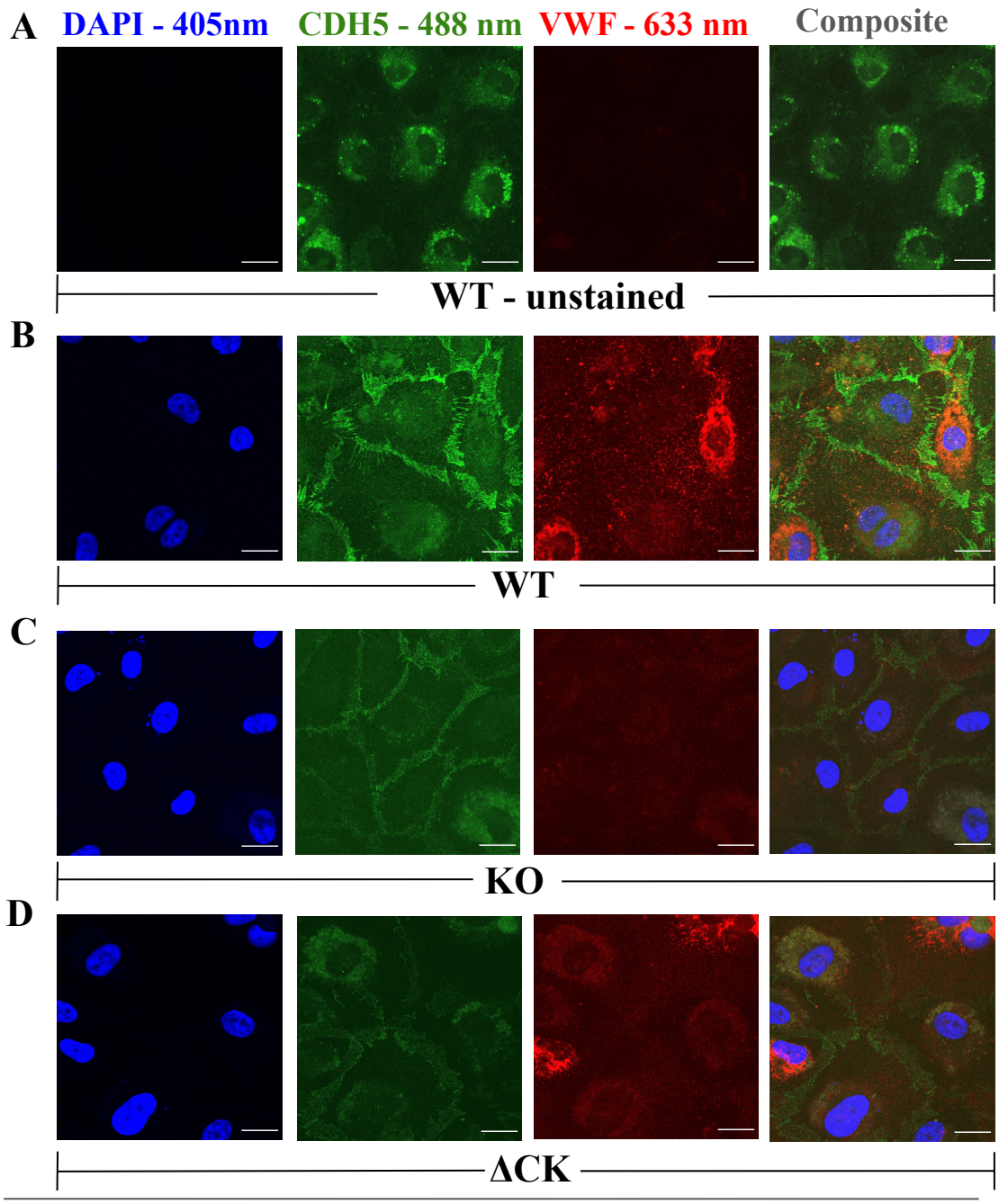


Figure 5.16 | iEC image analysis: component channels. Confocal images of iECs captured following excitation using lasers with a wavelength of 405, 488, and 633 nm, and merged composite of all three channels for (A) unstained WT (no DAPI, primary or secondary antibodies), (B) WT, (C) KO, and (D) ΔCK clones. The scale bar on each image represents 20 µm. The images were taken using x 63 objective with numerical aperture of 1.4 and x 2 zoom factor. In this figure, Figure 5.17, and Figure 5.18, the results were obtained from three biological samples (clones) following the CRISPR/Cas9 editing of the QOLG_3 hiPSC line (ΔCK, KO, WT). The iECs of each of these clones were cultured, stained and imaged by confocal microscopy in a single experiment. The number of images of each clone taken from non-overlapping positions is outlined on page 228 and the total number of corresponding cells evaluated is detailed in Figure 5.18A (page 232).

Figure 5.17

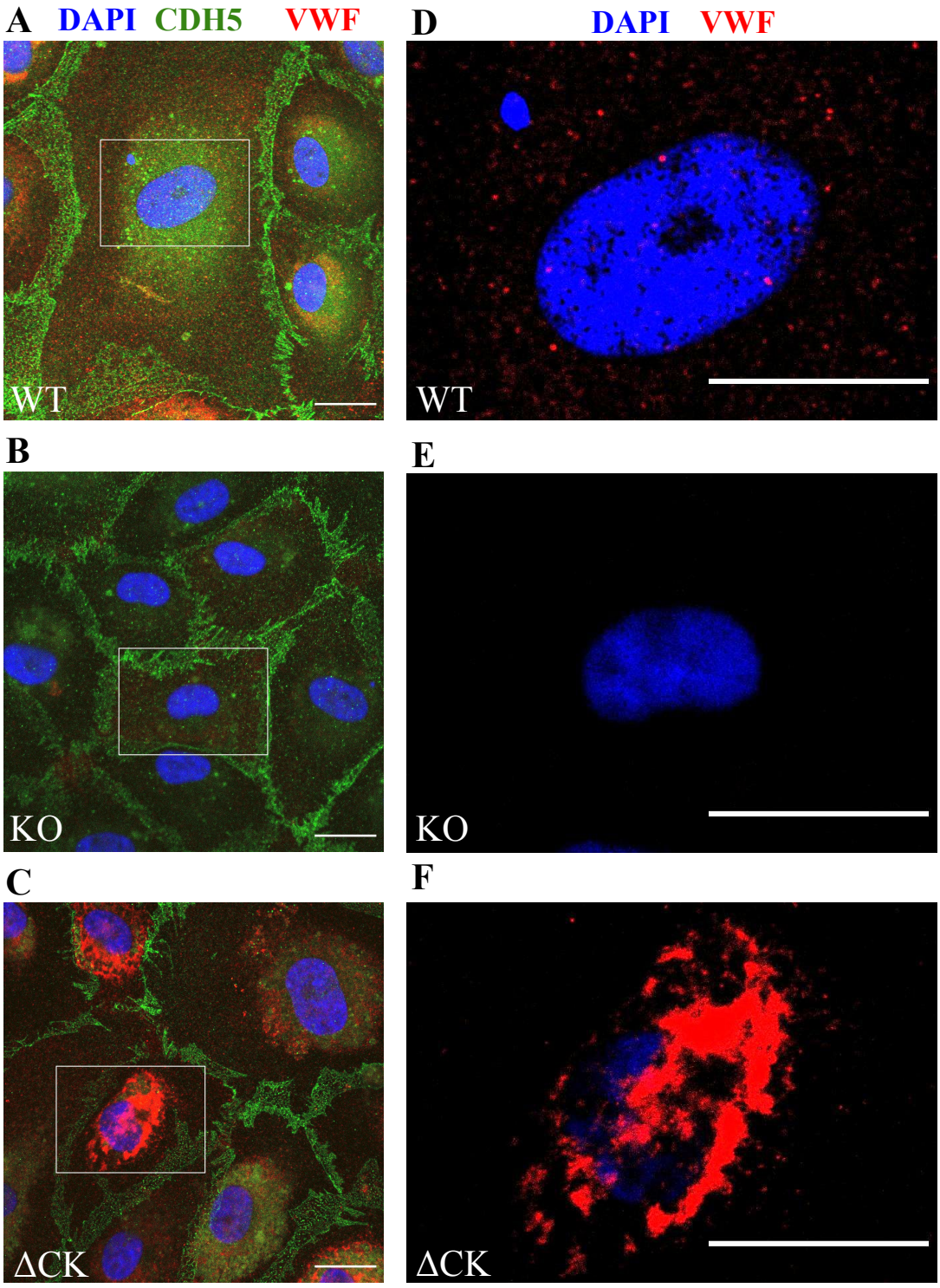


Figure 5.17 | Examples of intracellular VWF localisation. (A) - (C) Composite confocal images are shown for, respectively, WT, KO and ΔCK iECs which were stained with DAPI (blue), VWF (red), and CDH5 (green). (D) - (F) are zoomed in projections of the insets (in white boxes) of (A) - (C) respectively, showing a composite image of the blue and red channels only. The scale bar in all images represents 20 µm. The images were taken using x 63 objective with numerical aperture of 1.4 and x 2 zoom factor.

The ΔCK images showed that some cells expressed an intense cloud of VWF around the nucleus (Figure 5.16D, page 229; Figure 5.17C & F, page 230), in an intracellular position consistent with ER localisation. To assess this systematically, the 37 images from all clones were evaluated using the workflow outlined in Figure 5.18A (page 232). Four images (2 KO and 2 ΔCK) were excluded because they were too blurred to accurately delineate VWF localisation. In 71% of both KO and ΔCK iECs, VWF expression was absent in a significantly greater number of cells than WT (Fisher's Exact Test, $P < 1.05 \times 10^{-11}$ and $P < 2.11 \times 10^{-14}$ respectively) in which only 4% of cells did not express VWF (Figure 5.18B, page 232). In the ΔCK iECs that did express VWF (36/126 cells evaluated), in 72% of iECs this was expressed in a either perinuclear or combined perinuclear and punctate distribution. Conversely, in 53% of WT iECs, VWF was expressed in a punctate distribution, significantly more often than in ΔCK as this pattern was only found in 8% of iECs from this clone (Fisher's Exact Test, $P = 5.96 \times 10^{-10}$). These results indicate that the majority of WT iECs packaged mature VWF into WPBs. Moreover, ΔCK expressed VWF in fewer iECs, but where expressed it was generally retained in an immature (perinuclear) form.

What is more difficult to explain is why 29% of KO iECs were categorised as containing VWF in a punctate pattern, despite the sequencing results confirming the insertion of termination codons, in both alleles, ~ 40 amino acids from the ATG codon (Figure 5.14, page 225). My suspicion is that this results from non-specific binding and fluorescence from either the rabbit polyclonal primary VWF antibody and/or from anti-rabbit Alexa Fluor 633 secondary antibody. There are three reasons why I arrived at this conclusion. First, there was no red background fluorescence on unstained WT iECs (Figure 5.16A, page 229). Second, KO iECs did not express any VWF in a perinuclear position whereas both WT iECs and ECFCs did express some VWF in this location (Figure 5.16B, page 229; Figure 5.17A, page 230; Figure 5.5, page 206), as do the WT ECFCs of other investigators (Starke et al., 2013; Wang et al., 2013b). This is because in WT ECs, VWF has to progress through all steps of its biosynthetic pathway including transiting through the ER. Therefore, the absence of perinuclear staining in KO iECs would suggest that the

Figure 5.18

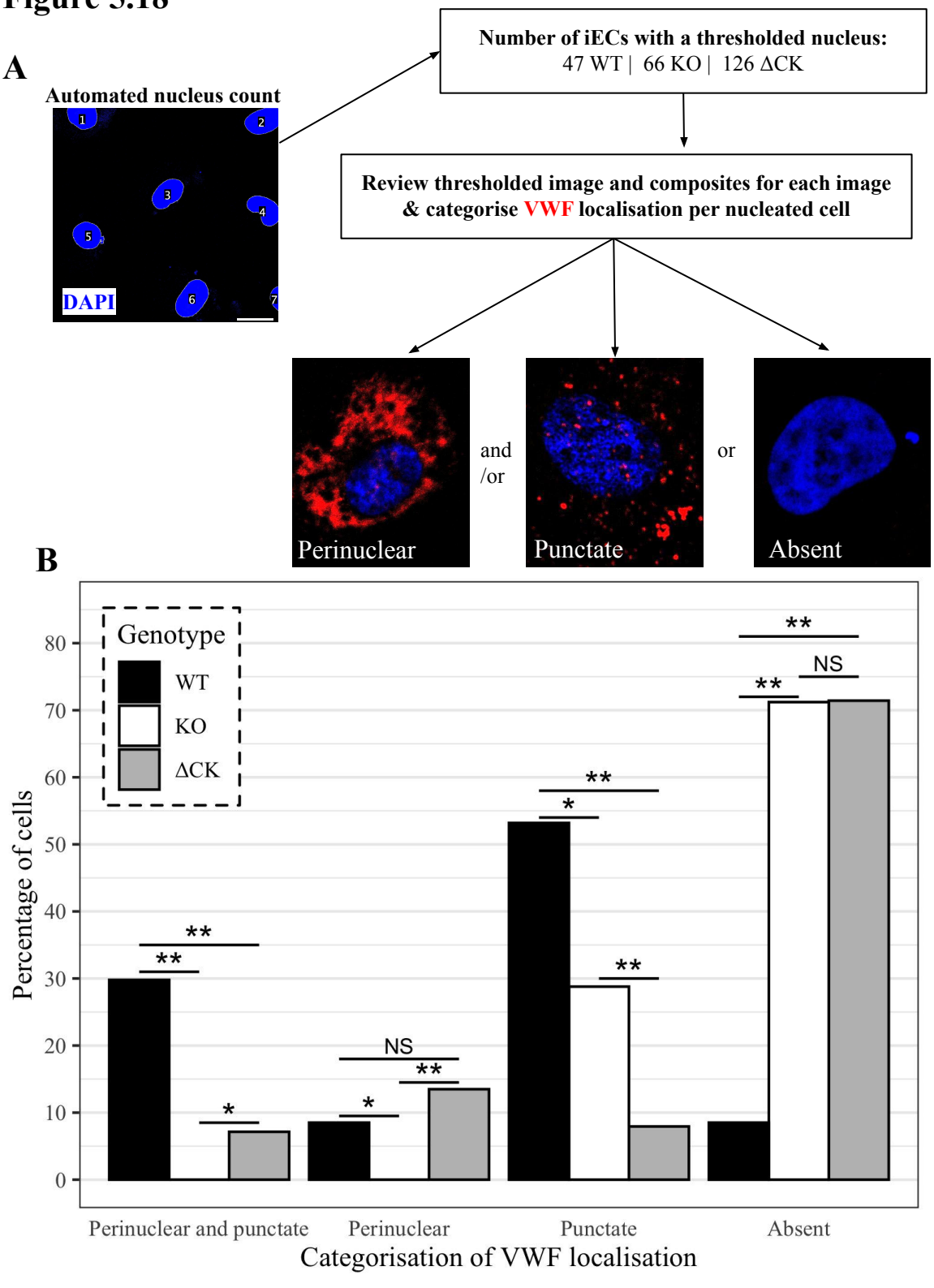


Figure 5.18 | Quantification of intracellular VWF localisation. (A) Workflow for evaluation of images. The scale bar represents 20 µm. (B) A bar graph of the number of iECs assigned to each of the four VWF localisation categories as a percentage of the total number of iECs for the given genotype. Abbreviations of the outcome of Fisher's Exact Test: NS = Not significant; * $P < 0.05$; ** $P < 0.01$.

punctate staining counted using my method was likely to be non-specific. Third, the non-specific fluorescence with the same polyclonal VWF antibody has been shown in a mouse VWF KO model which otherwise recapitulates the phenotype of VWD3 because of undetectable plasma VWF:Ag (Denis et al., 1998).

5.6. Conclusions

In NBR1 ECFCs, the presence of the c.8155+6T>A (p.Gly2706ValfsTer25) mutation on both alleles resulted in a greater number of cells in which VWF was absent and, where detected it was stuck in a perinuclear position consistent with ER retention. Despite the different cellular contexts (iEC versus ECFC), genetic backgrounds (European versus South Asian ancestry), and nature of the variants (CRISPR/Cas9 edited versus naturally occurring), biallelic c.8139_8140insA (p.Thr2714AsnfsTer4), 'ΔCK' phenocopied the abnormal intracellular distribution of VWF seen in NBR 1. The common denominator for both p.Gly2706ValfsTer25 and p.Thr2714AsnfsTer4 is that, based on their nucleotide sequence, the distal 100 amino acids of VWF are removed including the whole CK domain (which comprises 93 amino acids). Therefore, my results indicate that the intracellular phenotype I observed in both NBR 1 ECFCs and ΔCK iECs may be a more general reflection of the way ECs handle VWF lacking CK. As outlined in the next paragraphs this has implications for understanding of the importance of C-terminal dimerisation in the VWF biosynthetic pathway and for PAVVs immediately proximal to or involving CK.

As outlined in the introduction (Subsection 1.1.5, page 23), N-glycosylated and folded proVWF monomers form inter-unit disulfide bridges between three cysteine residues (2771, 2773, and 2811) located in the CK of two different molecules (Katsumi et al., 2000; Zhou & Springer, 2014). This tail-to-tail coupling is called C-terminal dimerisation (Sadler, 1998). What is still unresolved is whether CK-mediated dimerisation is a prerequisite for VWF from exiting the ER with some investigators arguing that it is (Voorberg et al., 1991), whereas others suggest that it is not (Wagner et al., 1991). However both of these studies were carried out in heterologous, non-human cells (from monkey kidney and mouse pituitary). My data suggests that in human endothelial cells, where the majority of VWF is produced, the removal of the CK is not compatible with ER

exit and packaging of VWF into WPBs. However the following assays on iECs would experimentally validate this: multimer gel electrophoresis and EndoH and PNGase on iEC lysates; colocalisation microscopy with antibodies directed against the ER, Golgi and WPBs.

The fact that the majority of both NBR 1's ECFCs and ΔCK iECs did not contain any detectable VWF remains unexplained. As discussed in Chapter 4 (Section 4.6, pages 193-194), nonsense mediated decay is not likely to be the mechanism for a termination codon close to the carboxy-terminal domain of VWF. Proteasomal degradation is plausible. Lactacystin is an inhibitor of the proteasome and when added to the medium of baby hamster kidney cells transfected with Cys1149Arg (a variant that causes ER retention through a CK-independent mechanism) it prolonged the degradation time of VWF by at least 16 hours (Bodó et al., 2001). Another potential mechanism is that ΔCK variants alter stochastic phenotype switching. This is the name given to the process by which ECs toggle in and out of *VWF* expression, due to alterations in methylation status of the *VWF* promoter attributed to the background noise of cellular processes (Yuan et al., 2016). Potential ways to explore this further would be to insert a fluorescence tag at the 3' end of the endogenous *VWF* locus in QOLG_3 hiPSCs and then perform time-lapse, live cell imaging. This could be coupled with bisulfite sequencing in order to assess the methylation state of the *VWF* promoter in ΔCK compared to both KO and WT lines.

In order to obtain a better understanding of the implications of my work in the context of VWD, I used VWDbase to extract PAVVs which were positioned at or 3' of the first nucleotide of exon 50. I obtained a list of 32 rare variants, of which 7 comprised the intron 50-51 donor splice site variants, including c.8155+6T>A (Figure 4.14, page 180), all of which would be expected to result in p.Gly2706ValfsTer25. Of the remaining 25 variants, four were also identified in homozygosity in patients with VWD3: p.Cys2715Ter (Kasatkar et al., 2014), p.His2748_Cys2750del (Baronciani et al., 2000), p.Cys2804Tyr (Baronciani et al., 2000), and p.Pro2808LeufsTer24 (Bowman et al., 2013; Bowman et al., 2017). Although based on a limited number of studies, these publications and others detailing relevant functional experiments (Schneppenheim et al., 1996; Wang et al., 2011)

allow a couple of conclusions to be drawn. First, with respect to the role of CK cysteine residues in dimerisation, the multitude of different cell systems used and mutations evaluated means that it is challenging to come to a conclusion about which are necessary for CK dimerisation and those which are redundant at least in some systems, as there are a number of instances of conflicting results. For example Cys2771: COS-7 cells transfected full length Cys2771Ser or Cys2771Tyr result in low and intermediate multimers (Enayat et al., 2001), whereas when these are transfected with a Cys2771Ala mutated CK fragment dimerisation failure is observed (Katsumi et al., 2000). Second, of the 32 variants identified, only p.Pro2808LeufsTer24 was investigated in endothelial cells showing a diffuse perinuclear VWF staining with minimal discernible WPBs (Bowman et al., 2017). To really understand what these variants are doing in vivo and answer important questions about the relative importance of different CK cysteine residues in dimerisation, there is a need to examine more of these variants in endothelial cells. My data shows that genome edited QOLG_3 hiPSCs with subsequent differentiation to iECs would be a suitable and more physiologically relevant context in which to do this.

There are several limitations of the work presented in this chapter. With regard to the ECFC experiments, although the immunophenotype was consistent with ECs, a positive control for the EC markers (such as HUVECs) and the hematopoietic markers (such as blood monocytes), would have provided further confirmation of this, as would additional functional assays such as the uptake of acetylated LDL and tube formation on matrigel (Decaris et al., 2009). As for the CRISPR/Cas9 and iEC experiments, I did not fully take into account the possibility of off-target effects of the CRISPR guides. One potential strategy that I could have used would have been to analyse the clones by WGS post single cell selection and expansion. In order to address the challenge of distinguishing punctate staining from background in the confocal microscopy of iECs, strategies I could have employed include dose titration of the primary VWF antibody and use of an isotype control.

6. Discussion

VWF was one of the human first genes that was successfully mapped to a chromosome, cloned, and sequenced (Ginsburg et al., 1985; Verweij et al., 1985). This early achievement and the subsequent wealth of sequence variants in *VWF* linked to VWD has been both a blessing and a curse. On the one hand, important structure-function relationships have been revealed by the consequences of casual VWD variants in specific domains, but on the other hand many variants were reported before the tools to critically appraise variant pathogenicity were available. I brought these tools to bear on the variants I collated into VWDbase, the largest ever assembled collection of *VWF* variants with a reported link to VWD. Leveraging the combination of WES data from over 500,000 UKB and gnomAD alleles with systematic multidisciplinary (MDT) scrutiny of published data revealed that nearly half of the variants previously identified as being pathogenic for VWD did not meet the criteria I set for this definition (VWF:Act or VWF:Ag level < 0.30 IU/ml and MAF < 1 in 10,000; Figure 3.5, page 117). However, because my analysis was not designed to determine the effect of *VWF* variants with MAFs ≥ 1 in 10,000, it is plausible that some of those which fall into this group do reduce VWF to a level where it is in the 0.30 - 0.49 IU/ml range, which, in the presence of bleeding, has been proposed to be diagnostic of VWD (James et al., 2021) (Table 1.2, page 40). Furthermore such variants could be acting in concert with others in *VWF* and other trans-acting genes that have been shown to affect VWF levels (Table 1.4, page 52). As discussed below, polygenic risk scores would be a potential way forward to take into account their effects.

One of the important take-home messages from the MAF analysis of UKB PAVVs across ancestral groups was that 19 (out of a total of 194 analysed) were rejected by the MDT as being pathogenic (Figure 3.9, page 126) because of their frequency of ≥ 1 in 10,000 in at least one of the four non-European ancestral super-populations evaluated. This highlights the need for authors and clinicians that report on potentially causal VWD variants to consider the (genetically determined) ancestry of the individual in whom the variant has been identified and consider its MAF in an appropriately matched population, especially now that NGS base gene panels, WES and WGS are in widespread use. The aggregated

ancestry-specific MAFs I determined for UKB PAVVs offer an interim solution until, in a decade or so, more data will be available thanks to the rapidly increasing number of individuals having their genome, or part of it, sequenced (for example, since my analysis the UKB has released WGS data for 200,000 participants).

A challenge for researchers analysing large scale population cohorts such as UKB is understanding how a variant, or group of variants, impact(s) on a phenotype of interest. With thrombotic sequelae (such as deep venous thrombosis) this is relatively straightforward because of the availability of defined tests (e.g. ultrasound doppler of leg veins) and ICD-10 codes. However, in the case of bleeding this is more difficult because of the diverse systems and situations in which bleeding occurs.

To tackle this problem I developed the ICD-BAT score. Inspired by the BAT questionnaires used to assess patients with suspected bleeding disorders in clinic, the ICD-BAT provided a way of mapping 97 ICD-10 BAT codes to relevant groups of bleeding symptoms (Figure 4.2, page 145). I used the ICD-BAT to demonstrate that in the ~ 118,000 UKB participants that I analysed (the UKB Bleeding Assessment Cohort [BAC]), 1 in 5 UKB participants had a hospital-coded bleeding episode during a 23 year follow-up period. I then illustrated how, even for UKB participants in the genetically accepted (ga) VWD group, who were heterozygous for PAVVs accepted by the MDT as being pathogenic (Figure 4.5, page 150), there was no detectable difference in the ICD-BAT score or bleeding-free survival compared to ~ 115,000 control participants. This may reflect an underrepresentation of the most deleterious alleles in the UK Biobank (Dr Luca Stefanucci, personal communication) and a generally healthier population (that is perhaps less prone to bleeding).

There were 401 participants in the UKB BAC who were in the gaVWD group. This is equivalent to ~ 1 in 300 individuals. Thus, the prevalence of gaVWD in UKB is close to estimates of population prevalence of VWD between ~ 1 in 200 and ~ 1 in 500 depending on whether a threshold of 0.50 IU/ml or 0.30 IU/ml is used (Table 1.2, page 40), respectively, but 20-times higher than UK referral-based prevalence of ~ 1 in 5,900. Based

on my clinical experience, VWD diagnosis is phenotype-driven, prompted by abnormal bleeding or a finding of reduced VWF levels (e.g. during screening of family members of individuals with VWD). Therefore, one potential explanation for the discrepancy between referral and population prevalence is that individuals heterozygous for pathogenic VWD variants are for the most part phenotypically silent, but in the presence of additional risk factors for lowered VWF levels and/or bleeding then they come to clinical attention and are referred on to a haemophilia centre. Under this multi-hit model, risk factors include O blood group and female sex, both of which are overrepresented in VWD1 populations of referral centres (Cumming et al., 2006; Federici, 2004).

The plasma proteome of UKB participants is being systematically evaluated using the high throughput assay, Olink®. The first results have recently been made available on bioRxiv for the first ~54,000 participants which confirm the association between *ABO* and VWF (Sun et al., 2022) In the future these results could be used to develop a polygenic risk score for VWF levels, akin to that developed for venous thromboembolism in the same cohort (Klarin et al., 2019), which would take into consideration the effect sizes of all significant protein quantitative trait loci (Table 1.4, page 52). If this method proves successful it may help to shed light on how the cumulative effect of common variants (with MAFs > 0.01) interplays with rare VWD-causing *VWF* variants to modulate VWF levels. Furthermore, with the ICD-BAT I have demonstrated that bleeding episodes can be quantitated from electronic health records in the UKB and that histo-group O participants had a significant increase in the bleeding score on this scale (Table 4.2, page 155), commensurate with the finding that the *O* allele reduces relative risk for thrombosis (Klarin et al., 2019). An approach such as the ICD-BAT could be used, genome-wide, to establish determinants for bleeding risk which could then provide additional explanations as to why some individuals with pathogenic VWD variants come to medical attention and some do not.

In Chapter 5 I show, for the first time, how CRISPR/Cas9-edited hiPSC-derived endothelial cells (iECs) can be used to model VWD, as exemplified by the perinuclear retention phenotype of the ΔCK clone. Although there are a number of additional assays needed to confirm my hypothesis of ER-retention of VWF in ΔCK (Section 5.6, pages

233-234), my findings did further strengthen previous observations that, in the context of VWD3, there are both true null alleles, which, when biallelic result in no VWF expression at all, and other alleles, such as c.8155+6T>A found in NBR1, which result in an abnormal distribution of intracellular VWF that fails to be released into the bloodstream. As discussed, this is likely to be the case for nonsense and frameshift variants just proximal to and within the CK domain (Section 5.6, pages 233-235). Therefore, I propose a molecular classification of VWD3 into 'cell-retained' and 'cell-absent' VWF phenotypes. Such a classification would help to delineate whether there are any repercussions from the (suspected) ER accumulation of VWF in the cell-retained group. These could include the activation of the unfolded protein response (reviewed in Walter and Ron, 2011) known to be relevant to human diseases (Chambers and Marciniak, 2014). I planned to assess this hypothesis during my PhD, but unfortunately laboratory closures during COVID lockdowns curtailed these plans. This is a relevant area for further research as there are a number of phenotypes in VWD where we have limited understanding - including a lack of protection against cardiovascular disease (Holm et al., 2018) and impaired wound healing (Ishihara et al., 2019) - that could be related to the EC phenotype in patients with VWD and the potential for this to be dysregulated.

The iEC approach I have developed for the exploration of *VWF* variants in the QOLG_3 hiPSC line has broader implications. As highlighted, there are examples of conflicting results obtained when variants or modifications to VWF are modelled in different, often non-human, non-EC, cell lines (Section 5.6, pages 234-235). Genome-edited QOLG_3 hiPSCs with subsequent differentiation to iECs provide a scalable system for the parallel investigation of multiple *VWF* variants against an isogenic control. Ideally, in the future, further WT lines would provide the means to assess variants in different genetic backgrounds, and deriving iECs from VWD patient hiPSCs would open the door to 'rescue' experiments in which a genetic variant is edited back to the WT allele thus providing orthogonal validation of its pathogenicity. One of the main barriers to this, which future studies will need to address, is the variable propensity for hiPSC lines to be differentiated to ECs as I showed in my optimisation experiments (Figure 5.7C, page 211).

To conclude, the work I present in this thesis demonstrates how phenotype and genotype data from hundreds of thousands of individuals can improve our understanding of rare, potentially causal, VWD variants. When contextualised in this way, I have highlighted that prior assumptions about pathogenicity do not always hold. WGS is being rolled out in both research and clinical settings at rapid pace, with increased capacity to link these data to clinical and laboratory information. As a result, it is incumbent upon scientists and clinicians working in the haemostasis field to regularly re-scrutinise the evidence base that supports whether a variant causes VWD. Furthermore, we should continue to develop and optimise in vitro methods that model VWD variants in human endothelial cells as these provide a unique method to discriminate between more subtle intracellular effects.

7. Appendices

7.1. Bleeding assessment tool score

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

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

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

Table 7.1 | ISTH-BAT score. Reproduced from Rodeghiero et al., 2010.

7.2. Referral-based VWD prevalence

Author[1]	Date	No. VWD patients	Country (region)[2]	Population (x 10^6, 1 d.p.)[3]	Prevalence (%, 2 s.f.)
WFH	2020	2	Afghanistan	38.9	0.0000051
WFH	2020	7	Albania	2.8	0.00025
WFH	2020	526	Algeria	43.9	0.0012
Lazzari[4]	1998	33	Argentina	33.0	0.00010
WFH	2020	397	Argentina	45.4	0.00087
WFH	2020	15	Armenia	3.0	0.00051
Rodgers	2011	103	Australia (South Australia)	1.3	0.0079
WFH	2020	2,324	Australia	25.7	0.0090
WFH	2020	2	Bahamas	0.4	0.00051
WFH	2020	5	Bangladesh	164.7	0.0000030
WFH	2020	5	Barbados	0.3	0.0017
WFH	2020	194	Belarus	9.4	0.0021
WFH	2020	2,202	Belgium	11.6	0.019
WFH	2020	3	Bolivia	11.7	0.000026

WFH	2020	7	Botswana	2.4	0.00030
IEHASC[4]	1998	57	Brazil	150.0	0.000038
WFH	2020	9,768	Brazil	212.6	0.0046
WFH	2020	0	Burkina Faso	20.9	0
WFH	2020	7	Cambodia	16.7	0.000042
WFH	2020	5	Cameroon	26.5	0.000019
James	2011	3,500	Canada	34.0	0.010
WFH	2020	4,709	Canada	38.0	0.012
Cabrera[5]	1989	83	Chile	NA	0.0113
Scroppo[4]	1998	256	Chile	14.0	0.0018
WFH	2020	708	Chile	19.1	0.0037
Riaza[4]	1997	47	Colombia	37.0	0.00013
WFH	2020	3,446	Colombia	50.9	0.0068
WFH	2020	93	Costa Rica	5.1	0.0018
WFH	2020	3	Côte d'Ivoire	26.4	0.000011
WFH	2020	473	Cuba	11.3	0.0042
WFH	2020	779	Czech Republic	10.7	0.0073
WFH	2020	2	Djibouti	1.0	0.0002
WFH	2020	52	Dominican Republic	10.8	0.00048
Weilbauer[4]	1998	27	Ecuador	12.0	0.00023
WFH	2020	99	Ecuador	17.6	0.00056
El Shinnawi[4]	1998	239	Egypt	62.0	0.00039
WFH	2020	614	Egypt	102.3	0.00060
WFH	2020	2	El Salvador	6.5	0.000031
WFH	2020	128	Estonia	1.3	0.0096
WFH	2020	0	Ethiopia	115.0	0
WFH	2020	563	Finland	5.5	0.010

Baudauer	2004	31	France (Basque)	0.3	0.010
WFH	2020	2,992	France	67.4	0.0044
Lenk	1988	111	Germany (South GDR[6])	10.9	0.0010
WFH	2020	3,498	Germany	83.2	0.0042
WFH	2020	55	Georgia	3.7	0.0015
WFH	2020	11	Ghana	31.1	0.000035
WFH	2020	1,210	Greece	10.7	0.011
WFH	2020	21	Honduras	9.9	0.00021
WFH	2020	1,418	Hungary	9.7	0.015
Srivastava[4]	1998	183	India	960.	0.0000190625
WFH	2020	743	India	1.4	0.000054
WFH	2020	20	Indonesia	273.5	0.0000073
Lak[4]	1998	600	Iran	60.0	0.0010
Mansouritorghabeh	2013	50	Iran (North-East)	5.5	0.00091
WFH	2020	1,755	Iran	84.0	0.0021
WFH	2020	671	Iraq	40.2	0.0017
WFH	2020	1,762	Ireland	5.0	0.035
WFH	2020	175	Israel	9.2	0.0019
WFH	2020	3,245	Italy	59.6	0.0054
WFH	2020	3	Jamaica	3.0	0.00010
WFH	2020	1,438	Japan	125.8	0.0011
Awidi	1992	65	Jordan	3.0	0.0022
WFH	2020	263	Jordan	10.2	0.0026
WFH	2020	37	Kenya	53.8	0.000069
WFH	2020	71	Latvia	1.9	0.0037

Djambas Khayat	2014	13	Lebanon (Bekaa Valley)	0.3	0.0043
WFH	2020	178	Lebanon	6.8	0.0026
Jurgutis[4]	1998	30	Lithuania	3.7	0.00081
WFH	2020	305	Lithuania	2.8	0.011
WFH	2020	2	Madagascar	27.7	0.0000072
Duraiswamy[4]	1997	21	Malaysia	21.0	0.0001
WFH	2020	133	Malaysia	32.3	0.00041
WFH	2020	23	Mali	20.3	0.00011
WFH	2020	46	Malta	0.5	0.0088
WFH	2020	1	Mauritania	4.6	0.000022
WFH	2020	1	Mauritius	1.3	0.000079
Zavelia Padilla-Romo	2021	36	Mexico (Guadalajara)	5.0	0.00072
WFH	2020	355	Mexico	128.9	0.00028
WFH	2020	14	Mongolia	3.3	0.00043
WFH	2020	3	Montenegro	0.6	0.00048
WFH	2020	173	Morocco	36.9	0.00047
WFH	2020	4	Mozambique	31.2	0.000013
WFH	2020	9	Nepal	29.1	0.000031
WFH	2020	460	Netherlands	17.4	0.0026
WFH	2020	90	New Zealand	5.1	0.0018
WFH	2020	60	Nicaragua	6.6	0.00091
WFH	2020	13	Nigeria	206.1	0.0000063
Berntorp	2005	1643	Nordic countries[7]	20.8	0.0079
WFH	2020	601	Norway	5.4	0.011
Khanduri[4]	1998	53	Oman	1.8	0.0030
Alkaabi	2019	140	Oman	NA	0.005

Borhany	2011	68	Pakistan	16.0	0.000425
Hussain	2018	172	Pakistan	193.0	0.000089
WFH	2020	389	Pakistan	220.9	0.00018
WFH	2020	59	Palestine	4.8	0.0012
WFH	2020	521	Panama	4.3	0.012
WFH	2020	4	Paraguay	7.1	0.000056
WFH	2020	209	Peru	33.0	0.00063
WFH	2020	46	Philippines	109.6	0.000042
Milewska[4]	1998	200	Poland	38.0	0.00053
WFH	2020	2,250	Poland	38.0	0.0059
WFH	2020	911	Portugal	10.3	0.0088
WFH	2020	44	Qatar	2.9	0.0015
WFH	2020	325	Romania	19.3	0.0017
WFH	2020	2,738	Russia	144.1	0.0019
Ayas[4]	1998	25	Saudi Arabia	12.0	0.00021
WFH	2020	396	Saudi Arabia	34.8	0.0011
WFH	2020	13	Senegal	16.7	0.000078
WFH	2020	317	Serbia	6.9	0.0046
Tien[4]	1998	36	Singapore	3.0	0.0012
WFH	2020	93	Singapore	5.7	0.0016
WFH	2020	765	Slovakia	5.4	0.014
Batarova[4]	1998	235	Slovakia	5.3	0.0044
Kubisz	2017	610	Slovakia	5.4	0.011
WFH	2020	189	Slovenia	2.1	0.0090
Karabus[4]	1998	283	South Africa	38.0	0.00074
WFH	2020	659	South Africa	59.3	0.0011
WFH	2020	152	South Korea	51.8	0.00029

WFH	2020	714	Spain	47.4	0.0015
WFH	2020	67	Sri Lanka	21.9	0.00031
WFH	2020	367	Sudan	43.8	0.00084
WFH	2020	3	Suriname	0.6	0.00051
Nilsson	1984	530	Sweden[8]	NA	0.007
WFH	2020	908	Sweden	10.4	0.0088
WFH	2020	149	Switzerland	8.6	0.0017
WFH	2020	133	Syria	17.5	0.00076
WFH	2020	6	Tanzania	59.7	0.000010
Chuansumrit[4]	1998	60	Thailand	60.0	0.0001
WFH	2020	150	Thailand	69.8	0.00021
WFH	2020	233	Tunisia	11.8	0.0020
WFH	2020	3	Uganda	45.7	0.0000066
WFH	2020	469	Ukraine	44.1	0.0011
WFH	2020	11,183	United Kingdom	67.2	0.017
WFH	2020	8,919	United States	329.5	0.0027
Soucie	2021	24238	United States	NA[9]	0.0085
Flood	2004	1000	United States (Milwaukee)	4.0	0.025
WFH	2020	263	Uruguay	3.5	0.0076
WFH	2020	177	Uzbekistan	34.2	0.00052
Diez-Ewald[10]	1991	96	Venezuela (Zulia & Maracaibo)	NA	0.005795
De Bosch[4]	1998	511	Venezuela	20.0	0.0026
WFH	2020	1,184	Venezuela	28.4	0.0042
WFH	2020	184	Vietnam	97.4	0.00019
Baklaza[4]	1998	415	Yugoslavia	10.0	0.0042
WFH	2020	5	Zambia	18.4	0.000027

Coutts[4]	1998	23	Zimbabwe	10.0	0.00023
WFH	2020	1	Zimbabwe	14.9	0.0000067

Table 7.2 | Referral-based prevalence of VWD. [1]First author of manuscript or report. [2]The region that was focussed on in the report is specified in parentheses, if this is not specified then the population of the whole country was the denominator. [3]The denominator population provided in the source. This was not necessarily the population of the entire country if the focus of the report was referrals from a specific region within that country as specified in the previous column. [4]From Table 4, Sadler et al., 2000. [5]Only the abstract was obtained; 11.3 per 100,000 prevalence was extracted from Berntorp and Önundarson, 2005. [6]German Democratic Republic. [7]Denmark, Finland, Sweden, and Iceland. [8]Only the abstract available which did not provide the data; therefore this was extracted from Table 2, Rodeghiero & Castaman, 2001. [9]US population over the 2012-2019 period used as a denominator. [10]Only the abstract was obtained. Figures provided per 1,000,000 participants in two regions: Zulia State and Maracaibo, which were then averaged.

7.3. Resources

Package	Version	Package	Version
Car	3.0-12	ordinal	2019.12-10
Circlize	0.4.14	patchwork	1.1.1
countrycode	1.3.0	pheatmap	1.0.12
DescTools	0.99.44	plotly	4.10.0
DiagrammeR	1.0.8	RcolorBrewer	1.1-2
Facetscales (github)	0.1.0.9000	readxl	1.3.1
ggfortify	0.4.14	rvest	1.0.1
ggpattern	0.2.0	seqinr	4.2-8
ggpubr	0.4.0	shiny	1.7.1
ggVennDiagram	1.2.0	shinydashboard	0.7.2
grid	3.6.3	survminer	0.4.9
hrbrthemes	0.8.0	survival	3.2-13
magrittr	2.0.1	tidyverse	1.3.1
maps	3.4.0	treemap	2.4-3
MatchIt	4.3.4	viridis	0.6.2
networkD3	0.4		

Table 7.3 | R packages. The table lists packages installed in addition to those supplied with base R. The majority of packages were installed from the Comprehensive R Archive Network (CRAN). The default dependencies for each of the packages in the table were installed at the same time as each package but are not listed separately.

Materials are listed in alphabetical order in the tables. To avoid duplication, materials common to multiple experiments are in Table 7.4. All subsequent tables follow the order of the laboratory experiments in Section 2.2.

Name	Supplier	Catalogue number
Accutase	Biolegend	423301
Agarose	Sigma-Aldrich	A5939
Ammonium chloride	Sigma-Aldrich	D8537
Antioxidant (NuPAGE®)	Invitrogen	NP0005
BioRad protein assay	BioRad	500-0006
BisTris 4-12%, 1.0mm, 15 well gels (NuPAGE®)	Invitrogen	NP0323Box
Blocking buffer (Odyssey)	LI-COR Biosciences	927-50000
Blot paper (Extra Thick) Blot Filter Paper, Precut, 19 x 18.5 cm	BioRad	1703969
BLUeye Pre-Stained Protein Ladder	Geneflow	S6-0024
BSA Protease-free heat shock treated powder	Fisher scientific	BP9703-100
C-Chip disposable Haemocytometer slides	VWR	631-1098
CDH5 mouse monoclonal IgG1 antibody (F8)	Insight Biotech	SC9989
CDH5-FITC ab (Mouse anti-human CD144 IgG1κ)	BD Pharmingen	560411
Chloroform	Invitrogen	15596026
CompBead Plus compensation particles (Anti-mouse)	BD Pharmingen	560497
DAPI	Sigma-Aldrich	10236276001
D-PBS	Sigma-Aldrich	D8537
DNA Gel Loading Dye (6X)	Thermo Fisher	R0611
DTT	NuPage	NP0004
EDTA (0.5M) solution	CIMR[1]	N/A
Endothelial Cell Growth Basal Medium-2	Lonza	CC-3156
EGM™-2 MV Microvascular Endothelial Cell Growth Medium-2 SingleQuots™	Lonza	CC-4147
Fast-Media® Amp Agar X-Gal	Invivogen	fas-am-x

FcR block	Miltenyi Biotec	130-059-901
Gelatin	VWR	440454B
Glycerine (≥99%, Electran Molecular biology grade)	VWR	444485B
Glycine powder	Fisher Scientific	BP381-1
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, AF633	Thermo Fisher	A21070
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, AF488	Thermo Fisher	A11001
Human albumin solution (20%)	Fisher Scientific	GEM-800-121-S
Ibidi 12 well chamber, removal	Ibidi	81201
Immobilon®-FL PVDF Membrane (pore size 0.45 µm)	Sigma	IPFL00010
IRDye goat anti rabbit antibody 680	LI-COR	925-68071
LDS Sample Buffer	Invitrogen	NP0007
Lowenstein broth	CIMR	N/A
Maxtract heavy phase lock tubes	Qiagen	129056
Methanol	Fisher Scientific	M/4056/17
Mouse IgG1 k-FITC ab [isotype control for CDH5 ab]	BD Pharmingen	555748
Mouse IgG1-APC ab [isotype control for PECAM1 ab]	Thermo Fisher	MA5-18093
Mowiol®	Sigma-Aldrich	81381
NuPAGE® MOPS SDS running buffer (20 x)	Invitrogen	NP0001
Odyssey® FC Imaging System	LI-COR	2800
PECAM1-APC ab (Mouse anti-human CD31 IgG1κ, clone WM-59)	Fisher Scientific	17-0319-41
Penicillin-Streptomycin (10,000 U/mL) (Gibco™)	Fisher Scientific	11548876
Phusion™ High-Fidelity (HF) DNA Polymerase	New England Biolabs (NEB)	M0530AA
Phusion™ HF 5 x buffer	NEB	B05185
Plasmid Maxi Kit	Qiagen	12163
Polypropylene flow cytometry tubes	SLS[2]	352002
QIAprep Spin Miniprep Kit	Qiagen	27104
Qubit™ broad range high sensitivity RNA Assay Kit	Thermo Fisher	Q32855

Quick-load® 100 bp DNA ladder	NEB	N05515
Saponin from quillaja bark	Sigma-Aldrich	S-7900
Semi-dry transfer cell (Trans-Blot®)	BioRad	1703940
SOC outgrowth medium	NEB	B9020
Sodium azide	Sigma-Aldrich	S8032
SYBR Safe DNA Gel Stain	Invitrogen	S33102
TBE	CIMR	N/A
TBS	CIMR	N/A
Trypan blue	Thermo Fisher	15250061
Tris (1M)	CIMR	N/A
Trizma base	Sigma	T1503
TRIzol™	Invitrogen	15596026
TrypLE™ express (1X), no phenol red	Thermo Fisher	12604021
Trypsin/EDTA solution	Lonza	CC-5012
Vacutainer® blood tubes (with 0.105 M buffered sodium citrate)	BD Pharmingen	367691
Von Willebrand factor antibody (Rabbit anti-human polyclonal)	DAKO	A0082

Table 7.4 | Common laboratory materials. [1]CIMR = Cambridge Institute of Medical Research, University of Cambridge. [2]SLS = Scientific Laboratory Supplies.

Name	Supplier	Catalogue number
Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit	Thermo Fisher	4368814
CD45 beads	Invitrogen	11153D

Table 7.5 | Platelet cDNA: experiment materials

Name	Supplier	Catalogue number
DpnI	NEB	R0176S
pcDNA3.1-WT-VWF	Addgene (a gift from Sriram Neelamegham)	124794

NEB®stable E.coli strain	NEB	C3040H
QuikChange XL Site-Directed Mutagenesis Kit	VWR	200516
XL10-Gold Ultracompetent E.coli	Fisher Scientific	200314

Table 7.6 | Site directed mutagenesis of p.Gly2706Ter: experiment materials

Name	Supplier	Catalogue number
Dulbecco's Modified Eagle Medium - high glucose	Sigma	D6429
Endoglycosidase H (EndoH)	NEB	P0702L
Heat-inactivated fetal bovine serum	Sigma	F9665
MEM Non-Essential Amino Acids Solution (100X) (Gibco™)	Fisher Scientific	12084947
Polyethyleneimine (PEI)	Sigma	408727
PNGaseF	NEB	P0704L
Tylosin solution (8mg/ml)	Sigma	T3397

Table 7.7 | p.Gly2706Ter transfection into HEK293T: experiment materials

Name	Supplier	Catalogue number
Acetic acid solution 2.0N	Sigma-Aldrich	A8976
CD14 AF700-conjugated ab (monoclonal mouse IgG1 clone # 134620)	R&D Systems	FAB3832N
CD45 AF405-conjugated ab (monoclonal mouse IgG1 clone # 998209)	R&D Systems	FAB14301V
Corning type 1 rat tail collagen	Fisher Scientific	354236
Ficoll® Paque Plus™	Sigma-Aldrich	17144002
Formaldehyde	Sigma-Aldrich	25249
Hyclone™ Defined Fetal Bovine Serum	Fisher Scientific	SH30071.02
Mouse IgG1 AF700-conjugated ab (monoclonal mouse IgG1 κ Clone # 11711) [isotype control for CD14 ab]	R&D Systems	IC002N
Mouse IgG1 AF405-conjugated ab (Monoclonal Mouse IgG1 κ Clone # 11711) [isotype control for CD45 ab]	R&D Systems	IC002V
Türk's solution	Sigma-Aldrich	1.09277.0100
Water for embryo transfer, sterile-filtered, suitable for mouse embryo culture	Sigma-Aldrich	W1503

Table 7.8 | ECFC-specific materials

Name	Supplier	Catalogue number
Apo-transferrin	Sigma-Aldrich	T1147
CD144 (VE-Cadherin) MicroBeads (referred to as CDH5-coated microbeads)	Miltenyi Biotec	130-097-857
CD34 mouse IgG2ak-PE	Miltenyi Biotec	130-113-179
Chemically defined lipid concentrate	Thermo Fisher	11905031
CHIR99021	SCI[1]	N/A
Dimethyl sulfoxide	Sigma-Aldrich	D2650
FGF2 50 µg/ml stock solution	SCI	N/A
Fibronectin	Corning	356008
F12 medium	Thermo Fisher	31765027
Forskolin	Sigma-Aldrich	F6886
IMDM medium	Thermo Fisher	21980032
IgMk FITC [isotype control for TRA-1-60 antibody]	BD Pharmingen	553474
Lab Armor™ beads	Thermo Fisher	A1254301
L-ascorbic acid	SCI	N/A
L-glutamine (200mM)	SCI	N/A
LS columns	Miltenyi Biotec	130-042-401
LY294002	Adooq BioScience	A01547
Matrigel	Corning	354230
MACS MultiStand	Miltenyi Biotec	130-042-303
MidiMACS™ separator	Miltenyi Biotec	130-042-302
Mouse IgG3k-PE [isotype control for SSEA-4]	BD Pharmingen	559926
Mouse IgG2a-PE [isotype control for CD34]	Miltenyi Biotec	130-081-002
KnockOut Serum Replacement	Thermo Fisher	10828-028
PVA	Sigma-Aldrich	P8136
SSEA-4 Mouse IgG3k-PE, clone MC813-70	BD Pharmingen	560128
StemFlex™ media (including supplement)	Thermo Fisher	A3349401

StemPro-34	Thermo Fisher	10639011
Stericup Quick Release-GP Sterile Vacuum Filtration System (0.22 µm pore size, polyethersulfone membrane)	Millipore	S2GPU02RE
TRA-1-60 Mouse IgM-FITC antibody	Millipore	FCMAB115F
30um pre-separation filter	Miltenyi Biotec	130-041-407
VEGFA (Human VEGF-165 Recombinant Protein)	Thermo Fisher	PHC9393
Vitronectin	Thermo Fisher	A14700
Y-27632	Millipore	SCM075

Table 7.9 | Differentiation of hiPSCs to endothelial cells (iECs). [1]Cambridge University Stem Cell Institute.

Name	Supplier	Catalogue number
KAPA Illumina SYBR Universal Lib Q. Kit	Roche	KK4824
KAPA stranded RNA sequencing kit with RiboErase	Roche	7962304001
HMVEC-L-Lung cryopreserved cells	Lonza	CC-2527
RNA 6000 Pico kit	Agilent	5067-1513
RNA clean XP beads	Beckman Coulter	A63987

Table 7.10 | RNA-seq: experiment materials

Name	Supplier	Catalogue number
Adrenaline 1mg/ml	MK medical	N/A
8W10E ECIS array	Ibidi	72010
GAPDH (14C10) Rabbit monoclonal antibody	Cell Signalling	2118
Thrombin	Sigma-Aldrich	T8885

Table 7.11 | QOLG_3 iEC VWF release and agonist response: experiment materials

Name	Supplier	Catalogue number
Cobalt dichloride ($CoCl_2$)	NEB	B0252S
DNeasy Blood & Tissue Kit	Qiagen	69582
Formaldehyde (16%, methanol free)	Thermo Fisher	28906

Human Stem Cell Nucleofector™ Kit 2	Lonza	VPH-5022
JM109 highly efficient competent cells	Promega	L2005
pGEM®-T-easy	Promega	A137A
Rapid ligation buffer	Promega	C661A
Terminal transferase	NEB	M03015L
Terminal transferase reaction buffer	NEB	B0315S
T4 DNA ligase	Promega	M180A
Zymo DNA Clean & Concentrator-5 columns	VWF	D4004

Table 7.12 | CRISPR/Cas9 of QOLG_3 hiPSCs and confocal imaging of iECs: experiment materials

7.4. VWDbase

In Table 7.13 below, POS refers to the GRCh38 position on chromosome 12. REF and ALT are the reference and alternate alleles. VWDbase assertion is in the right hand most column. PAVVs were defined as variants with a VWDbase summary assertion of 'Conflicting interpretations of pathogenicity' or Pathogenic (Figure 3.3, page 104). UKB PAVVs accepted by the MDT are in green, those with uncertain pathogenicity in grey, and rejected variants are in orange-red.

POS	REF	ALT	HGVSc	HGVSp	Assertion
6126975	T	C	c.-2555A>G	NA	Not provided
6126688	A	C	c.-2268T>G	NA	Not provided
6126464	C	G	c.-2044G>C	NA	Benign
6126347	A	C	c.-1927T>G	NA	Not provided
6126255	G	A	c.-1835C>T	NA	Not provided
6126233	T	C	c.-1813A>G	NA	Not provided
6125928	G	A	c.-1508C>T	NA	Pathogenic
6125927	G	A	c.-1507C>T	NA	Conflicting interpretation
6125911	C	T	c.-1491G>A	NA	Pathogenic
6125888	G	A	c.-1468C>T	NA	Not provided
6125837	C	T	c.-1417G>A	NA	Pathogenic
6125823	G	A	c.-1403C>T	NA	Pathogenic

6125811	T	C	c.-1391A>G	NA	Conflicting interpretation
6125731	C	T	c.-1311G>A	NA	Pathogenic
6125723	C	T	c.-1303G>A	NA	Benign
6125718	G	A	c.-1298C>T	NA	Conflicting interpretation
6125683	C	T	c.-1263G>A	NA	Pathogenic
6125524	A	C	c.-1104T>G	NA	Pathogenic
6125273	T	A	c.-853A>T	NA	Pathogenic
6125272	T	C	c.-852A>G	NA	Pathogenic
6125239	G	T	c.-819C>A	NA	Not provided
6125092	G	A	c.-672C>T	NA	Pathogenic
6125082	T	G	c.-662A>C	NA	Pathogenic
6125071	C	T	c.-651G>A	NA	Pathogenic
6125069	T	C	c.-649A>G	NA	Pathogenic
6124861	C	G	c.-441G>C	NA	Pathogenic
6124846	C	G	c.-426G>C	NA	Pathogenic
6124705	AAAAGGAAACAATG	A	c.-298_-286del	NA	Pathogenic
6124558	T	C	c.-138A>G	NA	Not provided
6124551	C	G	c.-131G>C	NA	Uncertain significance
6124548	C	T	c.-128G>A	NA	Pathogenic
6124532	A	T	c.-112T>A	NA	Benign
6124532	A	G	c.-112T>C	NA	Benign
6124527	G	C	c.-107C>G	NA	Uncertain significance
6124492	G	A	c.-72C>T	NA	Uncertain significance
6124418	T	G	c.-1+3A>C	NA	Pathogenic
6123334	C	C	c.1-138A>G	NA	Pathogenic
6123216	G	A	c.1-20C>T	NA	Benign
6123196	T	C	c.1A>G	p.Met1Val	Pathogenic
6123183	C	T	c.14G>A	p.Arg5Lys	Pathogenic
6123176	G	A	c.21C>T	p.Ala7=	Benign
6123146	C	CA	c.50dup	p.Leu17PhefsTer25	Pathogenic
6123142	C	T	c.55G>A	p.Gly19Arg	Pathogenic

6123134	G	T	c.55+8C>A	NA	Conflicting interpretation
6123127	C	T	c.55+15G>A	NA	Uncertain significance
6123073	T	C	c.55+69A>G	NA	Not provided
6121378	G	A	c.56-40C>T	NA	Benign
6121352	C	A	c.56-14G>T	NA	Uncertain significance
6121345	G	A	c.56-7C>T	NA	Uncertain significance
6121317	G	C	c.77C>G	p.Thr26Ser	Uncertain significance
6121294	G	C	c.100C>G	p.Arg34Gly	Pathogenic
6121294	G	A	c.100C>T	p.Arg34Ter	Pathogenic
6121293	CG	C	c.100del	p.Arg34AspfsTer49	Pathogenic
6121291	A	G	c.103T>C	p.Cys35Arg	Pathogenic
6121280	G	A	c.114C>T	p.Phe38=	Conflicting interpretation
6121279	C	T	c.115G>A	p.Gly39Arg	Pathogenic
6121255	C	G	c.139G>C	p.Asp47His	Pathogenic
6121254	T	A	c.140A>T	p.Asp47Val	Pathogenic
6121247	G	T	c.147C>A	p.Ser49Arg	Pathogenic
6121247	G	C	c.147C>G	p.Ser49Arg	Pathogenic
6121223	G	T	c.171C>A	p.Cys57Ter	Pathogenic
6121215	A	G	c.179T>C	p.Leu60Pro	Pathogenic
6121212	A	G	c.182T>C	p.Leu61Pro	Uncertain significance
6121202	GC	G	c.191del	p.Gly64AlafsTer19	Pathogenic
6121198	G	A	c.196C>T	p.Gln66Ter	Pathogenic
6121188	GA	G	c.205del	p.Ser69ProfsTer14	Pathogenic
6121182	G	T	c.212C>A	p.Ser71Ter	Pathogenic
6121174	C	T	c.220G>A	p.Gly74Arg	Pathogenic
6121074	C	T	c.220+100G>A	NA	Not provided
6111038	C	A	c.221-70G>T	NA	Not provided
6110960	G	A	c.229C>T	p.Gln77Ter	Pathogenic
6110953	C	A	c.236G>T	p.Gly79Val	Pathogenic
6110948	T	C	c.241A>G	p.Arg81Gly	Pathogenic
6110942	T	TA	c.246_247insT	p.Ser83Ter	Pathogenic
6110939	G	A	c.250C>T	p.Leu84Phe	Conflicting interpretation

6110936	A	G	c.253T>C	p.Ser85Pro	Pathogenic
6110934	G	A	c.255C>T	p.Ser85=	Uncertain significance
6110929	T	G	c.260A>C	p.Tyr87Ser	Pathogenic
6110912	CA	C	c.276del	p.Phe92LeufsTer11	Pathogenic
6110912	C	CA	c.276dup	p.Asp93Ter	Pathogenic
6110896	ACAAA	ATGT	c.289_292delinsACA	p.Phe97ThrfsTer6	Pathogenic
6110893	T	C	c.296A>G	p.Asn99Ser	Pathogenic
6110879	G	A	c.310C>T	p.Gln104Ter	Pathogenic
6110876	CCT	C	c.311_312del	p.Gln104ArgfsTer19	Pathogenic
6110867	T	A	c.322A>T	p.Arg108Ter	Pathogenic
6110580	C	CACTCT	c.324-2_326dup	NA	Pathogenic
6110568	T	C	c.338A>G	p.Tyr113Cys	Uncertain significance
6110529	TAC	TG	c.375_376delinsC	p.Tyr126ThrfsTer49	Pathogenic
6110521	G	T	c.385C>A	p.Leu129Met	Conflicting interpretation
6110518	ACAGCTTGTAGTACC	A	c.374_387del	p.Gly125ValfsTer3	Conflicting interpretation
6110516	G	A	c.390C>T	p.Ser130=	Benign
6110515	C	T	c.391G>A	p.Gly131Ser	Conflicting interpretation
6110493	G	T	c.413C>A	p.Ala138Asp	Pathogenic
6110485	C	T	c.421G>A	p.Asp141Asn	Pathogenic
6110485	C	A	c.421G>T	p.Asp141Tyr	Pathogenic
6110484	T	C	c.422A>G	p.Asp141Gly	Pathogenic
6110481	C	T	c.425G>A	p.Gly142Asp	Pathogenic
6110479	TGCCATCGATCCTG	T	c.414_426del	p.Arg139AlafsTer32	Pathogenic
6110476	C	T	c.430G>A	p.Gly144Ser	Uncertain significance
6110471	G	C	c.435C>G	p.Asn145Lys	Uncertain significance
6110466	T	C	c.440A>G	p.Gln147Arg	Pathogenic
6110457	A	T	c.449T>A	p.Leu150Gln	Pathogenic
6110457	A	G	c.449T>C	p.Leu150Pro	Pathogenic
6110439	T	C	c.467A>G	p.Asn156Ser	Uncertain significance

6110437	T	C	c.469A>G	p.Lys157Glu	Pathogenic
6110428	C	T	c.478G>A	p.Gly160Arg	Pathogenic
6110428	C	A	c.478G>T	p.Gly160Trp	Pathogenic
6110428	C	G	c.478G>C	p.Gly160Arg	Pathogenic
6110422	A	G	c.484T>C	p.Cys162Arg	Pathogenic
6110409	T	A	c.497A>T	p.Asn166Ile	Pathogenic
6110408	GT	G	c.497del	p.Asn166ThrfsTer9	Pathogenic
6110392	C	T	c.514G>A	p.Asp172Asn	Pathogenic
6110384	C	CTACCTTCTTGGGTCA	c.521_532+3dup	NA	Uncertain significance
6110357	A	G	c.532+17T>C	NA	Benign
6095627	T	G	c.533-43A>C	NA	Not provided
6095586	T	C	c.533-2A>G	NA	Pathogenic
6095571	C	T	c.546G>A	p.Ser182=	Conflicting interpretation
6095564	A	G	c.553T>C	p.Tyr185His	Uncertain significance
6095563	TA	T	c.553del	p.Tyr185MetfsTer9	Pathogenic
6095554	G	A	c.563C>T	p.Ala188Val	Pathogenic
6095513	G	A	c.604C>T	p.Arg202Trp	Conflicting interpretation
6095512	C	T	c.605G>A	p.Arg202Gln	Pathogenic
6095512	C	G	c.605G>C	p.Arg202Pro	Pathogenic
6095508	T	C	c.609A>G	p.Ala203=	Benign
6095471	C	T	c.646G>A	p.Glu216Lys	Conflicting interpretation
6095465	G	A	c.652C>T	p.Gln218Ter	Pathogenic
6095449	T	G	c.657+11A>C	NA	Benign
6075554	G	T	c.658-3C>A	NA	Conflicting interpretation
6075553	T	C	c.658-2A>G	NA	Pathogenic
6075544	C	T	c.665G>A	p.Trp222Ter	Pathogenic
6075543	C	T	c.666G>A	p.Trp222Ter	Pathogenic
6075514	G	A	c.695C>T	p.Ser232Leu	Conflicting interpretation
6075498	G	C	c.711C>G	p.Cys237Trp	Pathogenic

6075473	AAGGCTCG	A	c.729_735del	p.Glu244LeufsTer211	Pathogenic
6075447	CAA	C	c.760_761del	p.Leu254ValfsTer2	Pathogenic
6075438	ACACTCACACAAAGT	A	c.757_770del	p.Thr253CysfsTer45	Pathogenic
6075416	AG	A	c.792del	p.Cys265AlafsTer192	Uncertain significance
6075397	TACTCCAGGAGGGCAGGGCAGGCGC	T	c.788_811del	p.Cys263_Glu270del	Pathogenic
6075396	G	T	c.813C>A	p.Tyr271Ter	Pathogenic
6075396	G	C	c.813C>G	p.Tyr271Ter	Pathogenic
6075395	C	G	c.814G>C	p.Ala272Pro	Uncertain significance
6075392	G	A	c.817C>T	p.Arg273Trp	Pathogenic
6075391	C	G	c.818G>C	p.Arg273Pro	Pathogenic
6075386	A	T	c.823T>A	p.Cys275Ser	Pathogenic
6075386	A	G	c.823T>C	p.Cys275Arg	Pathogenic
6075363	C	T	c.846G>A	p.Leu282=	Uncertain significance
6075354	C	T	c.855G>A	p.Trp285Ter	Pathogenic
6075351	G	A	c.858C>T	p.Thr286=	Conflicting interpretation
6075334	C	T	c.874+1G>A	NA	Conflicting interpretation
6075333	A	G	c.874+2T>C	NA	Pathogenic
6075330	C	T	c.874+5G>A	NA	Pathogenic
6075328	G	A	c.874+7C>T	NA	Uncertain significance
6075327	C	T	c.874+8G>A	NA	Conflicting interpretation
6073746	A	C	c.875-5T>G	NA	Pathogenic
6073737	TG	T	c.878del	p.Pro293GlnfsTer164	Pathogenic
6073733	A	T	c.883T>A	p.Cys295Ser	Pathogenic
6073722	A	AC	c.893dup	p.Met299TyrfsTer4	Pathogenic
6073714	T	TTAG	c.901_902insCTA	p.Tyr301delinsSerAsn	Pathogenic
6073714	T	C	c.902A>G	p.Tyr301Cys	Pathogenic

6073713	A	T	c.903T>A	p.Tyr301Ter	Pathogenic
6073692	G	A	c.924C>T	p.Cys308=	Uncertain significance
6073662	A	T	c.954T>A	p.Asn318Lys	Benign
6073662	A	AC	c.953_954insG	p.Asn318LysfsTer2	Pathogenic
6073654	C	T	c.962G>A	p.Cys321Tyr	Pathogenic
6073646	G	A	c.970C>T	p.Arg324Ter	Pathogenic
6073645	C	T	c.971G>A	p.Arg324Gln	Pathogenic
6073645	C	G	c.971G>C	p.Arg324Pro	Pathogenic
6073642	C	A	c.974G>T	p.Cys325Phe	Pathogenic
6073627	C	CT	c.988dup	p.Ser330LysfsTer4	Pathogenic
6073623	GC	TT	c.992_993delinsAA	p.Cys331Ter	Pathogenic
6073623	G	T	c.993C>A	p.Cys331Ter	Pathogenic
6073616	T	A	c.997+3A>T	NA	Pathogenic
6072488	G	A	c.998-46C>T	NA	Not provided
6072469	G	A	c.998-27C>T	NA	Benign
6072439	C	T	c.1001G>A	p.Gly334Glu	Conflicting interpretation
6072414	G	GCTCCACGC	c.1025_1032dup	p.Cys342TrpfsTer118	Pathogenic
6072413	C	T	c.1027G>A	p.Val343Met	Pathogenic
6072403	G	A	c.1037C>T	p.Thr346Ile	Conflicting interpretation
6072390	G	T	c.1050C>A	p.Cys350Ter	Pathogenic
6072389	C	T	c.1051G>A	p.Val351Met	Uncertain significance
6072388	AC	A	c.1051del	p.Val351CysfsTer106	Pathogenic
6072376	T	C	c.1064A>G	p.Lys355Arg	Pathogenic
6072369	G	T	c.1071C>A	p.Tyr357Ter	Pathogenic
6072363	G	A	c.1077C>T	p.Pro359=	Conflicting interpretation
6072347	G	A	c.1093C>T	p.Arg365Ter	Pathogenic
6072342	G	A	c.1098C>T	p.Asp366=	Uncertain significance
6072341	A	C	c.1099T>G	p.Cys367Gly	Pathogenic
6072331	C	T	c.1109G>A	p.Cys370Tyr	Pathogenic
6072329	A	G	c.1109+2T>C	NA	Pathogenic
6072284	A	T	c.1109+47T>A	NA	Pathogenic

6071369	A	T	c.1110-26T>A	NA	Not provided
6071348	T	TG	c.1110-6dup	NA	Benign
6071348	T	TG	c.1110-7dup	NA	Conflicting interpretation
6071345	T	C	c.1110-2A>G	NA	Pathogenic
6071344	C	T	c.1110-1G>A	NA	Pathogenic
6071336	G	A	c.1117C>T	p.Arg373Ter	Pathogenic
6071322	C	A	c.1131G>T	p.Trp377Cys	Pathogenic
6071318	A	C	c.1135T>G	p.Cys379Gly	Pathogenic
6071311	T	C	c.1142A>G	p.Asn381Ser	Uncertain significance
6071295	A	G	c.1156+2T>C	NA	Pathogenic
6071270	G	A	c.1156+27C>T	NA	Not provided
6071255	G	A	c.1156+42C>T	NA	Not provided
6065279	G	A	c.1157-6C>T	NA	Benign
6065274	C	T	c.1157-1G>A	NA	Pathogenic
6065257	T	A	c.1173A>T	p.Thr391=	Benign
6065248	T	G	c.1182A>C	p.Ser394=	Benign
6065225	C	T	c.1205G>A	p.Arg402Lys	Uncertain significance
6065221	G	C	c.1209C>G	p.Tyr403Ter	Pathogenic
6065216	G	GTGGGAT	c.1213_1214insATCCCA	p.Phe404_Thr405insAsnPro	Pathogenic
6065202	A	T	c.1228T>A	p.Cys410Ser	Pathogenic
6065175	G	A	c.1255C>T	p.Gln419Ter	Pathogenic
6065159	GAGA	G	c.1268_1270del	p.Phe423del	Pathogenic
6065150	A	T	c.1280T>A	p.Ile427Asn	Pathogenic
6065144	G	A	c.1286C>T	p.Thr429Ile	Uncertain significance
6065135	A	G	c.1293+2T>C	NA	Pathogenic
6065051	G	A	c.1293+86C>T	NA	Pathogenic
6065028	A	G	c.1293+109T>C	NA	Not provided
6064372	G	A	c.1306C>T	p.Arg436Cys	Uncertain significance
6064369	C	A	c.1309G>T	p.Asp437Tyr	Pathogenic
6064367	G	A	c.1311C>T	p.Asp437=	Uncertain significance
6064354	G	A	c.1324C>T	p.Arg442Cys	Uncertain significance

6064351	AGCGGGTGCACACAGCGTC	A	c.1309_1326del	p.Asp437_Arg442del	Pathogenic
6064349	G	A	c.1329C>T	p.Ser443=	Benign
6064348	C	T	c.1330G>A	p.Val444Ile	Uncertain significance
6064339	G	A	c.1339C>T	p.Arg447Trp	Pathogenic
6064338	CG	C	c.1339del	p.Arg447GlyfsTer10	Pathogenic
6064293	GC	G	c.1384del	p.Ala462GlnfsTer15	Pathogenic
6064268	G	A	c.1410C>T	p.Asp470=	Benign
6064267	C	T	c.1411G>A	p.Val471Ile	Benign
6064080	G	T	c.1432+166C>A	NA	Not provided
6063064	A	G	c.1433-10T>C	NA	Benign
6063054	C	T	c.1433G>A	p.Gly478Asp	Uncertain significance
6063041	G	C	c.1446C>G	p.Ile482Met	Conflicting interpretation
6063036	T	C	c.1451A>G	p.His484Arg	Benign
6063036	TG	CT	c.1450_1451delinsAG	p.His484Ser	Pathogenic
6063024	G	C	c.1463C>G	p.Ala488Gly	Uncertain significance
6063020	G	A	c.1467C>T	p.Ser489=	Benign
6063015	C	G	c.1472G>C	p.Arg491Pro	Pathogenic
6063004	C	T	c.1483G>A	p.Gly495Arg	Uncertain significance
6063000	TC	T	c.1486del	p.Glu496ArgfsTer15	Pathogenic
6062990	C	G	c.1497G>C	p.Gln499His	Conflicting interpretation
6062984	G	C	c.1503C>G	p.Asp501Glu	Uncertain significance
6062973	C	T	c.1514G>A	p.Arg505His	Uncertain significance
6062972	G	A	c.1515C>T	p.Arg505=	Benign
6062953	C	T	c.1533+1G>A	NA	Pathogenic
6062953	C	A	c.1533+1G>T	NA	Pathogenic
6062939	C	T	c.1533+15G>A	NA	Conflicting interpretation
6058050	G	T	c.1534-6C>A	NA	Uncertain significance
6058047	G	T	c.1534-3C>A	NA	Pathogenic
6058041	ACAG	A	c.1534_1536del	p.Leu512del	Pathogenic

6058030	A	G	c.1548T>C	p.Tyr516=	Benign
6058030	A	T	c.1548T>A	p.Tyr516Ter	Pathogenic
6058026	C	A	c.1552G>T	p.Gly518Trp	Uncertain significance
6058026	CGGCATAGACGGGGGACAGCTGCAGGAGAGAC	CTG	c.1534-13_1551delinsCA	NA	Pathogenic
6058016	C	G	c.1562G>C	p.Cys521Ser	Uncertain significance
6058015	GC	AG	c.1562_1563delinsCT	p.Cys521Ser	Uncertain significance
6058015	G	A	c.1563C>T	p.Cys521=	Benign
6058007	C	T	c.1571G>A	p.Cys524Tyr	Pathogenic
6058005	C	T	c.1573G>A	p.Gly525Arg	Pathogenic
6057995	T	C	c.1583A>G	p.Asn528Ser	Pathogenic
6057990	T	C	c.1588A>G	p.Asn530Asp	Uncertain significance
6057988	G	C	c.1590C>G	p.Asn530Lys	Uncertain significance
6057987	G	A	c.1591C>T	p.Gln531Ter	Pathogenic
6057982	G	A	c.1596C>T	p.Gly532=	Uncertain significance
6057971	A	G	c.1607T>C	p.Leu536Pro	Pathogenic
6057965	G	A	c.1613C>T	p.Pro538Leu	Uncertain significance
6057964	G	A	c.1614C>T	p.Pro538=	Conflicting interpretation
6057953	G	C	c.1625C>G	p.Ala542Gly	Conflicting interpretation
6057952	C	T	c.1626G>A	p.Ala542=	Benign
6057948	G	T	c.1630C>A	p.Pro544Thr	Uncertain significance
6057932	A	G	c.1646T>C	p.Phe549Ser	Pathogenic
6057930	C	T	c.1648G>A	p.Gly550Arg	Pathogenic
6057924	C	T	c.1654G>A	p.Ala552Thr	Pathogenic
6057920	C	CA	c.1657dup	p.Trp553LeufsTer97	Pathogenic
6057919	C	T	c.1659G>A	p.Trp553Ter	Pathogenic
6057915	G	A	c.1663C>T	p.Leu555=	Uncertain significance
6057910	G	A	c.1668C>T	p.His556=	Benign
6057909	CG	C	c.1668del	p.His556GlnfsTer21	Pathogenic

6057906	C	A	c.1672G>T	p.Asp558Tyr	Pathogenic
6057899	T	C	c.1679A>G	p.Gln560Arg	Uncertain significance
6057885	G	A	c.1693C>T	p.Gln565Ter	Pathogenic
6057870	A	G	c.1708T>C	p.Cys570Arg	Pathogenic
6057869	C	G	c.1709G>C	p.Cys570Ser	Pathogenic
6057857	G	C	c.1721C>G	p.Pro574Arg	Pathogenic
6057850	C	A	c.1728G>T	p.Met576Ile	Conflicting interpretation
6057846	T	G	c.1729+3A>C	NA	Pathogenic
6057846	T	C	c.1729+3A>G	NA	Pathogenic
6057083	AGAGGAGC	A	c.1730-18_1730-12del	NA	Uncertain significance
6057082	G	T	c.1730-10C>A	NA	Pathogenic
6057077	G	A	c.1730-5C>T	NA	Conflicting interpretation
6057074	T	C	c.1730-2A>G	NA	Pathogenic
6057073	C	G	c.1730-1G>C	NA	Pathogenic
6057051	C	A	c.1751G>T	p.Cys584Phe	Pathogenic
6057050	G	A	c.1752C>T	p.Cys584=	Uncertain significance
6057049	C	T	c.1753G>A	p.Ala585Thr	Uncertain significance
6057040	T	G	c.1762A>C	p.Thr588Pro	Pathogenic
6057036	GACGTCAGGACCGCGCA	GCG	c.1750_1765delinsCG	p.Cys584ArgfsTer61	Pathogenic
6057032	G	C	c.1770C>G	p.Pro590=	Benign
6057021	G	C	c.1781C>G	p.Ala594Gly	Pathogenic
6057016	G	T	c.1786C>A	p.His596Asn	Pathogenic
6057008	G	A	c.1794C>T	p.Ala598=	Benign
6056990	G	T	c.1812C>A	p.Tyr604Ter	Pathogenic
6056990	G	C	c.1812C>G	p.Tyr604Ter	Pathogenic
6056985	C	T	c.1817G>A	p.Arg606Gln	Uncertain significance
6056972	G	T	c.1830C>A	p.Tyr610Ter	Pathogenic
6056961	G	A	c.1841C>T	p.Ser614Phe	Uncertain significance
6056955	G	A	c.1847C>T	p.Ser616Leu	Pathogenic

6056944	C	A	c.1858G>T	p.Glu620Ter	Pathogenic
6056940	C	G	c.1862G>C	p.Cys621Ser	Uncertain significance
6056939	G	T	c.1863C>A	p.Cys621Ter	Pathogenic
6056934	C	T	c.1868G>A	p.Cys623Tyr	Pathogenic
6056933	G	C	c.1869C>G	p.Cys623Trp	Pathogenic
6056933	G	A	c.1869C>T	p.Cys623=	Benign
6056932	C	T	c.1870G>A	p.Gly624Ser	Conflicting interpretation
6056930	G	GCGC	c.1871_1873dup	p.Gly624dup	Pathogenic
6056928	G	GCGC	c.1868_1870insGCG	p.Gly624dup	Not provided
6056916	T	C	c.1886A>G	p.Tyr629Cys	Pathogenic
6056910	G	A	c.1892C>T	p.Ala631Val	Conflicting interpretation
6056905	A	G	c.1897T>C	p.Cys633Arg	Pathogenic
6056901	G	A	c.1901C>T	p.Ala634Val	Uncertain significance
6056890	CG	C	c.1911del	p.Val638CysfsTer13	Pathogenic
6056887	G	A	c.1915C>T	p.Arg639Cys	Uncertain significance
6056880	G	A	c.1922C>T	p.Ala641Val	Conflicting interpretation
6056876	C	T	c.1926G>A	p.Trp642Ter	Pathogenic
6056872	C	A	c.1930G>T	p.Glu644Ter	Pathogenic
6056856	CCACAGCGGCCTGG	C	c.1933_1945del	p.Pro645SerfsTer2	Pathogenic
6056851	ACGCACCACAGCGGCCTGGCT	A	c.1931_1945+5del	NA	Pathogenic
6056842	A	T	c.1945+15T>A	NA	Benign
6056835	T	C	c.1945+22A>G	NA	Uncertain significance
6056833	G	A	c.1945+24C>T	NA	Benign
6052799	G	GAAA	c.1946-17_1946-16insTTT	NA	Not provided
6052798	A	AGAG	c.1946-16_1946-15insCTC	NA	Not provided
6052797	G	GAGA	c.1946-17_1946-15dup	NA	Benign

6052796	G	GAGA	c.1946-14_1946-13insTCT	NA	Benign
6052787	G	A	c.1946-4C>T	NA	Conflicting interpretation
6052784	C	T	c.1946-1G>A	NA	Pathogenic
6052765	C	T	c.1964G>A	p.Gly655Asp	Uncertain significance
6052755	G	C	c.1974C>G	p.Tyr658Ter	Pathogenic
6052748	A	G	c.1981T>C	p.Cys661Arg	Uncertain significance
6052736	A	AG	c.1992dup	p.Cys665LeufsTer13	Pathogenic
6052720	C	T	c.2009G>A	p.Arg670His	Uncertain significance
6052709	AAGAG	A	c.2016_2019del	p.Ser673ThrfsTer67	Pathogenic
6052708	TAA	T	c.2019_2020del	p.Tyr674ProfsTer3	Pathogenic
6052704	C	T	c.2025G>A	p.Pro675=	Uncertain significance
6052669	C	T	c.2060G>A	p.Cys687Tyr	Pathogenic
6052658	G	T	c.2071C>A	p.Pro691Thr	Uncertain significance
6052657	G	T	c.2072C>A	p.Pro691Gln	Uncertain significance
6052656	TG	T	c.2072del	p.Pro691GlnfsTer50	Not provided
6052656	TGG	TC	c.2071_2072delinsG	p.Pro691GlufsTer50	Pathogenic
6052640	C	G	c.2089G>C	p.Glu697Gln	Pathogenic
6052636	C	T	c.2093G>A	p.Arg698Lys	Benign
6052631	C	A	c.2098G>T	p.Asp700Tyr	Uncertain significance
6052626	G	A	c.2103C>T	p.Cys701=	Uncertain significance
6052625	C	T	c.2104G>A	p.Val702Met	Uncertain significance
6052620	G	A	c.2109C>T	p.Pro703=	Benign
6052613	G	A	c.2116C>T	p.Gln706Ter	Pathogenic
6052610	A	G	c.2119T>C	p.Cys707Arg	Pathogenic
6052603	CAG	C	c.2124_2125del	p.Cys709LeufsTer3	Pathogenic
6052583	A	C	c.2146T>G	p.Phe716Val	Uncertain significance
6052571	CT	C	c.2157del	p.Asp720ThrfsTer21	Pathogenic
6046847	C	G	c.2187-30G>C	NA	Benign
6046797	A	G	c.2207T>C	p.Met736Thr	Pathogenic
6046796	C	A	c.2208G>T	p.Met736Ile	Pathogenic
6046786	T	TCATG	c.2217_2220dup	p.Met740HisfsTer12	Pathogenic

6046784	C	T	c.2220G>A	p.Met740Ile	Conflicting interpretation
6046782	C	CCATG	c.2221_2222insCATG	p.Ser741ThrfsTer11	Pathogenic
6046733	CAG	C	c.2269_2270del	p.Leu757ValfsTer22	Pathogenic
6046726	G	T	c.2278C>A	p.Arg760Ser	Uncertain significance
6046726	G	A	c.2278C>T	p.Arg760Cys	Pathogenic
6046725	C	T	c.2279G>A	p.Arg760His	Conflicting interpretation
6046719	T	C	c.2281+4A>G	NA	Pathogenic
6046710	G	A	c.2281+13C>T	NA	Uncertain significance
6044493	G	T	c.2282-42C>A	NA	Benign
6044466	A	T	c.2282-15T>A	NA	Benign
6044453	T	C	c.2282-2A>G	NA	Pathogenic
6044449	T	C	c.2284A>G	p.Lys762Glu	Conflicting interpretation
6044446	T	C	c.2287A>G	p.Arg763Gly	Pathogenic
6044445	C	T	c.2288G>A	p.Arg763Lys	Uncertain significance
6044445	C	A	c.2288G>T	p.Arg763Met	Pathogenic
6044444	C	G	c.2289G>C	p.Arg763Ser	Pathogenic
6044444	C	T	c.2289G>A	p.Arg763=	Uncertain significance
6044443	T	TC	c.2289dup	p.Ser764GlufsTer16	Pathogenic
6044434	A	G	c.2299T>C	p.Cys767Arg	Uncertain significance
6044430	C	T	c.2303G>A	p.Arg768Gln	Pathogenic
6044422	T	C	c.2311A>G	p.Met771Val	Pathogenic
6044420	C	A	c.2313G>T	p.Met771Ile	Pathogenic
6044420	C	T	c.2313G>A	p.Met771Ile	Pathogenic
6044401	C	T	c.2332G>A	p.Ala778Thr	Uncertain significance
6044393	G	C	c.2340C>G	p.Asn780Lys	Uncertain significance
6044389	G	A	c.2344C>T	p.Arg782Trp	Pathogenic
6044388	C	T	c.2345G>A	p.Arg782Gln	Conflicting interpretation
6044379	C	T	c.2354G>A	p.Gly785Glu	Pathogenic
6044374	C	T	c.2359G>A	p.Glu787Lys	Pathogenic
6044371	A	G	c.2362T>C	p.Cys788Arg	Pathogenic
6044370	C	T	c.2363G>A	p.Cys788Tyr	Pathogenic

6044368	T	G	c.2365A>C	p.Thr789Pro	Pathogenic
6044368	T	C	c.2365A>G	p.Thr789Ala	Conflicting interpretation
6044363	T	G	c.2370A>C	p.Lys790Asn	Uncertain significance
6044361	G	A	c.2372C>T	p.Thr791Met	Pathogenic
6044360	C	T	c.2373G>A	p.Thr791=	Uncertain significance
6044356	G	A	c.2377C>T	p.Gln793Ter	Pathogenic
6044349	T	C	c.2384A>G	p.Tyr795Cys	Pathogenic
6044348	A	G	c.2385T>C	p.Tyr795=	Benign
6044343	A	C	c.2390T>G	p.Leu797Arg	Pathogenic
6044337	C	A	c.2396G>T	p.Cys799Phe	Pathogenic
6044337	C	T	c.2396G>A	p.Cys799Tyr	Pathogenic
6044335	T	C	c.2398A>G	p.Met800Val	Pathogenic
6044322	C	A	c.2411G>T	p.Cys804Phe	Pathogenic
6044314	CAG	C	c.2417_2418del	p.Ser806TrpfsTer12	Pathogenic
6044310	C	T	c.2423G>A	p.Cys808Tyr	Pathogenic
6044307	A	G	c.2426T>C	p.Leu809Pro	Pathogenic
6044303	G	T	c.2430C>A	p.Cys810Ter	Pathogenic
6044298	G	A	c.2435C>T	p.Pro812Leu	Conflicting interpretation
6044297	CG	C	c.2435del	p.Pro812ArgfsTer31	Pathogenic
6044297	C	CG	c.2435dup	p.Met814HisfsTer5	Pathogenic
6044294	G	GC	c.2438dup	p.Met814HisfsTer5	Pathogenic
6044291	C	T	c.2442G>A	p.Met814Ile	Pathogenic
6044290	C	T	c.2442+1G>A	NA	Uncertain significance
6044287	T	C	c.2442+4A>G	NA	Uncertain significance
6036492	C	G	c.2443-1G>C	NA	Pathogenic
6036489	G	A	c.2445C>T	p.Val815=	Uncertain significance
6036488	G	A	c.2446C>T	p.Arg816Trp	Pathogenic
6036487	C	T	c.2447G>A	p.Arg816Gln	Pathogenic
6036483	A	T	c.2451T>A	p.His817Gln	Conflicting interpretation
6036457	C	T	c.2477G>A	p.Arg826Lys	Conflicting interpretation
6036454	C	T	c.2480G>A	p.Cys827Tyr	Pathogenic
6036424	G	T	c.2510C>A	p.Ala837Asp	Benign

6036417	TC	T	c.2516del	p.Gly839GlufsTer4	Pathogenic
6036416	C	A	c.2518G>T	p.Glu840Ter	Pathogenic
6036409	A	T	c.2525T>A	p.Val842Glu	Uncertain significance
6036393	G	GT	c.2540dup	p.Asn847LysfsTer18	Pathogenic
6036388	CA	C	c.2545del	p.Cys849ValfsTer60	Pathogenic
6036388	C	T	c.2546G>A	p.Cys849Tyr	Pathogenic
6036385	C	G	c.2546+3G>C	NA	Pathogenic
6036363	G	A	c.2546+25C>T	NA	Benign
6036333	C	A	c.2546+55G>T	NA	Pathogenic
6036256	C	T	c.2546+132G>A	NA	Not provided
6034839	A	T	c.2547-13T>A	NA	Pathogenic
6034831	G	A	c.2547-5C>T	NA	Uncertain significance
6034818	T	C	c.2555A>G	p.Gln852Arg	Benign
6034818	T	T	c.2555G>A	NA	Benign
6034813	G	A	c.2560C>T	p.Arg854Trp	Pathogenic
6034812	C	T	c.2561G>A	p.Arg854Gln	Pathogenic
6034806	C	T	c.2567G>A	p.Trp856Ter	Pathogenic
6034805	C	T	c.2568G>A	p.Trp856Ter	Pathogenic
6034803	T	C	c.2570A>G	p.Asn857Ser	Conflicting interpretation
6034800	C	A	c.2573G>T	p.Cys858Phe	Pathogenic
6034799	G	C	c.2574C>G	p.Cys858Trp	Pathogenic
6034788	ACATGGTCTGTG	A	c.2574_2584del	p.Thr859ValfsTer2	Pathogenic
6034787	C	A	c.2586G>T	p.Val862=	Benign
6034748	G	A	c.2625C>T	p.Tyr875=	Uncertain significance
6034738	C	T	c.2635G>A	p.Asp879Asn	Pathogenic
6034736	G	T	c.2637C>A	p.Asp879Glu	Pathogenic
6034731	AG	A	c.2641del	p.Leu881SerfsTer28	Pathogenic
6034731	AG	C	c.2641_2642delinsG	p.Leu881AlafsTer28	Pathogenic
6034723	G	GCAAA	c.2649_2650insTTTG	p.Leu884PhefsTer19	Pathogenic
6034708	A	G	c.2665T>C	p.Cys889Arg	Pathogenic

6034700	G	A	c.2673C>T	p.Tyr891=	Uncertain significance
6034690	G	A	c.2683C>T	p.Gln895Ter	Pathogenic
6034689	T	C	c.2684A>G	p.Gln895Arg	Pathogenic
6034688	C	G	c.2685G>C	p.Gln895His	Pathogenic
6034686	A	G	c.2685+2T>C	NA	Pathogenic
6034686	A	C	c.2685+2T>G	NA	Pathogenic
6034541	C	T	c.2685+147G>A	NA	Not provided
6031585	A	C	c.2686-7T>G	NA	Uncertain significance
6031580	T	C	c.2686-2A>G	NA	Pathogenic
6031579	C	T	c.2686-1G>A	NA	Pathogenic
6031579	C	G	c.2686-1G>C	NA	Pathogenic
6031578	C	A	c.2686G>T	p.Asp896Tyr	Pathogenic
6031530	T	TA	c.2733dup	p.Lys912Ter	Pathogenic
6031529	T	TA	c.2734_2735insT	p.Lys912IlefsTer24	Not provided
6031520	C	T	c.2744G>A	p.Ser915Asn	Uncertain significance
6031513	G	GGAGG	c.2750_2753dup	p.Ser918LeufsTer19	Pathogenic
6031501	G	T	c.2763C>A	p.Cys921Ter	Pathogenic
6031494	G	A	c.2770C>T	p.Arg924Trp	Conflicting interpretation
6031493	C	T	c.2771G>A	p.Arg924Gln	Conflicting interpretation
6031473	C	A	c.2791G>T	p.Gly931Ter	Pathogenic
6031443	C	T	c.2820+1G>A	NA	Pathogenic
6031443	C	G	c.2820+1G>C	NA	Pathogenic
6029636	C	T	c.2821-148G>A	NA	Not provided
6029611	T	G	c.2821-123A>C	NA	Pathogenic
6029576	T	C	c.2821-88A>G	NA	Not provided
6029566	T	G	c.2821-78A>C	NA	Not provided
6029484	T	C	c.2825A>G	p.Asn942Ser	Benign
6029442	A	G	c.2867T>C	p.Val956Ala	Pathogenic
6029431	G	A	c.2878C>T	p.Arg960Trp	Conflicting interpretation
6029429	CCG	TCA	c.2878_2880delinsTGA	p.Arg960Ter	Pathogenic
6029429	C	T	c.2880G>A	p.Arg960=	Benign

6029409	C	T	c.2900G>A	p.Gly967Asp	Conflicting interpretation
6029409	C	A	c.2900G>T	p.Gly967Val	Pathogenic
6029400	AG	A	c.2908del	p.Leu970SerfsTer9	Uncertain significance
6029388	C	T	c.2921G>A	p.Trp974Ter	Pathogenic
6029383	G	A	c.2926C>T	p.Arg976Cys	Pathogenic
6029373	C	T	c.2936G>A	p.Ser979Asn	Pathogenic
6029366	G	A	c.2943C>T	p.Ser981=	Benign
6029365	C	G	c.2944G>C	p.Val982Leu	Pathogenic
6029345	G	T	c.2964C>A	p.Tyr988Ter	Pathogenic
6029341	C	G	c.2967+1G>C	NA	Pathogenic
6026060	T	C	c.2968-14A>G	NA	Pathogenic

6026058	A	G	c.2968-12T>C	NA	Uncertain significance
6026033	C	T	c.2981G>A	p.Gly994Asp	Uncertain significance
6026028	A	C	c.2986T>G	p.Cys996Gly	Pathogenic
6026027	C	G	c.2987G>C	p.Cys996Ser	Pathogenic
6026025	C	T	c.2989G>A	p.Gly997Arg	Pathogenic
6025971	C	A	c.3043G>T	p.Glu1015Ter	Pathogenic
6025952	A	C	c.3062T>G	p.Phe1021Cys	Pathogenic
6025941	AG	A	c.3072del	p.Trp1025GlyfsTer3	Pathogenic
6025925	T	C	c.3089A>G	p.Gln1030Arg	Uncertain significance
6025922	C	G	c.3092G>C	p.Cys1031Ser	Pathogenic
6025916	T	TGTC	c.3097_3099dup	p.Asp1033delinsGlyHis	Uncertain significance
6025910	CTGG	C	c.3101_3103del	p.Thr1034del	Conflicting interpretation
6025904	A	C	c.3108+2T>G	NA	Pathogenic
6025901	C	T	c.3108+5G>A	NA	Conflicting interpretation
6025821	C	A	c.3108+85G>T	NA	Benign
6025783	C	T	c.3109-90G>A	NA	Benign
6025723	G	A	c.3109-30C>T	NA	Benign
6025698	G	A	c.3109-5C>T	NA	Uncertain significance
6025658	G	A	c.3144C>T	p.Asn1048=	Benign

6025650	A	G	c.3152T>C	p.Met1051Thr	Pathogenic
6025643	C	A	c.3159G>T	p.Gln1053His	Pathogenic
6025640	C	G	c.3162G>C	p.Thr1054=	Uncertain significance
6025639	T	C	c.3163A>G	p.Met1055Val	Benign
6025638	A	T	c.3164T>A	p.Met1055Lys	Pathogenic
6025624	A	G	c.3178T>C	p.Cys1060Arg	Pathogenic
6025624	A	C	c.3178T>G	p.Cys1060Gly	Uncertain significance
6025623	C	T	c.3179G>A	p.Cys1060Tyr	Pathogenic
6025622	AC	A	c.3179del	p.Cys1060LeufsTer59	Pathogenic
6025604	G	A	c.3198C>T	p.Asp1066=	Uncertain significance
6025603	C	T	c.3199G>A	p.Val1067Ile	Benign
6025600	AGACGT	A	c.3197_3201del	p.Asp1066ValfsTer25	Pathogenic
6025590	C	A	c.3212G>T	p.Cys1071Phe	Pathogenic
6025549	G	A	c.3222+31C>T	NA	Benign
6023793	A	AGGCTCGGGGTCCACCTGCAAA	c.3223-7_3236dup	NA	Pathogenic
6023779	G	A	c.3231C>T	p.Pro1077=	Benign
6023778	C	T	c.3232G>A	p.Glu1078Lys	Pathogenic
6023772	AT	A	c.3237del	p.Tyr1080IlefsTer39	Pathogenic
6023770	A	G	c.3240T>C	p.Tyr1080=	Benign
6023759	C	T	c.3251G>A	p.Cys1084Tyr	Uncertain significance
6023752	G	A	c.3258C>T	p.Tyr1086=	Benign
6023751	C	CA	c.3258_3259insT	p.Asp1087Ter	Pathogenic
6023739	A	G	c.3271T>C	p.Cys1091Arg	Pathogenic
6023729	A	G	c.3281T>C	p.Ile1094Thr	Pathogenic
6023719	G	A	c.3291C>T	p.Cys1097=	Conflicting interpretation
6023714	CA	GG	c.3295_3296delinsCC	p.Cys1099Pro	Pathogenic
6023711	AAGCAGGCGCAGTCC	A	c.3269_3298del	p.Ser1090_Cys1099del	Pathogenic

	CCAATGGACTCACAGG				
6023709	A	G	c.3301T>C	p.Cys1101Arg	Conflicting interpretation
6023707	G	C	c.3303C>G	p.Cys1101Trp	Conflicting interpretation
6023707	G	T	c.3303C>A	p.Cys1101Ter	Pathogenic
6023696	G	T	c.3314C>A	p.Ala1105Asp	Pathogenic
6023691	A	G	c.3319T>C	p.Tyr1107His	Pathogenic
6023690	T	C	c.3320A>G	p.Tyr1107Cys	Conflicting interpretation
6023688	C	G	c.3322G>C	p.Ala1108Pro	Pathogenic
6023685	G	C	c.3325C>G	p.His1109Asp	Uncertain significance
6023678	C	T	c.3332G>A	p.Cys1111Tyr	Pathogenic
6023651	C	G	c.3359G>C	p.Trp1120Ser	Pathogenic
6023650	C	T	c.3360G>A	p.Trp1120Ter	Pathogenic
6023648	C	A	c.3362G>T	p.Arg1121Met	Pathogenic
6023647	C	A	c.3363G>T	p.Arg1121Ser	Pathogenic
6023645	G	A	c.3365C>T	p.Thr1122Met	Conflicting interpretation
6023633	C	A	c.3377G>T	p.Cys1126Phe	Pathogenic
6023633	C	T	c.3377G>A	p.Cys1126Tyr	Uncertain significance
6023630	C	T	c.3379+1G>A	NA	Pathogenic
6023626	C	T	c.3379+5G>A	NA	Pathogenic
6023624	T	G	c.3379+7A>C	NA	Benign
6023619	G	A	c.3379+12C>T	NA	Uncertain significance
6023618	C	T	c.3379+13G>A	NA	Benign
6023604	T	A	c.3379+27A>T	NA	Not provided
6022902	T	C	c.3380-4A>G	NA	Pathogenic
6022900	T	C	c.3380-2A>G	NA	Pathogenic
6022891	GCT	G	c.3385_3386del	p.Ser1129LeufsTer12	Pathogenic
6022890	A	G	c.3388T>C	p.Cys1130Arg	Pathogenic
6022890	A	C	c.3388T>G	p.Cys1130Gly	Pathogenic
6022889	C	A	c.3389G>T	p.Cys1130Phe	Pathogenic
6022889	C	T	c.3389G>A	p.Cys1130Tyr	Pathogenic
6022888	G	A	c.3390C>T	p.Cys1130=	Pathogenic

6022888	G	C	c.3390C>G	p.Cys1130Trp	Conflicting interpretation
6022865	T	TTT	c.3412_3413dup	p.Asn1138LysfsTer78	Pathogenic
6022864	G	A	c.3414C>T	p.Asn1138=	Benign
6022852	A	G	c.3426T>C	p.Cys1142=	Conflicting interpretation
6022848	A	C	c.3430T>G	p.Trp1144Gly	Pathogenic
6022845	G	A	c.3433C>T	p.Arg1145Cys	Pathogenic
6022841	T	C	c.3437A>G	p.Tyr1146Cys	Pathogenic
6022833	A	G	c.3445T>C	p.Cys1149Arg	Pathogenic
6022832	C	T	c.3446G>A	p.Cys1149Tyr	Pathogenic
6022830	C	G	c.3448G>C	p.Ala1150Pro	Pathogenic
6022820	C	T	c.3458G>A	p.Cys1153Tyr	Pathogenic
6022818	G	A	c.3460C>T	p.Gln1154Ter	Pathogenic
6022811	G	A	c.3467C>T	p.Thr1156Met	Pathogenic
6022808	C	A	c.3470G>T	p.Cys1157Phe	Pathogenic
6022798	AG	A	c.3479del	p.Pro1160LeufsTer55	Pathogenic
6022797	C	T	c.3481G>A	p.Glu1161Lys	Pathogenic
6022793	G	A	c.3485C>T	p.Pro1162Leu	Benign
6022792	TG	CA	c.3485_3486inv	p.Pro1162Leu	Conflicting interpretation
6022792	T	C	c.3486A>G	p.Pro1162=	Benign
6022785	A	G	c.3493T>C	p.Cys1165Arg	Pathogenic
6022784	C	A	c.3494G>T	p.Cys1165Phe	Pathogenic
6022771	A	C	c.3507T>G	p.Cys1169Trp	Pathogenic
6022767	C	G	c.3511G>C	p.Glu1171Gln	Pathogenic
6022764	C	A	c.3514G>T	p.Gly1172Cys	Pathogenic
6022763	C	A	c.3515G>T	p.Gly1172Val	Pathogenic
6022761	A	G	c.3517T>C	p.Cys1173Arg	Pathogenic
6022760	C	A	c.3518G>T	p.Cys1173Phe	Pathogenic
6022740	C	T	c.3538G>A	p.Gly1180Arg	Pathogenic
6022739	C	T	c.3538+1G>A	NA	Pathogenic
6022739	C	G	c.3538+1G>C	NA	Pathogenic
6022734	C	A	c.3538+6G>T	NA	Uncertain significance
6022720	C	T	c.3538+20G>A	NA	Pathogenic

6022084	G	A	c.3539-49C>T	NA	Benign
6022070	C	G	c.3539-35G>C	NA	Not provided
6022068	C	T	c.3539-33G>A	NA	Benign
6022050	T	C	c.3539-15A>G	NA	Uncertain significance
6022036	CT	C	c.3539-2del	NA	Pathogenic
6022013	CA	C	c.3560del	p.Leu1187CysfsTer28	Pathogenic
6022006	A	G	c.3568T>C	p.Cys1190Arg	Pathogenic
6022005	C	G	c.3569G>C	p.Cys1190Ser	Pathogenic
6022005	C	T	c.3569G>A	p.Cys1190Tyr	Pathogenic
6021995	A	G	c.3579T>C	p.Pro1193=	Benign
6021991	C	A	c.3583G>T	p.Asp1195Tyr	Conflicting interpretation
6021988	A	G	c.3586T>C	p.Cys1196Arg	Pathogenic
6021961	G	A	c.3613C>T	p.Arg1205Cys	Conflicting interpretation
6021961	G	T	c.3613C>A	p.Arg1205Ser	Pathogenic
6021960	C	T	c.3614G>A	p.Arg1205His	Pathogenic
6021960	C	A	c.3614G>T	p.Arg1205Leu	Pathogenic
6021951	GA	G	c.3622del	p.Ser1208GlnfsTer7	Pathogenic
6021917	AG	A	c.3656del	p.Pro1219LeufsTer34	Pathogenic
6021901	A	C	c.3673T>G	p.Cys1225Gly	Pathogenic
6019818	T	C	c.3675-75A>G	NA	Benign
6019757	C	T	c.3675-14G>A	NA	Conflicting interpretation
6019744	C	T	c.3675-1G>A	NA	Pathogenic
6019739	A	G	c.3679T>C	p.Cys1227Arg	Pathogenic
6019732	A	C	c.3686T>G	p.Val1229Gly	Conflicting interpretation
6019727	T	G	c.3691A>C	p.Asn1231His	Pathogenic
6019726	T	G	c.3692A>C	p.Asn1231Thr	Conflicting interpretation
6019726	T	C	c.3692A>G	p.Asn1231Ser	Pathogenic
6019717	C	T	c.3701G>A	p.Cys1234Tyr	Pathogenic
6019716	A	C	c.3702T>G	p.Cys1234Trp	Pathogenic
6019706	G	A	c.3712C>T	p.Gln1238Ter	Pathogenic
6019699	G	A	c.3719C>T	p.Pro1240Leu	Conflicting interpretation
6019698	C	T	c.3720G>A	p.Pro1240=	Uncertain significance

6019681	G	GGG	c.3736_3737dup	p.Pro1247LeufsTer7	Pathogenic
6019671	A	G	c.3747T>C	p.Asp1249=	Benign
6019669	G	T	c.3749C>A	p.Ala1250Asp	Pathogenic
6019650	A	G	c.3768T>C	p.Thr1256=	Uncertain significance
6019645	T	C	c.3773A>G	p.Tyr1258Cys	Pathogenic
6019630	G	A	c.3788C>T	p.Ser1263Leu	Pathogenic
6019629	C	T	c.3789G>A	p.Ser1263=	Benign
6019623	C	T	c.3795G>A	p.Pro1265=	Benign
6019621	G	T	c.3797C>A	p.Pro1266Gln	Pathogenic
6019621	G	A	c.3797C>T	p.Pro1266Leu	Conflicting interpretation
6019618	A	T	c.3800T>A	p.Leu1267Ter	Pathogenic
6019616	G	T	c.3802C>A	p.His1268Asn	Pathogenic
6019616	G	C	c.3802C>G	p.His1268Asp	Pathogenic
6019611	A	AAGTAGAAAT	c.3806_3814dup	p.Asp1269delinsGluPheLeuLeu	Pathogenic
6019604	A	G	c.3814T>C	p.Cys1272Arg	Pathogenic
6019604	A	C	c.3814T>G	p.Cys1272Gly	Pathogenic
6019603	C	G	c.3815G>C	p.Cys1272Ser	Pathogenic
6019603	C	A	c.3815G>T	p.Cys1272Phe	Pathogenic
6019603	C	T	c.3815G>A	p.Cys1272Tyr	Pathogenic
6019602	G	C	c.3816C>G	p.Cys1272Trp	Pathogenic
6019591	A	G	c.3827T>C	p.Leu1276Pro	Pathogenic
6019591	A	C	c.3827T>G	p.Leu1276Arg	Pathogenic
6019589	C	G	c.3829G>C	p.Asp1277His	Conflicting interpretation
6019588	T	C	c.3830A>G	p.Asp1277Gly	Pathogenic
6019585	A	C	c.3833T>G	p.Leu1278Arg	Pathogenic
6019585	A	G	c.3833T>C	p.Leu1278Pro	Pathogenic
6019584	CAGG	C	c.3831_3833del	p.Asp1277_Leu1278delinsGlu	Pathogenic
6019583	C	T	c.3835G>A	p.Val1279Ile	Conflicting interpretation
6019583	C	A	c.3835G>T	p.Val1279Phe	Pathogenic
6019581	G	A	c.3837C>T	p.Val1279=	Benign

6019578	G	GAGCAGGA	c.3839_3845dup	p.Leu1281ProfsTer14	Pathogenic
6019576	A	G	c.3842T>C	p.Leu1281Pro	Uncertain significance
6019573	A	C	c.3845T>G	p.Leu1282Arg	Pathogenic
6019571	C	A	c.3847G>T	p.Asp1283Tyr	Pathogenic
6019565	A	G	c.3853T>C	p.Ser1285Pro	Pathogenic
6019564	G	A	c.3854C>T	p.Ser1285Phe	Pathogenic
6019556	G	C	c.3862C>G	p.Leu1288Val	Pathogenic
6019555	A	C	c.3863T>G	p.Leu1288Arg	Pathogenic
6019550	C	T	c.3868G>A	p.Glu1290Lys	Uncertain significance
6019542	C	A	c.3876G>T	p.Glu1292Asp	Pathogenic
6019541	A	G	c.3877T>C	p.Phe1293Leu	Pathogenic
6019531	A	G	c.3887T>C	p.Leu1296Pro	Pathogenic
6019520	CA	C	c.3897del	p.Phe1299LeufsTer5	Pathogenic
6019513	T	C	c.3905A>G	p.Asp1302Gly	Pathogenic
6019510	A	ACAT	c.3907_3909dup	p.Met1303delinsAsnVal	Not provided
6019508	T	C	c.3910A>G	p.Met1304Val	Pathogenic
6019507	A	ACAT	c.3910_3912dup	p.Met1304delinsAsnVal	Pathogenic
6019507	A	C	c.3911T>G	p.Met1304Arg	Pathogenic
6019502	G	A	c.3916C>T	p.Arg1306Trp	Pathogenic
6019501	C	T	c.3917G>A	p.Arg1306Gln	Pathogenic
6019501	C	A	c.3917G>T	p.Arg1306Leu	Pathogenic
6019501	C	G	c.3917G>C	p.Arg1306Pro	Pathogenic
6019498	A	G	c.3920T>C	p.Leu1307Pro	Pathogenic
6019498	A	C	c.3920T>G	p.Leu1307Arg	Pathogenic
6019496	G	A	c.3922C>T	p.Arg1308Cys	Pathogenic
6019496	G	T	c.3922C>A	p.Arg1308Ser	Pathogenic
6019495	C	T	c.3923G>A	p.Arg1308His	Pathogenic
6019495	C	G	c.3923G>C	p.Arg1308Pro	Pathogenic
6019495	C	A	c.3923G>T	p.Arg1308Leu	Pathogenic

6019493	T	C	c.3925A>G	p.Ile1309Val	Pathogenic
6019490	A	G	c.3928T>C	p.Ser1310Pro	Pathogenic
6019489	G	A	c.3929C>T	p.Ser1310Phe	Pathogenic
6019487	G	A	c.3931C>T	p.Gln1311Ter	Pathogenic
6019482	C	A	c.3936G>T	p.Lys1312Asn	Uncertain significance
6019479	C	G	c.3939G>C	p.Trp1313Cys	Pathogenic
6019479	C	T	c.3939G>A	p.Trp1313Ter	Pathogenic
6019478	C	G	c.3940G>C	p.Val1314Leu	Pathogenic
6019478	C	A	c.3940G>T	p.Val1314Phe	Pathogenic
6019477	A	T	c.3941T>A	p.Val1314Asp	Pathogenic
6019477	AC	A	c.3940del	p.Val1314SerfsTer34	Pathogenic
6019475	G	A	c.3943C>T	p.Arg1315Cys	Pathogenic
6019475	G	C	c.3943C>G	p.Arg1315Gly	Pathogenic
6019474	C	T	c.3944G>A	p.Arg1315His	Conflicting interpretation
6019474	C	A	c.3944G>T	p.Arg1315Leu	Pathogenic
6019474	C	G	c.3944G>C	p.Arg1315Pro	Pathogenic
6019472	C	T	c.3946G>A	p.Val1316Met	Pathogenic
6019466	C	T	c.3952G>A	p.Val1318Met	Conflicting interpretation
6019457	A	T	c.3961T>A	p.Tyr1321Asn	Pathogenic
6019457	A	C	c.3961T>G	p.Tyr1321Asp	Pathogenic
6019456	T	C	c.3962A>G	p.Tyr1321Cys	Pathogenic
6019453	T	G	c.3965A>C	p.His1322Pro	Conflicting interpretation
6019451	CGTG	C	c.3964_3966del	p.His1322del	Pathogenic
6019448	C	T	c.3970G>A	p.Gly1324Ser	Pathogenic
6019447	C	G	c.3971G>C	p.Gly1324Ala	Pathogenic
6019444	G	A	c.3974C>T	p.Ser1325Phe	Pathogenic
6019440	G	A	c.3978C>T	p.His1326=	Benign
6019418	G	A	c.4000C>T	p.Arg1334Trp	Conflicting interpretation
6019412	G	A	c.4006C>T	p.Arg1336Ter	Pathogenic
6019411	C	T	c.4007G>A	p.Arg1336Gln	Uncertain significance
6019408	G	A	c.4010C>T	p.Pro1337Leu	Pathogenic
6019406	A	C	c.4012T>G	p.Ser1338Ala	Uncertain significance

6019405	G	C	c.4013C>G	p.Ser1338Ter	Pathogenic
6019399	A	G	c.4019T>C	p.Leu1340Pro	Pathogenic
6019397	G	A	c.4021C>T	p.Arg1341Trp	Pathogenic
6019396	C	T	c.4022G>A	p.Arg1341Gln	Pathogenic
6019396	C	A	c.4022G>T	p.Arg1341Leu	Pathogenic
6019396	C	G	c.4022G>C	p.Arg1341Pro	Pathogenic
6019394	G	A	c.4024C>T	p.Arg1342Cys	Conflicting interpretation
6019391	T	C	c.4027A>G	p.Ile1343Val	Uncertain significance
6019382	G	A	c.4036C>T	p.Gln1346Ter	Pathogenic
6019372	T	C	c.4046A>G	p.Tyr1349Cys	Pathogenic
6019368	C	T	c.4050G>A	p.Ala1350=	Benign
6019354	G	T	c.4064C>A	p.Ala1355Asp	Pathogenic
6019345	C	T	c.4073G>A	p.Ser1358Asn	Pathogenic
6019343	C	T	c.4075G>A	p.Glu1359Lys	Pathogenic
6019339	A	G	c.4079T>C	p.Val1360Ala	Pathogenic
6019336	A	G	c.4082T>C	p.Leu1361Ser	Pathogenic
6019336	A	C	c.4082T>G	p.Leu1361Trp	Pathogenic
6019333	T	G	c.4085A>C	p.Lys1362Thr	Pathogenic
6019330	T	C	c.4088A>G	p.Tyr1363Cys	Pathogenic
6019324	AGT	A	c.4092_4093del	p.Leu1365ValfsTer11	Pathogenic
6019324	AGT	C	c.4094T>G	p.Leu1365ValfsTer11	Pathogenic
6019324	AGT	G	c.4094T>C	p.Leu1365ValfsTer11	Pathogenic
6019315	A	G	c.4103T>C	p.Ile1368Thr	Uncertain significance
6019313	A	T	c.4105T>A	p.Phe1369Ile	Pathogenic
6019313	A	G	c.4105T>C	p.Phe1369Leu	Pathogenic
6019303	A	C	c.4115T>G	p.Ile1372Ser	Pathogenic
6019301	C	A	c.4117G>T	p.Asp1373Tyr	Pathogenic
6019298	G	A	c.4120C>T	p.Arg1374Cys	Pathogenic
6019298	G	T	c.4120C>A	p.Arg1374Ser	Pathogenic
6019297	C	T	c.4121G>A	p.Arg1374His	Pathogenic
6019297	C	A	c.4121G>T	p.Arg1374Leu	Pathogenic
6019295	G	A	c.4123C>T	p.Pro1375Ser	Uncertain significance

6019288	G	A	c.4130C>T	p.Ala1377Val	Conflicting interpretation
6019285	G	A	c.4133C>T	p.Ser1378Phe	Pathogenic
6019283	GGGAGG	AGGAGA	c.4130_4135delinsTCTCCT	p.Ala1377_Arg1379delinsValSerCys	Pathogenic
6019283	G	A	c.4135C>T	p.Arg1379Cys	Pathogenic
6019282	C	A	c.4136G>T	p.Arg1379Leu	Pathogenic
6019280	T	C	c.4138A>G	p.Ile1380Val	Benign
6019277	T	C	c.4141A>G	p.Thr1381Ala	Benign
6019277	T	T	c.4141G>A	NA	Benign
6019273	A	G	c.4145T>C	p.Leu1382Pro	Pathogenic
6019272	C	A	c.4146G>T	p.Leu1382=	Conflicting interpretation
6019270	A	C	c.4148T>G	p.Leu1383Arg	Pathogenic
6019270	A	G	c.4148T>C	p.Leu1383Pro	Pathogenic
6019261	G	T	c.4157C>A	p.Ala1386Asp	Pathogenic
6019258	C	A	c.4160G>T	p.Ser1387Ile	Pathogenic
6019256	G	A	c.4162C>T	p.Gln1388Ter	Pathogenic
6019253	C	T	c.4165G>A	p.Glu1389Lys	Pathogenic
6019245	T	C	c.4173A>G	p.Gln1391=	Benign
6019243	C	T	c.4175G>A	p.Arg1392Gln	Uncertain significance
6019237	G	A	c.4181C>T	p.Ser1394Phe	Pathogenic
6019235	G	A	c.4183C>T	p.Arg1395Trp	Pathogenic
6019223	G	A	c.4195C>T	p.Arg1399Cys	Conflicting interpretation
6019222	C	T	c.4196G>A	p.Arg1399His	Conflicting interpretation
6019217	C	T	c.4201G>A	p.Val1401Ile	Uncertain significance
6019213	T	G	c.4205A>C	p.Gln1402Pro	Pathogenic
6019213	T	C	c.4205A>G	p.Gln1402Arg	Uncertain significance
6019212	C	T	c.4206G>A	p.Gln1402=	Benign
6019212	CTGGACGTAGCGGACAAAGTTCCGGGACATCCGT	C	c.4173_4205del	p.Arg1392_Gln1402del	Pathogenic
6019193	CCTT	C	c.4222_4224del	p.Lys1408del	Pathogenic

6019193	C	A	c.4225G>T	p.Val1409Phe	Pathogenic
6019180	G	A	c.4238C>T	p.Pro1413Leu	Conflicting interpretation
6019180	G	C	c.4238C>G	p.Pro1413Arg	Pathogenic
6019180	G	T	c.4238C>A	p.Pro1413Gln	Uncertain significance
6019177	A	C	c.4241T>G	p.Val1414Gly	Conflicting interpretation
6019174	C	T	c.4244G>A	p.Gly1415Asp	Pathogenic
6019171	A	T	c.4247T>A	p.Ile1416Asn	Pathogenic
6019171	A	G	c.4247T>C	p.Ile1416Thr	Pathogenic
6019169	C	A	c.4249G>T	p.Gly1417Trp	Pathogenic
6019163	G	T	c.4255C>A	p.His1419Asn	Pathogenic
6019161	A	C	c.4257T>G	p.His1419Gln	Conflicting interpretation
6019155	G	C	c.4263C>G	p.Asn1421Lys	Pathogenic
6019145	T	A	c.4273A>T	p.Ile1425Phe	Pathogenic
6019142	G	A	c.4276C>T	p.Arg1426Cys	Pathogenic
6019114	T	C	c.4304A>G	p.Asn1435Ser	Benign
6019109	C	T	c.4309G>A	p.Ala1437Thr	Pathogenic
6019105	A	C	c.4313T>G	p.Phe1438Cys	Pathogenic
6019103	C	T	c.4315G>A	p.Val1439Met	Pathogenic
6019094	T	C	c.4324A>G	p.Ser1442Gly	Pathogenic
6019093	C	CTCCACACT	c.4324_4331dup	p.Ser1442LysfsTer86	Pathogenic
6019081	A	G	c.4337T>C	p.Leu1446Pro	Pathogenic
6019079	C	G	c.4339G>C	p.Glu1447Gln	Pathogenic
6019060	A	T	c.4358T>A	p.Ile1453Asn	Pathogenic
6019059	G	A	c.4359C>T	p.Ile1453=	Benign
6019058	C	T	c.4360G>A	p.Val1454Ile	Uncertain significance
6019050	G	T	c.4368C>A	p.Tyr1456Ter	Pathogenic
6019050	G	C	c.4368C>G	p.Tyr1456Ter	Pathogenic
6019045	C	T	c.4373G>A	p.Cys1458Tyr	Pathogenic
6019040	G	A	c.4378C>T	p.Leu1460Phe	Pathogenic
6019040	G	C	c.4378C>G	p.Leu1460Val	Pathogenic
6019039	A	G	c.4379T>C	p.Leu1460Pro	Pathogenic

6019037	C	T	c.4381G>A	p.Ala1461Thr	Uncertain significance
6019036	G	T	c.4382C>A	p.Ala1461Asp	Pathogenic
6019036	G	A	c.4382C>T	p.Ala1461Val	Pathogenic
6019034	G	C	c.4384C>G	p.Pro1462Ala	Pathogenic
6019019	G	A	c.4399C>T	p.Pro1467Ser	Pathogenic
6019004	C	CG	c.4413dup	p.Asp1472ArgfsTer40	Pathogenic
6019004	C	G	c.4414G>C	p.Asp1472His	Benign
6019004	C	T	c.4414G>A	p.Asp1472Asn	Uncertain significance
6019003	TC	TGG	c.4414delinsCC	p.Asp1472ProfsTer40	Not provided
6019002	G	GC	c.4415_4416insG	p.Asp1472GlufsTer40	Not provided
6018995	G	A	c.4423C>T	p.Gln1475Ter	Pathogenic
6018988	G	A	c.4430C>T	p.Thr1477Ile	Pathogenic
6018975	C	A	c.4443G>T	p.Gly1481=	Benign
6018972	GAG	GC	c.4444_4445delinsG	p.Leu1482AlafsTer43	Pathogenic
6018971	AGA	AC	c.4445_4446delinsG	p.Leu1482ArgfsTer43	Pathogenic
6018964	AC	A	c.4453del	p.Val1485PhefsTer40	Pathogenic
6018961	G	A	c.4457C>T	p.Ser1486Leu	Benign
6018961	G	T	c.4457C>A	p.Ser1486Ter	Pathogenic
6018960	C	T	c.4458G>A	p.Ser1486=	Benign
6018945	C	G	c.4473G>C	p.Lys1491Asn	Uncertain significance
6018935	T	G	c.4483A>C	p.Met1495Leu	Pathogenic
6018926	C	T	c.4492G>A	p.Asp1498Asn	Pathogenic
6018925	T	C	c.4493A>G	p.Asp1498Gly	Conflicting interpretation
6018922	A	T	c.4496T>A	p.Val1499Glu	Pathogenic
6018920	C	T	c.4498G>A	p.Ala1500Thr	Pathogenic
6018919	G	T	c.4499C>A	p.Ala1500Glu	Pathogenic
6018919	G	A	c.4499C>T	p.Ala1500Val	Conflicting interpretation
6018910	A	C	c.4508T>G	p.Leu1503Arg	Pathogenic
6018910	A	T	c.4508T>A	p.Leu1503Gln	Pathogenic
6018910	A	G	c.4508T>C	p.Leu1503Pro	Pathogenic
6018905	C	T	c.4513G>A	p.Gly1505Arg	Pathogenic
6018905	C	G	c.4513G>C	p.Gly1505Arg	Pathogenic

6018904	C	T	c.4514G>A	p.Gly1505Glu	Pathogenic
6018901	G	A	c.4517C>T	p.Ser1506Leu	Pathogenic
6018893	T	C	c.4525A>G	p.Ile1509Val	Pathogenic
6018877	A	C	c.4541T>G	p.Phe1514Cys	Pathogenic
6018866	T	C	c.4552A>G	p.Lys1518Glu	Pathogenic
6018860	ACTC	A	c.4555_4557del	p.Glu1519del	Pathogenic
6018847	AC	A	c.4570del	p.Val1524Ter	Pathogenic
6018847	AC	C	c.4571del	p.Val1524GlyfsTer23	Pathogenic
6018844	A	C	c.4574T>G	p.Ile1525Ser	Pathogenic
6018842	G	A	c.4576C>T	p.Gln1526Ter	Pathogenic
6018840	C	A	c.4578G>T	p.Gln1526His	Pathogenic
6018838	C	T	c.4580G>A	p.Arg1527Gln	Pathogenic
6018836	T	C	c.4582A>G	p.Met1528Val	Pathogenic
6018826	CCCACAT	C	c.4586_4591del	p.Asp1529_Val1530del	Pathogenic
6018808	A	C	c.4610T>G	p.Val1537Gly	Pathogenic
6018806	TGACGTG	T	c.4606_4611del	p.His1536_Val1537del	Pathogenic
6018805	GTGACGTGGA	G	c.4604_4612del	p.Ile1535_Val1537del	Pathogenic
6018802	A	T	c.4616T>A	p.Val1539Glu	Pathogenic
6018799	A	G	c.4619T>C	p.Leu1540Pro	Pathogenic
6018798	C	G	c.4620G>C	p.Leu1540=	Not provided
6018796	T	A	c.4622A>T	p.Gln1541Leu	Pathogenic
6018796	T	C	c.4622A>G	p.Gln1541Arg	Uncertain significance
6018793	TAC	T	c.4623_4624del	p.Tyr1542LeufsTer45	Pathogenic
6018793	T	C	c.4625A>G	p.Tyr1542Cys	Pathogenic
6018792	G	C	c.4626C>G	p.Tyr1542Ter	Pathogenic
6018791	A	G	c.4627T>C	p.Ser1543Pro	Pathogenic
6018790	G	A	c.4628C>T	p.Ser1543Phe	Pathogenic
6018781	A	T	c.4637T>A	p.Val1546Glu	Uncertain significance
6018781	AC	A	c.4636del	p.Val1546Ter	Pathogenic

6018781	A	C	c.4637T>G	p.Val1546Gly	Pathogenic
6018777	A	G	c.4641T>C	p.Thr1547=	Benign
6018773	C	T	c.4645G>A	p.Glu1549Lys	Pathogenic
6018773	C	A	c.4645G>T	p.Glu1549Ter	Pathogenic
6018772	TCCACAGTCA	T	c.4637_4645del	p.Val1546_Val1548del	Pathogenic
6018766	GGGT	G	c.4649_4651del	p.Tyr1550_Pro1551delinsSer	Pathogenic
6018753	T	G	c.4665A>C	p.Ala1555=	Benign
6018752	G	A	c.4666C>T	p.Gln1556Ter	Pathogenic
6018751	T	C	c.4667A>G	p.Gln1556Arg	Pathogenic
6018739	T	TGTC	c.4678_4680dup	p.Asp1560delinsGlyHis	Pathogenic
6018738	G	C	c.4680C>G	p.Asp1560Glu	Uncertain significance
6018733	A	G	c.4685T>C	p.Leu1562Pro	Pathogenic
6018728	G	A	c.4690C>T	p.Arg1564Trp	Conflicting interpretation
6018727	C	G	c.4691G>C	p.Arg1564Pro	Pathogenic
6018725	C	A	c.4693G>T	p.Val1565Leu	Benign
6018722	G	A	c.4696C>T	p.Arg1566Ter	Pathogenic
6018718	TC	T	c.4699del	p.Glu1567ArgfsTer126	Pathogenic
6018715	A	T	c.4703T>A	p.Ile1568Asn	Pathogenic
6018710	AGCG	A	c.4705_4707del	p.Arg1569del	Pathogenic
6018702	G	A	c.4716C>T	p.Gly1572=	Benign
6018701	C	T	c.4717G>A	p.Gly1573Ser	Pathogenic
6018692	TC	T	c.4725del	p.Thr1576ProfsTer117	Pathogenic
6018692	T	G	c.4726A>C	p.Thr1576Pro	Pathogenic
6018691	G	T	c.4727C>A	p.Thr1576Asn	Uncertain significance
6018686	T	G	c.4732A>C	p.Thr1578Pro	Uncertain significance
6018685	G	T	c.4733C>A	p.Thr1578Asn	Pathogenic
6018683	C	T	c.4735G>A	p.Gly1579Arg	Conflicting interpretation
6018680	G	C	c.4738C>G	p.Leu1580Val	Pathogenic

6018679	A	G	c.4739T>C	p.Leu1580Pro	Pathogenic
6018673	A	G	c.4745T>C	p.Leu1582Pro	Pathogenic
6018671	G	A	c.4747C>T	p.Arg1583Trp	Conflicting interpretation
6018670	C	T	c.4748G>A	p.Arg1583Gln	Pathogenic
6018667	T	C	c.4751A>G	p.Tyr1584Cys	Conflicting interpretation
6018643	A	T	c.4775T>A	p.Val1592Asp	Pathogenic
6018629	G	C	c.4789C>G	p.Arg1597Gly	Pathogenic
6018629	G	A	c.4789C>T	p.Arg1597Trp	Pathogenic
6018628	C	T	c.4790G>A	p.Arg1597Gln	Pathogenic
6018628	C	A	c.4790G>T	p.Arg1597Leu	Pathogenic
6018628	C	G	c.4790G>C	p.Arg1597Pro	Pathogenic
6018618	C	T	c.4800G>A	p.Ala1600=	Benign
6018610	A	G	c.4808T>C	p.Leu1603Pro	Pathogenic
6018608	C	A	c.4810G>T	p.Val1604Phe	Pathogenic
6018598	A	T	c.4820T>A	p.Val1607Asp	Pathogenic
6018596	T	G	c.4822A>C	p.Thr1608Pro	Pathogenic
6018594	G	A	c.4824C>T	p.Thr1608=	Benign
6018593	C	T	c.4825G>A	p.Gly1609Arg	Pathogenic
6018584	C	G	c.4834G>C	p.Ala1612Pro	Uncertain significance
6018581	A	G	c.4837T>C	p.Ser1613Pro	Pathogenic
6018578	C	T	c.4840G>A	p.Asp1614Asn	Conflicting interpretation
6018577	T	C	c.4841A>G	p.Asp1614Gly	Pathogenic
6018576	A	C	c.4842T>G	p.Asp1614Glu	Benign
6018568	T	C	c.4850A>G	p.Lys1617Arg	Pathogenic
6018552	G	A	c.4866C>T	p.Asp1622=	Pathogenic
6018545	C	G	c.4873G>C	p.Val1625Leu	Uncertain significance
6018544	A	C	c.4874T>G	p.Val1625Gly	Pathogenic
6018538	G	T	c.4880C>A	p.Pro1627His	Pathogenic
6018535	A	G	c.4883T>C	p.Ile1628Thr	Pathogenic
6018535	A	T	c.4883T>A	p.Ile1628Asn	Pathogenic
6018533	C	T	c.4885G>A	p.Gly1629Arg	Pathogenic
6018533	C	G	c.4885G>C	p.Gly1629Arg	Pathogenic

6018532	C	T	c.4886G>A	p.Gly1629Glu	Pathogenic
6018531	TC	T	c.4886del	p.Gly1629GlufsTer64	Not provided
6018530	C	T	c.4888G>A	p.Val1630Met	Pathogenic
6018529	A	C	c.4889T>G	p.Val1630Gly	Pathogenic
6018529	A	T	c.4889T>A	p.Val1630Glu	Pathogenic
6018526	C	T	c.4892G>A	p.Gly1631Asp	Pathogenic
6018514	T	A	c.4904A>T	p.Asn1635Ile	Pathogenic
6018514	T	G	c.4904A>C	p.Asn1635Thr	Pathogenic
6018512	C	T	c.4906G>A	p.Val1636Met	Uncertain significance
6018511	A	G	c.4907T>C	p.Val1636Ala	Uncertain significance
6018506	C	T	c.4912G>A	p.Glu1638Lys	Pathogenic
6018502	A	G	c.4916T>C	p.Leu1639Pro	Pathogenic
6018492	A	G	c.4926T>C	p.Ile1642=	Uncertain significance
6018491	C	T	c.4927G>A	p.Gly1643Ser	Conflicting interpretation
6018487	C	T	c.4931G>A	p.Trp1644Ter	Pathogenic
6018478	G	T	c.4940C>A	p.Ala1647Asp	Pathogenic
6018476	G	A	c.4942C>T	p.Pro1648Ser	Pathogenic
6018475	G	C	c.4943C>G	p.Pro1648Arg	Pathogenic
6018474	A	G	c.4944T>C	p.Pro1648=	Uncertain significance
6018473	TA	T	c.4944del	p.Ile1649SerfsTer44	Conflicting interpretation
6018472	A	T	c.4946T>A	p.Ile1649Asn	Pathogenic
6018458	A	G	c.4960T>C	p.Phe1654Leu	Pathogenic
6018449	G	T	c.4969C>A	p.Leu1657Ile	Pathogenic
6018448	A	T	c.4970T>A	p.Leu1657His	Pathogenic
6018443	G	A	c.4975C>T	p.Arg1659Ter	Pathogenic
6018442	C	CG	c.4975dup	p.Arg1659ProfsTer5	Pathogenic
6018424	A	T	c.4994T>A	p.Val1665Glu	Pathogenic
6018424	A	C	c.4994T>G	p.Val1665Gly	Pathogenic
6018417	C	T	c.5001G>A	p.Gln1667=	Not provided
6018417	C	G	c.5001G>C	p.Gln1667His	Pathogenic
6018414	C	A	c.5004G>T	p.Arg1668Ser	Pathogenic
6018404	C	T	c.5014G>A	p.Gly1672Arg	Conflicting interpretation

6018399	C	G	c.5019G>C	p.Glu1673Asp	Pathogenic
6018394	AG	TA	c.5023_5024delinsTA	p.Leu1675Ter	Not provided
6018374	G	A	c.5044C>T	p.Pro1682Ser	Uncertain significance
6018371	CA	C	c.5046del	p.Ala1683HisfsTer10	Pathogenic
6018369	T	G	c.5049A>C	p.Ala1683=	Uncertain significance
6018364	C	T	c.5053+1G>A	NA	Pathogenic
6018364	C	A	c.5053+1G>T	NA	Pathogenic
6018362	T	C	c.5053+3A>G	NA	Pathogenic
6018328	C	T	c.5053+37G>A	NA	Benign
6016851	G	T	c.5073C>A	p.Asp1691Glu	Pathogenic
6016837	A	C	c.5087T>G	p.Leu1696Arg	Pathogenic
6016836	CAGG	C	c.5085_5087del	p.Leu1696del	Pathogenic
6016828	G	A	c.5096C>T	p.Ser1699Phe	Pathogenic
6016826	AG	A	c.5097del	p.Ser1700ProfsTer11	Pathogenic
6016785	G	A	c.5139C>T	p.Phe1713=	Benign
6016784	C	G	c.5140G>C	p.Ala1714Pro	Pathogenic
6016778	C	G	c.5146G>C	p.Ala1716Pro	Pathogenic
6016759	T	C	c.5165A>G	p.Asn1722Ser	Uncertain significance
6016751	C	T	c.5170+3G>A	NA	Uncertain significance
6016749	C	T	c.5170+5G>A	NA	Uncertain significance
6016744	G	A	c.5170+10C>T	NA	Conflicting interpretation
6016740	C	G	c.5170+14G>C	NA	Uncertain significance
6016664	G	A	c.5171-8C>T	NA	Uncertain significance
6016664	GA	G	c.5171-9del	NA	Benign
6016663	G	A	c.5171-7C>T	NA	Uncertain significance
6016658	T	C	c.5171-2A>G	NA	Pathogenic
6016654	G	A	c.5173C>T	p.Pro1725Ser	Benign
6016650	C	T	c.5177G>A	p.Arg1726His	Uncertain significance
6016646	G	GAA	c.5180_5181insTT	p.Thr1728SerfsTer29	Pathogenic
6016645	T	A	c.5182A>T	p.Thr1728Ser	Pathogenic

6016640	CTGAG	C	c.5183_5186del	p.Thr1728ArgfsTer27	Pathogenic
6016638	A	C	c.5189T>G	p.Val1730Gly	Pathogenic
6016636	A	T	c.5191T>A	p.Ser1731Thr	Conflicting interpretation
6016629	A	G	c.5198T>C	p.Leu1733Pro	Pathogenic
6016627	G	A	c.5200C>T	p.Gln1734Ter	Pathogenic
6016624	A	C	c.5203T>G	p.Tyr1735Asp	Pathogenic
6016611	G	A	c.5216C>T	p.Thr1739Ile	Uncertain significance
6016600	C	T	c.5227G>A	p.Val1743Met	Uncertain significance
6016592	C	A	c.5235G>T	p.Trp1745Cys	Pathogenic
6016554	A	G	c.5273T>C	p.Val1758Ala	Pathogenic
6016550	G	A	c.5277C>T	p.Asp1759=	Conflicting interpretation
6016549	C	T	c.5278G>A	p.Val1760Ile	Conflicting interpretation
6016545	A	T	c.5282T>A	p.Met1761Lys	Pathogenic
6016516	C	T	c.5311G>A	p.Gly1771Arg	Pathogenic
6016516	CG	C	c.5310del	p.Ile1770MetfsTer12	Pathogenic
6016370	C	T	c.5312-138G>A	NA	Not provided
6016279	C	G	c.5312-47G>C	NA	Benign
6016251	T	G	c.5312-19A>C	NA	Conflicting interpretation
6016251	T	C	c.5312-19A>G	NA	Pathogenic
6016232	CCT	C	c.5312-2_5312-1del	NA	Pathogenic
6016231	C	A	c.5313G>T	p.Gly1771=	Benign
6016229	T	TTC	c.5314_5315dup	NA	Pathogenic
6016223	A	G	c.5321T>C	p.Leu1774Ser	Pathogenic
6016209	G	A	c.5335C>T	p.Arg1779Ter	Pathogenic
6016208	C	A	c.5336G>T	p.Arg1779Leu	Pathogenic
6016206	A	G	c.5338T>C	p.Tyr1780His	Uncertain significance
6016197	A	C	c.5347T>G	p.Ser1783Ala	Pathogenic
6016188	G	C	c.5356C>G	p.His1786Asp	Pathogenic
6016176	G	A	c.5368C>T	p.Pro1790Ser	Pathogenic
6016175	G	A	c.5369C>T	p.Pro1790Leu	Uncertain significance
6016165	T	C	c.5379A>G	p.Ser1793=	Uncertain significance

6016164	T	C	c.5380A>G	p.Lys1794Glu	Pathogenic
6016160	G	A	c.5384C>T	p.Ala1795Val	Uncertain significance
6016138	G	A	c.5406C>T	p.Asp1802=	Uncertain significance
6016128	CCA	C	c.5414_5415del	p.Val1805GlyfsTer8	Pathogenic
6016102	G	T	c.5442C>A	p.Ala1814=	Uncertain significance
6016091	T	C	c.5453A>G	p.Asn1818Ser	Conflicting interpretation
6016088	C	T	c.5455+1G>A	NA	Pathogenic
6016087	A	G	c.5455+2T>C	NA	Pathogenic
6013707	T	C	c.5456-62A>G	NA	Not provided
6013636	A	T	c.5465T>A	p.Val1822Glu	Pathogenic
6013636	A	C	c.5465T>G	p.Val1822Gly	Pathogenic
6013630	G	T	c.5471C>A	p.Pro1824His	Pathogenic
6013630	G	C	c.5471C>G	p.Pro1824Arg	Pathogenic
6013613	G	A	c.5488C>T	p.Arg1830Cys	Pathogenic
6013608	G	A	c.5493C>T	p.Tyr1831=	Uncertain significance
6013592	G	A	c.5509C>T	p.Arg1837Trp	Pathogenic
6013586	A	G	c.5515T>C	p.Leu1839=	Benign
6013580	C	CT	c.5520dup	p.Gly1841ArgfsTer37	Pathogenic
6013579	C	A	c.5522G>T	p.Gly1841Val	Uncertain significance
6013568	C	T	c.5533G>A	p.Asp1845Asn	Uncertain significance
6013556	C	T	c.5545G>A	p.Val1849Met	Not provided
6013552	T	A	c.5549A>T	p.Lys1850Met	Pathogenic
6013544	G	A	c.5557C>T	p.Arg1853Ter	Pathogenic
6013485	GCA	G	c.5614_5615del	p.Cys1872LeufsTer5	Pathogenic
6013471	T	C	c.5620+10A>G	NA	Uncertain significance
6013431	A	G	c.5620+50T>C	NA	Benign
6012115	C	A	c.5636G>T	p.Cys1879Phe	Pathogenic
6012090	C	A	c.5661G>T	p.Lys1887Asn	Pathogenic
6012085	A	G	c.5664+2T>C	NA	Pathogenic
6012082	C	T	c.5664+5G>A	NA	Pathogenic
6012072	C	T	c.5664+15G>A	NA	Benign
6012054	C	T	c.5664+33G>A	NA	Benign

6012049	T	A	c.5664+38A>T	NA	Benign
6011981	A	G	c.5664+106T>C	NA	Benign
6011959	C	A	c.5664+128G>T	NA	Benign
6011912	C	T	c.5665-118G>A	NA	Benign
6011830	A	G	c.5665-36T>C	NA	Not provided
6011803	G	T	c.5665-9C>A	NA	Uncertain significance
6011792	G	A	c.5667C>T	p.Pro1889=	Benign
6011792	G	C	c.5667C>G	p.Pro1889=	Uncertain significance
6011791	C	T	c.5668G>A	p.Gly1890Arg	Conflicting interpretation
6011790	C	T	c.5669G>A	p.Gly1890Glu	Pathogenic
6011767	G	A	c.5692C>T	p.Gln1898Ter	Pathogenic
6011764	A	G	c.5695T>C	p.Cys1899Arg	Pathogenic
6011760	T	G	c.5699A>C	p.His1900Pro	Pathogenic
6011712	C	T	c.5747G>A	p.Arg1916Gln	Not provided
6011691	A	G	c.5768T>C	p.Leu1923Pro	Pathogenic
6011680	A	G	c.5779T>C	p.Cys1927Arg	Conflicting interpretation
6011675	AGG	A	c.5782_5783del	p.Pro1928Ter	Pathogenic
6011674	T	A	c.5785A>T	p.Asn1929Tyr	Uncertain significance
6011668	G	A	c.5791C>T	p.Gln1931Ter	Pathogenic
6011666	C	G	c.5793G>C	p.Gln1931His	Pathogenic
6011661	G	A	c.5798C>T	p.Pro1933Leu	Pathogenic
6011658	A	C	c.5801T>G	p.Val1934Gly	Pathogenic
6011657	A	C	c.5802T>G	p.Val1934=	Benign
6011645	C	G	c.5814G>C	p.Glu1938Asp	Pathogenic
6011622	C	A	c.5837G>T	p.Cys1946Phe	Pathogenic
6011616	C	G	c.5842+1G>C	NA	Pathogenic
6011612	C	T	c.5842+5G>A	NA	Pathogenic
6011586	G	A	c.5842+31C>T	NA	Not provided
5996230	G	C	c.5843-8C>G	NA	Benign
5996221	G	A	c.5844C>T	p.Cys1948=	Benign
5996216	C	T	c.5849G>A	p.Cys1950Tyr	Uncertain significance
5996214	T	C	c.5851A>G	p.Thr1951Ala	Conflicting interpretation

5996199	G	A	c.5866C>T	p.Arg1956Trp	Uncertain significance
5996194	GT	G	c.5870del	p.His1957ProfsTer3	Pathogenic
5996189	A	C	c.5876T>G	p.Val1959Gly	Uncertain significance
5996175	G	T	c.5890C>A	p.Gln1964Lys	Pathogenic
5996124	C	A	c.5941G>T	p.Glu1981Ter	Pathogenic
5996050	G	A	c.6015C>T	p.Ile2005=	Benign
5996040	G	A	c.6025C>T	p.His2009Tyr	Uncertain significance
5996026	G	A	c.6039C>T	p.Ser2013=	Benign
5996022	C	T	c.6043G>A	p.Glu2015Lys	Uncertain significance
5994604	T	C	c.6067A>G	p.Thr2023Ala	Pathogenic
5994603	G	A	c.6068C>T	p.Thr2023Met	Pathogenic
5994602	C	T	c.6069G>A	p.Thr2023=	Benign
5994572	G	A	c.6099C>T	p.Tyr2033=	Benign
5994572	G	C	c.6099C>G	p.Tyr2033Ter	Pathogenic
5994567	C	T	c.6104G>A	p.Gly2035Asp	Pathogenic
5994540	C	T	c.6131G>A	p.Gly2044Asp	Pathogenic
5994488	GA	G	c.6182del	p.Phe2061SerfsTer38	Pathogenic
5994488	G	C	c.6183C>G	p.Phe2061Leu	Uncertain significance
5994484	G	A	c.6187C>T	p.Pro2063Ser	Conflicting interpretation
5994475	T	C	c.6196A>G	p.Asn2066Asp	Uncertain significance
5994474	T	C	c.6197A>G	p.Asn2066Ser	Conflicting interpretation
5994434	T	C	c.6237A>G	p.Ser2079=	Not provided
5994423	C	T	c.6248G>A	p.Gly2083Asp	Pathogenic
5994417	C	A	c.6254G>T	p.Cys2085Phe	Pathogenic
5994417	C	T	c.6254G>A	p.Cys2085Tyr	Pathogenic
5994412	T	TA	c.6256+2dup	NA	Conflicting interpretation
5994204	C	T	c.6257-1G>A	NA	Pathogenic
5994157	G	T	c.6303C>A	p.Gly2101=	Uncertain significance
5994149	G	A	c.6311C>T	p.Thr2104Ile	Pathogenic
5994146	G	A	c.6314C>T	p.Thr2105Ile	Uncertain significance
5994139	C	T	c.6321G>A	p.Trp2107Ter	Pathogenic
5994115	A	T	c.6345T>A	p.Thr2115=	Benign

5994108	G	A	c.6352C>T	p.Arg2118Trp	Pathogenic
5994107	C	T	c.6353G>A	p.Arg2118Gln	Uncertain significance
5994083	A	G	c.6377T>C	p.Ile2126Thr	Uncertain significance
5994075	C	A	c.6385G>T	p.Glu2129Ter	Pathogenic
5994049	GGAGCTGTCGGGGAC	G	c.6397_6410del	p.Val2133ProfsTer12	Pathogenic
5994042	G	A	c.6418C>T	p.Gln2140Ter	Pathogenic
5994039	C	T	c.6421G>A	p.Val2141Ile	Uncertain significance
5994036	G	A	c.6424C>T	p.Leu2142Phe	Conflicting interpretation
5994027	G	C	c.6433C>G	p.Pro2145Ala	Pathogenic
5994027	G	A	c.6433C>T	p.Pro2145Ser	Conflicting interpretation
5994011	C	T	c.6449G>A	p.Cys2150Tyr	Pathogenic
5994010	G	C	c.6450C>G	p.Cys2150Trp	Pathogenic
5994009	G	A	c.6451C>T	p.His2151Tyr	Pathogenic
5994003	C	A	c.6457G>T	p.Val2153Phe	Pathogenic
5993981	T	C	c.6479A>G	p.Tyr2160Cys	Uncertain significance
5993976	TG	T	c.6483del	p.Ile2162SerfsTer15	Pathogenic
5993972	C	CGATCACCTCACACACTTGCTCCTGGTGGCAACTGTCCTGCTGGCA	c.6487_6531dup	p.Cys2163delinsLeuProAlaGlyGlnLeuProProGlyAlaSerValTer	Pathogenic
5993972	C	T	c.6488G>A	p.Cys2163Tyr	Pathogenic
5993970	G	A	c.6490C>T	p.Gln2164Ter	Pathogenic
5993940	A	C	c.6520T>G	p.Cys2174Gly	Conflicting interpretation
5993928	C	A	c.6532G>T	p.Ala2178Ser	Benign
5993924	G	A	c.6536C>T	p.Ser2179Phe	Pathogenic
5993922	A	G	c.6538T>C	p.Tyr2180His	Uncertain significance
5993920	A	C	c.6540T>G	p.Tyr2180Ter	Pathogenic
5993909	C	T	c.6551G>A	p.Cys2184Tyr	Pathogenic

5993909	C	A	c.6551G>T	p.Cys2184Phe	Pathogenic
5993909	C	G	c.6551G>C	p.Cys2184Ser	Pathogenic
5993907	G	A	c.6553C>T	p.Arg2185Trp	Conflicting interpretation
5993906	C	T	c.6554G>A	p.Arg2185Gln	Conflicting interpretation
5993891	C	T	c.6569G>A	p.Cys2190Tyr	Pathogenic
5993890	G	C	c.6570C>G	p.Cys2190Trp	Pathogenic
5993888	A	G	c.6572T>C	p.Val2191Ala	Uncertain significance
5993883	A	G	c.6577T>C	p.Trp2193Arg	Conflicting interpretation
5993881	C	G	c.6579G>C	p.Trp2193Cys	Pathogenic
5993861	C	T	c.6598+1G>A	NA	Pathogenic
5992038	T	A	c.6599-20A>T	NA	Conflicting interpretation
5992022	T	TA	c.6599-5dup	NA	Benign
5992006	G	A	c.6611C>T	p.Pro2204Leu	Uncertain significance
5991997	A	G	c.6620T>C	p.Leu2207Pro	Pathogenic
5991983	A	G	c.6634T>C	p.Cys2212Arg	Pathogenic
5991982	C	T	c.6635G>A	p.Cys2212Tyr	Pathogenic
5991938	A	AG	c.6678dup	p.Cys2227LeufsTer15	Pathogenic
5991920	C	T	c.6697G>A	p.Glu2233Lys	Pathogenic
5991920	C	G	c.6697G>C	p.Glu2233Gln	Not provided
5991919	T	C	c.6698A>G	p.Glu2233Gly	Pathogenic
5991919	T	A	c.6698A>T	p.Glu2233Val	Pathogenic
5991917	C	CCCT	c.6699_6702dup	p.Glu2233_Gly2234insArg	Pathogenic
5991908	A	G	c.6709T>C	p.Cys2237Arg	Pathogenic
5991907	C	A	c.6710G>T	p.Cys2237Phe	Pathogenic
5991874	C	T	c.6743G>A	p.Cys2248Tyr	Pathogenic
5991861	C	T	c.6756G>A	p.Glu2252=	Conflicting interpretation
5991856	C	T	c.6761G>A	p.Cys2254Tyr	Pathogenic
5991855	G	C	c.6762C>G	p.Cys2254Trp	Uncertain significance
5991849	C	CCACT	c.6767_6770dup	p.Cys2257ValfsTer5	Pathogenic
5991848	A	T	c.6769T>A	p.Cys2257Ser	Pathogenic
5991846	G	T	c.6771C>A	p.Cys2257Ter	Pathogenic

5991819	C	G	c.6798G>C	p.Gln2266His	Pathogenic
5991818	C	G	c.6798+1G>C	NA	Pathogenic
5991818	C	A	c.6798+1G>T	NA	Pathogenic
5991814	C	T	c.6798+5G>A	NA	Uncertain significance
5991813	T	C	c.6798+6A>G	NA	Uncertain significance
5991808	C	T	c.6798+11G>A	NA	Benign
5991806	C	T	c.6798+13G>A	NA	Uncertain significance
5991805	G	A	c.6798+14C>T	NA	Benign
5991787	C	T	c.6798+32G>A	NA	Not provided
5985712	C	T	c.6799-47G>A	NA	Not provided
5985692	G	A	c.6799-27C>T	NA	Not provided
5985679	G	A	c.6799-14C>T	NA	Benign
5985653	A	C	c.6811T>G	p.Trp2271Gly	Pathogenic
5985618	T	C	c.6846A>G	p.Thr2282=	Benign
5985617	A	G	c.6847T>C	p.Cys2283Arg	Pathogenic
5985607	CCGCT	C	c.6853_6856del	p.Ser2285GlyfsTer24	Pathogenic
5985605	G	A	c.6859C>T	p.Arg2287Trp	Conflicting interpretation
5985604	C	T	c.6860G>A	p.Arg2287Gln	Pathogenic
5985596	T	A	c.6868A>T	p.Asn2290Tyr	Pathogenic
5985574	G	A	c.6890C>T	p.Pro2297Leu	Conflicting interpretation
5985571	G	A	c.6893C>T	p.Thr2298Met	Uncertain significance
5985553	G	A	c.6901+10C>T	NA	Benign
5985134	C	T	c.6902-15G>A	NA	Benign
5985124	A	T	c.6902-5T>A	NA	Benign
5985113	G	A	c.6908C>T	p.Thr2303Met	Uncertain significance
5985111	ACGTGGGAG	A	c.6902_6909del	p.Ala2301ValfsTer5	Pathogenic
5985110	C	T	c.6911G>A	p.Cys2304Tyr	Pathogenic
5985090	G	A	c.6931C>T	p.Arg2311Cys	Uncertain significance
5985089	C	T	c.6932G>A	p.Arg2311His	Pathogenic
5985084	G	A	c.6937C>T	p.Arg2313Cys	Conflicting interpretation
5985083	C	T	c.6938G>A	p.Arg2313His	Conflicting interpretation

5985048	A	T	c.6973T>A	p.Cys2325Ser	Pathogenic
5985034	G	A	c.6976+11C>T	NA	Uncertain significance
5984934	C	T	c.6976+111G>A	NA	Not provided
5983255	C	G	c.6977-1G>C	NA	Pathogenic
5983252	A	T	c.6979T>A	p.Cys2327Ser	Pathogenic
5983251	C	T	c.6980G>A	p.Cys2327Tyr	Pathogenic
5983250	A	C	c.6981T>G	p.Cys2327Trp	Pathogenic
5983242	A	C	c.6989T>G	p.Val2330Gly	Pathogenic
5983224	G	A	c.7007C>T	p.Pro2336Leu	Uncertain significance
5983213	A	G	c.7018T>C	p.Cys2340Arg	Pathogenic
5983206	C	T	c.7025G>A	p.Arg2342His	Conflicting interpretation
5983203	C	A	c.7028G>T	p.Gly2343Val	Pathogenic
5983175	G	A	c.7056C>T	p.Gly2352=	Pathogenic
5983174	C	T	c.7057G>A	p.Glu2353Lys	Pathogenic
5983172	C	G	c.7059G>C	p.Glu2353Asp	Uncertain significance
5983161	T	A	c.7070A>T	p.Asn2357Ile	Pathogenic
5983151	G	T	c.7080C>A	p.Cys2360Ter	Pathogenic
5983144	C	A	c.7081+6G>T	NA	Pathogenic
5982004	C	G	c.7082-13G>C	NA	Conflicting interpretation
5981998	G	A	c.7082-7C>T	NA	Benign
5981993	T	C	c.7082-2A>G	NA	Pathogenic
5981988	C	A	c.7085G>T	p.Cys2362Phe	Pathogenic
5981987	G	T	c.7086C>A	p.Cys2362Ter	Pathogenic
5981987	G	C	c.7086C>G	p.Cys2362Trp	Uncertain significance
5981955	G	A	c.7118C>T	p.Pro2373Leu	Pathogenic
5981942	C	CG	c.7130dup	p.His2378AlafsTer13	Pathogenic
5981938	G	A	c.7135C>T	p.Arg2379Cys	Conflicting interpretation
5981933	C	CA	c.7139dup	p.Leu2380PhefsTer11	Pathogenic
5981933	C	A	c.7140G>T	p.Leu2380Phe	Uncertain significance
5981923	G	A	c.7150C>T	p.Arg2384Trp	Pathogenic
5981922	C	T	c.7151G>A	p.Arg2384Gln	Uncertain significance
5981921	C	A	c.7152G>T	p.Arg2384=	Benign

5981900	C	CA	c.7172_7173insT	p.Glu2391AspfsTer3	Pathogenic
5981897	A	C	c.7176T>G	p.Tyr2392Ter	Pathogenic
5981891	A	C	c.7182T>G	p.Cys2394Trp	Pathogenic
5981889	G	A	c.7184C>T	p.Ala2395Val	Uncertain significance
5981873	GT	G	c.7199del	p.Asn2400ThrfsTer4	Pathogenic
5981853	A	G	c.7220T>C	p.Leu2407Pro	Conflicting interpretation
5981834	A	G	c.7239T>C	p.Thr2413=	Benign
5981833	C	T	c.7240G>A	p.Ala2414Thr	Uncertain significance
5981819	ACA	A	c.7252_7253del	NA	Pathogenic
5981785	C	T	c.7287+1G>A	NA	Pathogenic
5976328	C	T	c.7288-68G>A	NA	Pathogenic
5976279	G	A	c.7288-19C>T	NA	Benign
5976269	A	T	c.7288-9T>A	NA	Uncertain significance
5976269	A	C	c.7288-9T>G	NA	Uncertain significance
5976256	C	T	c.7292G>A	p.Cys2431Tyr	Pathogenic
5976252	GAC	G	c.7294_7295del	p.Val2432ProfsTer42	Pathogenic
5976248	G	A	c.7300C>T	p.Arg2434Ter	Pathogenic
5976227	C	A	c.7321G>T	p.Gly2441Cys	Pathogenic
5976216	C	T	c.7332G>A	p.Trp2444Ter	Pathogenic
5976214	TC	T	c.7333del	p.Glu2445ArgfsTer16	Pathogenic
5976205	C	T	c.7343G>A	p.Cys2448Tyr	Pathogenic
5976204	G	A	c.7344C>T	p.Cys2448=	Benign
5976204	G	T	c.7344C>A	p.Cys2448Ter	Pathogenic
5976196	C	T	c.7352G>A	p.Cys2451Tyr	Pathogenic
5976187	G	T	c.7361C>A	p.Thr2454Asn	Pathogenic
5976186	G	A	c.7362C>T	p.Thr2454=	Uncertain significance
5976171	GGCATCCTCCATGTCGGT	G	c.7360_7376del	p.Thr2454ArgfsTer15	Pathogenic
5976170	C	CCGCGGAGGCCCATCACG	c.7377_7393dup	p.Val2465delinsAlaTer	Not provided

5976158	G	A	c.7390C>T	p.Arg2464Cys	Pathogenic
5976155	C	T	c.7393G>A	p.Val2465Met	Conflicting interpretation
5976149	G	A	c.7399C>T	p.Gln2467Ter	Pathogenic
5976148	T	G	c.7400A>C	p.Gln2467Pro	Pathogenic
5976146	A	G	c.7402T>C	p.Cys2468Arg	Pathogenic
5976143	A	G	c.7405T>C	p.Ser2469Pro	Conflicting interpretation
5976140	G	A	c.7408C>T	p.Gln2470Ter	Pathogenic
5976129	A	C	c.7419T>G	p.Cys2473Trp	Pathogenic
5976119	A	G	c.7429T>C	p.Cys2477Arg	Uncertain significance
5976118	C	G	c.7430G>C	p.Cys2477Ser	Pathogenic
5976118	C	T	c.7430G>A	p.Cys2477Tyr	Pathogenic
5976111	C	T	c.7437G>A	p.Ser2479=	Pathogenic
5976110	C	T	c.7437+1G>A	NA	Pathogenic
5976109	A	G	c.7437+2T>C	NA	Pathogenic
5976106	C	T	c.7437+5G>A	NA	Benign
5976106	C	A	c.7437+5G>T	NA	Pathogenic
5971878	C	T	c.7438-169G>A	NA	Not provided
5971698	G	GT	c.7448dup	p.Tyr2483Ter	Pathogenic
5971697	C	CT	c.7449_7450insA	p.Val2484SerfsTer4	Not provided
5971697	C	T	c.7450G>A	p.Val2484Ile	Pathogenic
5971690	T	C	c.7457A>G	p.His2486Arg	Uncertain significance
5971683	G	A	c.7464C>T	p.Gly2488=	Pathogenic
5971676	A	G	c.7471T>C	p.Cys2491Arg	Pathogenic
5971663	AG	A	c.7483del	p.Leu2495CysfsTer8	Uncertain significance
5971658	A	G	c.7489T>C	p.Ser2497Pro	Pathogenic
5971654	G	T	c.7493C>A	p.Ala2498Asp	Conflicting interpretation
5971633	G	T	c.7514C>A	p.Ser2505Ter	Pathogenic
5971621	TCC	T	c.7524_7525del	p.Asp2509LeufsTer23	Pathogenic
5971621	TC	T	c.7525del	p.Asp2509ThrfsTer28	Pathogenic
5971577	C	T	c.7548+22G>A	NA	Pathogenic
5969471	A	T	c.7549-80T>A	NA	Pathogenic

5969394	G	T	c.7549-3C>A	NA	Pathogenic
5969391	C	A	c.7549G>T	p.Val2517Phe	Pathogenic
5969388	C	T	c.7552G>A	p.Gly2518Ser	Pathogenic
5969387	C	G	c.7553G>C	p.Gly2518Ala	Uncertain significance
5969382	G	A	c.7558C>T	p.Gln2520Ter	Pathogenic
5969381	T	G	c.7559A>C	p.Gln2520Pro	Pathogenic
5969360	G	T	c.7580C>A	p.Pro2527His	Pathogenic
5969358	AGGGGTTCTCC	A	c.7572_7581del	p.Glu2525AlafsTer9	Pathogenic
5969357	C	T	c.7583G>A	p.Cys2528Tyr	Pathogenic
5969341	A	T	c.7599T>A	p.Cys2533Ter	Not provided
5969337	G	A	c.7603C>T	p.Arg2535Ter	Pathogenic
5969336	C	T	c.7604G>A	p.Arg2535Gln	Pathogenic
5969336	C	G	c.7604G>C	p.Arg2535Pro	Pathogenic
5969321	A	G	c.7619T>C	p.Val2540Ala	Uncertain significance
5969315	A	T	c.7625T>A	p.Ile2542Lys	Uncertain significance
5969313	G	A	c.7627C>T	p.Gln2543Ter	Pathogenic
5969310	G	A	c.7630C>T	p.Gln2544Ter	Pathogenic
5969304	T	A	c.7636A>T	p.Asn2546Tyr	Conflicting interpretation
5969289	G	A	c.7651C>T	p.Gln2551Ter	Pathogenic
5969288	TGG	T	c.7650_7651del	p.Gln2551AlafsTer15	Pathogenic
5969287	C	CA	c.7652_7653insT	p.Gln2551HisfsTer16	Pathogenic
5969275	A	ACT	c.7664_7665insAG	p.Cys2557SerfsTer8	Pathogenic
5969271	A	G	c.7669T>C	p.Cys2557Arg	Pathogenic
5969265	A	AG	c.7674dup	p.Ser2559LeufsTer8	Pathogenic
5969263	CGAGGG	C	c.7672_7676del	p.Pro2558GlyfsTer7	Pathogenic
5969261	C	A	c.7679G>T	p.Gly2560Val	Pathogenic
5969260	GCCCGAGGGGCAGA	G	c.7667_7679del	p.Val2556AlafsTer4	Pathogenic
5969258	A	T	c.7682T>A	p.Phe2561Tyr	Benign
5969256	GA	G	c.7683del	p.Gln2562SerfsTer2	Pathogenic
5969242	C	T	c.7698G>A	p.Lys2566=	Uncertain significance

5969237	G	T	c.7703C>A	p.Ser2568Ter	Pathogenic
5969217	G	A	c.7723C>T	p.Arg2575Cys	Pathogenic
5969216	C	T	c.7724G>A	p.Arg2575His	Pathogenic
5969210	CCACAG	C	c.7725_7729del	p.Cys2576AlafsTer59	Pathogenic
5969206	C	T	c.7729+5G>A	NA	Pathogenic
5969204	G	A	c.7729+7C>T	NA	Pathogenic
5969203	T	C	c.7729+8A>G	NA	Uncertain significance
5969184	A	G	c.7729+27T>C	NA	Pathogenic
5968344	C	A	c.7730-177G>T	NA	Pathogenic
5968223	G	A	c.7730-56C>T	NA	Pathogenic
5968171	G	C	c.7730-4C>G	NA	Conflicting interpretation
5968168	C	G	c.7730-1G>C	NA	Pathogenic
5968168	C	A	c.7730-1G>T	NA	Pathogenic
5968165	G	A	c.7732C>T	p.Arg2578Cys	Pathogenic
5968126	C	T	c.7770+1G>A	NA	Pathogenic
5968126	C	A	c.7770+1G>T	NA	Pathogenic
5968114	G	T	c.7770+13C>A	NA	Uncertain significance
5967689	C	T	c.7771-87G>A	NA	Pathogenic
5967688	C	T	c.7771-86G>A	NA	Pathogenic
5967684	C	T	c.7771-82G>A	NA	Pathogenic
5967651	C	T	c.7771-49G>A	NA	Not provided
5967642	C	T	c.7771-40G>A	NA	Not provided
5967615	G	A	c.7771-13C>T	NA	Benign
5967612	G	A	c.7771-10C>T	NA	Benign
5967600	G	A	c.7773C>T	p.Pro2591=	Not provided
5967591	A	T	c.7782T>A	p.Thr2594=	Benign
5967583	A	T	c.7790T>A	p.Ile2597Asn	Uncertain significance
5967573	G	A	c.7800C>T	p.Cys2600=	Conflicting interpretation
5967524	G	T	c.7849C>A	p.Leu2617Met	Uncertain significance
5967517	C	T	c.7856G>A	p.Cys2619Tyr	Pathogenic
5967509	TC	T	c.7863del	p.Thr2622ProfsTer32	Pathogenic
5967489	G	C	c.7884C>G	p.Pro2628=	Pathogenic

5967474	A	G	c.7887+12T>C	NA	Conflicting interpretation
5953659	G	T	c.7888-65C>A	NA	Pathogenic
5953576	T	A	c.7906A>T	p.Asn2636Tyr	Pathogenic
5953546	G	T	c.7936C>A	p.Pro2646Thr	Pathogenic
5953542	G	A	c.7940C>T	p.Thr2647Met	Conflicting interpretation
5953535	G	A	c.7947C>T	p.Cys2649=	Uncertain significance
5953522	TTAGC	T	c.7956_7959del	p.Leu2653GlufsTer6	Pathogenic
5953513	G	A	c.7969C>T	p.Gln2657Ter	Pathogenic
5953497	T	C	c.7985A>G	p.Lys2662Arg	Pathogenic
5953491	C	T	c.7986+5G>A	NA	Uncertain significance
5952519	G	A	c.7987C>T	p.Arg2663Cys	Pathogenic
5952518	C	G	c.7988G>C	p.Arg2663Pro	Conflicting interpretation
5952518	C	T	c.7988G>A	p.Arg2663His	Uncertain significance
5952509	G	A	c.7997C>T	p.Thr2666Met	Benign
5952495	A	C	c.8011T>G	p.Cys2671Gly	Pathogenic
5952494	C	T	c.8012G>A	p.Cys2671Tyr	Pathogenic
5952479	C	A	c.8027G>T	p.Cys2676Phe	Pathogenic
5952479	C	T	c.8027G>A	p.Cys2676Tyr	Pathogenic
5952470	T	C	c.8036A>G	p.Asn2679Ser	Conflicting interpretation
5952461	CCT	C	c.8043_8044del	p.Gly2682ArgfsTer15	Pathogenic
5952454	G	T	c.8052C>A	p.Tyr2684Ter	Uncertain significance
5952428	C	T	c.8078G>A	p.Cys2693Tyr	Pathogenic
5952427	G	A	c.8079C>T	p.Cys2693=	Benign
5952427	G	T	c.8079C>A	p.Cys2693Ter	Pathogenic
5952422	G	C	c.8084C>G	p.Pro2695Arg	Conflicting interpretation
5952412	T	C	c.8094A>G	p.Glu2698=	Benign
5952410	T	C	c.8096A>G	p.His2699Arg	Uncertain significance
5952393	C	T	c.8113G>A	p.Gly2705Arg	Benign
5952390	C	T	c.8115+1G>A	NA	Pathogenic
5951903	T	G	c.8116-20A>C	NA	Benign
5951884	CT	C	c.8116-2del	NA	Pathogenic
5951854	G	T	c.8145C>A	p.Cys2715Ter	Pathogenic

5951844	A	G	c.8155T>C	p.Cys2719Arg	Uncertain significance
5951843	C	A	c.8155+1G>T	NA	Pathogenic
5951841	C	G	c.8155+3G>C	NA	Pathogenic
5951841	C	T	c.8155+3G>A	NA	Pathogenic
5951841	C	A	c.8155+3G>T	NA	Pathogenic
5951839	C	G	c.8155+5G>C	NA	Pathogenic
5951838	A	T	c.8155+6T>A	NA	Pathogenic
5951838	A	G	c.8155+6T>C	NA	Pathogenic
5951836	G	A	c.8155+8C>T	NA	Uncertain significance
5951794	G	A	c.8155+50C>T	NA	Benign
5949925	G	A	c.8156-42C>T	NA	Not provided
5949879	CT	C	c.8159del	p.Glu2720GlyfsTer24	Pathogenic
5949879	C	T	c.8160G>A	p.Glu2720=	Uncertain significance
5949875	G	C	c.8164C>G	p.Pro2722Ala	Pathogenic
5949864	G	A	c.8175C>T	p.Asn2725=	Benign
5949845	G	A	c.8194C>T	p.Gln2732Ter	Pathogenic
5949844	TGCAGCCTG	T	c.8187_8194del	p.Arg2730ValfsTer8	Pathogenic
5949824	A	G	c.8215T>C	p.Cys2739Arg	Uncertain significance
5949823	C	T	c.8216G>A	p.Cys2739Tyr	Pathogenic
5949786	CTGGCAGTAG	C	c.8244_8252del	p.His2748_Cys2750del	Pathogenic
5949754	A	G	c.8253+32T>C	NA	Benign
5949747	G	A	c.8253+39C>T	NA	Benign
5949213	A	G	c.8254-10T>C	NA	Pathogenic
5949208	A	C	c.8254-5T>G	NA	Pathogenic
5949195	A	C	c.8262T>G	p.Cys2754Trp	Pathogenic
5949184	G	A	c.8273C>T	p.Ala2758Val	Uncertain significance
5949182	T	C	c.8275A>G	p.Met2759Val	Uncertain significance
5949181	A	G	c.8276T>C	p.Met2759Thr	Uncertain significance
5949150	G	T	c.8307C>A	p.Asp2769Glu	Uncertain significance
5949146	A	G	c.8311T>C	p.Cys2771Arg	Pathogenic

5949146	A	T	c.8311T>A	p.Cys2771Ser	Pathogenic
5949145	C	T	c.8312G>A	p.Cys2771Tyr	Pathogenic
5949140	AG	A	c.8316del	p.Cys2773AlafsTer76	Pathogenic
5949140	A	G	c.8317T>C	p.Cys2773Arg	Pathogenic
5949139	C	G	c.8318G>C	p.Cys2773Ser	Conflicting interpretation
5949133	G	C	c.8324C>G	p.Ser2775Cys	Pathogenic
5949132	A	G	c.8325T>C	p.Ser2775=	Benign
5949130	G	A	c.8327C>T	p.Pro2776Leu	Pathogenic
5949125	G	A	c.8332C>T	p.Arg2778Trp	Uncertain significance
5949124	C	T	c.8333G>A	p.Arg2778Gln	Uncertain significance
5949121	G	A	c.8336C>T	p.Thr2779Met	Uncertain significance
5949116	G	A	c.8341C>T	p.Pro2781Ser	Pathogenic
5949110	G	A	c.8347C>T	p.Gln2783Ter	Pathogenic
5949100	A	G	c.8357T>C	p.Leu2786Pro	Pathogenic
5949091	G	C	c.8366C>G	p.Thr2789Ser	Conflicting interpretation
5949079	A	G	c.8378T>C	p.Val2793Ala	Uncertain significance
5949069	A	C	c.8388T>G	p.His2796Gln	Uncertain significance
5949055	G	T	c.8402C>A	p.Ala2801Asp	Pathogenic
5949046	C	T	c.8411G>A	p.Cys2804Tyr	Pathogenic
5949044	T	TGGGGA	c.8412_8413insTCCCC	p.Lys2805SerfsTer46	Not provided
5949041	A	G	c.8416T>C	p.Cys2806Arg	Uncertain significance
5949037	G	GGGGA	c.8419_8422dup	p.Pro2808LeufsTer24	Pathogenic

Table 7.13 | VWDbase

7.5. ICD-BAT codes

ICD-10 code	ICD-10 code description
BAT|Epistaxis	
R04.0	Epistaxis
BAT|Cutaneous bleeding	
R23.3	Spontaneous ecchymoses

D69.2	Other non-thrombocytopenic purpura
BAT|Haematuria	
R31	unspecified haematuria
BAT|Gastrointestinal bleeding	
K92.0	Haematemesis
K92.1	Melaena
K92.2	Gastrointestinal haemorrhage, unspecified
K62.5	Haemorrhage of anus and rectum
K29.0	Acute haemorrhagic gastritis
I85.0	Oesophageal varices with bleeding
I98.3	Oesophageal varices with bleeding in diseases classified elsewhere
K22.6	Gastro-oesophageal laceration-haemorrhage syndrome
K25.0	Gastric ulcer ; Acute with haemorrhage
K25.2	Gastric ulcer ; Acute with both haemorrhage and perforation
K25.4	Gastric ulcer ; Chronic or unspecified with haemorrhage
K25.6	Gastric ulcer ; Chronic or unspecified with both haemorrhage and perforation
K26.0	Duodenal ulcer ; Acute with haemorrhage
K26.2	Duodenal ulcer ; Acute with both haemorrhage and perforation
K26.4	Duodenal ulcer ; Chronic or unspecified with haemorrhage
K26.6	Duodenal ulcer ; Chronic or unspecified with both haemorrhage and perforation
K27.0	Peptic ulcer, site unspecified ; Acute with haemorrhage
K27.2	Peptic ulcer, site unspecified ; Acute with both haemorrhage and perforation
K27.4	Peptic ulcer, site unspecified ; Chronic or unspecified with haemorrhage
K27.6	Peptic ulcer, site unspecified ; Chronic or unspecified with both haemorrhage and perforation
K28.0	Gastrojejunal ulcer ; Acute with haemorrhage
K28.2	Gastrojejunal ulcer ; Acute with both haemorrhage and perforation
K28.4	Gastrojejunal ulcer ; Chronic or unspecified with haemorrhage
K28.6	Gastrojejunal ulcer ; Chronic or unspecified with both haemorrhage and perforation
K29.0	Acute haemorrhagic gastritis
K62.5	Haemorrhage of anus and rectum
BAT|Oral cavity bleeding	

R04.1	haemorrhage from throat
BAT|Bleeding after tooth/teeth extraction and bleeding after surgery or major trauma	
T81.0	Haemorrhage and haematoma complicating a procedure, not elsewhere classified
BAT|Menorrhagia	
N92.0	Excessive and frequent menstruation with regular cycle
N92.1	Excessive and frequent menstruation with irregular cycle
N92.2	Excessive menstruation at puberty
N92.4	Excessive bleeding in the premenopausal period
BAT|Post-partum bleeding	
O72.0	Third stage haemorrhage
O72.1	Other immediate postpartum haemorrhage
O72.2	Delayed and secondary postpartum haemorrhage
BAT|Hemarthrosis (spontaneous)	
M25.0	Haemarthrosis (Excl.: current injury - see injury of joint by body region)
BAT|Central nervous system (CNS) bleeding	
I61.0	Intracerebral haemorrhage in hemisphere, subcortical
I61.1	Intracerebral haemorrhage in hemisphere, cortical
I61.2	Intracerebral haemorrhage in hemisphere, unspecified
I61.3	Intracerebral haemorrhage in brain stem
I61.4	Intracerebral haemorrhage in cerebellum
I61.5	Intracerebral haemorrhage, intraventricular
I61.6	Intracerebral haemorrhage, multiple localized
I61.8	Other intracerebral haemorrhage
I61.9	Intracerebral haemorrhage, unspecified
S06.4	Epidural haemorrhage
S06.5	Traumatic subdural haemorrhage
S06.6	Traumatic subarachnoid haemorrhage
I60.0	Subarachnoid haemorrhage from carotid siphon and bifurcation
I60.1	Subarachnoid haemorrhage from middle cerebral artery
I60.2	Subarachnoid haemorrhage from anterior communicating artery
I60.3	Subarachnoid haemorrhage from posterior communicating artery

I60.4	Subarachnoid haemorrhage from basilar artery
I60.5	Subarachnoid haemorrhage from vertebral artery
I60.6	Subarachnoid haemorrhage from other intracranial arteries
I60.7	Subarachnoid haemorrhage from intracranial artery, unspecified
I60.8	Other subarachnoid haemorrhage
I60.9	Subarachnoid haemorrhage, unspecified
I62.0	Nontraumatic subdural haemorrhage
I62.1	Nontraumatic extradural haemorrhage
I62.9	Intracranial haemorrhage (nontraumatic), unspecified
Additional	Traumatic bleeding
T79.2	Traumatic secondary and recurrent haemorrhage
Additional	Respiratory system bleeding
P26.1	Massive pulmonary haemorrhage originating in the perinatal period
R04.2	Haemoptysis
R04.8	Haemorrhage from other sites in respiratory passages
R04.9	Haemorrhage from respiratory passages, unspecified
Additional	Other obstetric bleeding
O20.8	Other haemorrhage in early pregnancy
O20.9	Haemorrhage in early pregnancy, unspecified
O46.8	Other antepartum haemorrhage
O46.9	Antepartum haemorrhage, unspecified
O67.8	Other intrapartum haemorrhage
O67.9	Intrapartum haemorrhage, unspecified
O71.7	Obstetric haematoma of pelvis
O90.2	Haematoma of obstetric wound
Additional	Other gynaecological bleeding
N83.7	Haematoma of broad ligament
N93.8	Other specified abnormal uterine and vaginal bleeding
N93.9	Abnormal uterine and vaginal bleeding, unspecified
O03.1	Spontaneous abortion; Incomplete, complicated by delayed or excessive haemorrhage
O03.6	Spontaneous abortion ; Complete or unspecified, complicated by delayed or excessive

	haemorrhage
O04.1	Medical abortion ; Incomplete, complicated by delayed or excessive haemorrhage
O04.6	Medical abortion ; Complete or unspecified, complicated by delayed or excessive haemorrhage
O05.1	Other abortion ; Incomplete, complicated by delayed or excessive haemorrhage
O05.6	Other abortion ; Complete or unspecified, complicated by delayed or excessive haemorrhage
O06.1	Unspecified abortion ; Incomplete, complicated by delayed or excessive haemorrhage
O06.6	Unspecified abortion ; Complete or unspecified, complicated by delayed or excessive haemorrhage
O07.1	Failed medical abortion, complicated by delayed or excessive haemorrhage
O07.6	Other and unspecified failed attempted abortion, complicated by delayed or excessive haemorrhage
O08.1	Delayed or excessive haemorrhage following abortion and ectopic and molar pregnancy
Additional	Cardiac bleeding
I23.0	Haemopericardium as current complication following acute myocardial infarction
I31.2	Haemopericardium, not elsewhere classified
S26.0	Injury of heart with haemopericardium
Additional	Eye bleeding
H35.6	Retinal haemorrhage
H43.1	Vitreous haemorrhage

Table 7.14 | ICD-10 codes used to generate the ICD-BAT score.

8. References

Aasen, T., Raya, A., Barrero, M. J., Garreta, E., Consiglio, A., Gonzalez, F., Vassena, R., Bilić, J., Pekarik, V., Tiscornia, G., Edel, M., Boue, S., & Belmonte, J. C. (2008). Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. *Nature biotechnology*, *26*(11), 1276-1284. https://doi.org/10.1038/nbt.1503

Abou−Ismail, M. Y., Ogunbayo, G. O., Secic, M., & Kouides, P. A. (2018). Outgrowing the laboratory diagnosis of type 1 von Willebrand disease: A two decade study. *American journal of hematology*, *93*(2), 232-237. https://doi.org/10.1002/ajh.24962

Abrahimi, P., Chang, W. G., Kluger, M. S., Qyang, Y., Telleides, G., Saltzmann, W. M., & Pober, J. S. (2015). Efficient gene disruption in cultured primary human endothelial cells by CRISPR/Cas9. *Circulation research*, *117*(2), 121-128. https://doi.org/10.1161/CIRCRESAHA.117.306290

Abshire, T. C., Federici, A. B., Alvarez, M. T., Bowen, J., Carcao, M. D., Cox Gill, J., Key, N. S., Kouides, P. A., Kurnik, K., Lail, A. E., Leebeek, F. W., Makris, M., Mannucci, P. M., Winikoff, R., Berntorp, E., & VWD PN. (2013). Prophylaxis in severe forms of von Willebrand's disease: results from the von Willebrand Disease Prophylaxis Network (VWD PN). *Haemophilia*, *19*, 76-81. https://doi.org/10.1111/j.1365-2516.2012.02916.x

Abu-Douleh, E., Al-Numair, N., Albanyan, A., Alsuliman, A., Bayoumi, N., & Owaidah, T. (2018). Prevalence of von Willebrand disease among university students in Riyadh, Saudi Arabia. *J Appl Hematol*, *9*, 136-139.

Adams, W. J., Zhang, Y., Cloutier, J., Kuichmanchi, P., Newton, G., Sehrawat, S., Aird, W. C., Mayadas, T. N., Luscinskas, F. W., & García-Cardeña, G. (2013). Functional vascular endothelium derived from human induced pluripotent stem cells. *Stem cell reports*, *1*(2), 105-113. https://doi.org/10.1016/j.stemcr.2013.06.007

Adcock, D. M., Strandberg, K., Shima, M., & Marlar, R. A. (2018). Advantages, disadvantages and optimization of one−stage and chromogenic factor activity assays in haemophilia A and B. *International journal of laboratory hematology*, *40*(6), 621-629. https://doi.org/10.1111/ijlh.12877

Adzhubei, I. A., Schmidt, S., Peshkin, L., Ramensky, V. E., Gerasimova, A., Bork, P., Kondrashov, A. S., & Sunyaev, S. R. (2010). A method and server for predicting damaging missense mutations. *Nature methods*, *7*(4), 248-249. https://doi.org/10.1038/nmeth0410-248

Afzal, S., Zaidi, S. T., Merchant, H. A., Babar, Z. U., & Hasan, S. S. (2021). Prescribing trends of oral anticoagulants in England over the last decade: a focus on new and old drugs and adverse events reporting. *Journal of thrombosis and thrombolysis*, *52*(2), 646-653. https://doi.org/10.1007/s11239-021-02416-4

Agrawal, R., & Prabakaran, S. (2020). Big data in digital healthcare: lessons learnt and recommendations for general practice. *Heredity*, *124*(4), 525-534. https://doi.org/10.1038/s41437-020-0303-2

Ahlberg, Å., & Silwer, J. (1970). Arthropathy in Von Willebrand's Disease. *Acta Orthopaedica Scandinavica*, *41*(5), 539-544. https://doi.org/10.3109/17453677008991542

Ahmad, F., Budde, U., Jan, R., Oyen, F., Kannan, M., Saxena, R., & Schneppenheim, R. (2013). Phenotypic and molecular characterisation of type 3 von Willebrand disease in a cohort of Indian patients. *Thrombosis and haemostasis*, *109*(4), 652-660. https://doi.org/10.1160/TH12-10-0737

Ahmad, F., Kannan, M., Kishor, K., & Saxena, R. (2010). Coinheritance of severe von Willebrand disease with Glanzmann thrombasthenia. *Clin Appl Thromb Hemost*, *16*(5), 529-32. https://doi.org/10.1177/1076029609360527

Ahmad, F., Oyen, F., Jan, R., Budde, U., Schneppenheim, R., & Saxena, R. (2014). Germline de novo mutations and linkage markers vs. DNA sequencing for carrier detection in Von Willebrand disease. *Haemophilia*, *20*(4), e311-e317. https://doi.org/10.1111/hae.12441

Ahmed, S., Yadegari, H., Naz, A., Biswas, A., Budde, U., Saqlain, N., Amanat, S., Tariq, S., Raziq, F., Masood, S., Pavlova, A., Sultan Shamsi, T., & Oldenburg, J. (2019). Characterization of the mutation spectrum in a Pakistani cohort of type 3 von Willebrand disease. *Haemophilia*, *25*(6), 1035-1044. https://doi.org/10.1111/hae.13841

Aird, W. C. (2007). Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. *Circulation research*, *100*(2), 158-173. https://doi.org/10.1161/01.RES.0000255691.76142.4a

Aird, W. C. (2012). Endothelial cell heterogeneity. *Cold Spring Harbor perspectives in medicine*, *2*(1), a006429. https://doi.org/10.1101/cshperspect.a006429

Alfares, A., Aloraini, T., Alissa, A., Al Qudsi, A., Alahmad, A., Al Mutairi, F., Alswaid, A., Alothaim, A., Eyaid, W., Albalwi, M., Alturki, S., & Alfadhel, M. (2018). Whole-genome sequencing offers additional but limited clinical utility compared with reanalysis of whole-exome sequencing. *Genetics in Medicine*, *20*(11), 1328-1333. https://doi.org/10.1038/gim.2018.41

Alkaabi, S., Alzadjali, A., Wasifuddin, M., Alghaithi, I. S., Al-Khabori, M., Pathare, A., & Wali, Y. (2019). Prevalence, Clinical Characteristics and Outcome of Von Willebrand Disease in Oman. *Blood*, *134 (Supplement 1)*, 4939. https://doi.org/10.1182/blood-2019-123915

Allain, J. P., Cooper, H. A., Wagner, R. H., & Brinkhous, K. M. (1975). Platelets fixed with paraformaldehyde: a new reagent for assay of von Willebrand factor and platelet aggregating factor. *The Journal of laboratory and clinical medicine*, *85*(2), 318-328. https://doi.org/10.5555/uri:pii:0022214375904370

Allen, S., Goodeve, A. C., Peake, I. R., & Daly, M. E. (2001). Endoplasmic reticulum retention and prolonged association of a von Willebrand's disease-causing von Willebrand factor variant with ERp57 and calnexin. *Biochemical and biophysical research communications*, *280*(2), 448-453. https://doi.org/10.1006/bbrc.2000.4139

Andersen, P., & Gill, R. (1982). Cox's regression model for counting processes, a large sample study. *Annals of Statistics*, *10*, 1100-1120.

Anderson, E. M., Haupt, A., Schiel, J. A., Chou, E., Machado, H. B., Strezoska, Ž., Langer, S., McClelland, S., Birmingham, A., Vermeulen, A., & van Brabant Smith, A. (2015). Systematic analysis of CRISPR–Cas9 mismatch tolerance reveals low levels of off-target activity. *Journal of biotechnology*, *211*, 56-65. https://doi.org/10.1016/j.jbiotec.2015.06.427

Apweiler, R., Bairoch, A., Wu, C. H., Barker, W. C., Boeckmann, B., Ferro, S., Gasteiger, E., Huang, H., Lopez, R., Magrane, M., Martin, M. J., Natale, D. A., O'Donovan, C., Redaschi, N., & Yeh, L.-S. L. (2004). UniProt: the universal protein knowledgebase. *Nucleic acids research*, *32*(suppl_1), D115-D119. https://doi.org/10.1093/nar/gkh131

The Assay of Factor VIII Related Antigen by an Immuno-Enzymatic Method. (1980). *Thrombosis and haemostasis*, *43*(01), 71. https://doi.org/10.1055/s-0038-1650019

Assmus, J., Kleffe, J., Schmitt, A. O., & Brockmann, G. A. (2013). Equivalent indels–ambiguous functional classes and redundancy in databases. *Plos one*, *8*(5), e62803. https://doi.org/10.1371/journal.pone.0062803

Atiq, F., Meijer, K., Eikenboom, J., Fijnvandraat, K., Mauser−Bunschoten, E. P., van Galen, K. P., Nijziel, M. R., Ypma, P. F., de Meris, J., Laros-van Gorkom, B. A., van der Bom, J. G., de Maat, M. P., Cnossen, M. H., Leebeek, F. W., & The WiN study group. (2018). Comorbidities associated with higher von Willebrand factor (VWF) levels may explain the age−related increase of VWF in von Willebrand disease. *British journal of haematology*, *182*(1), 93-105. https://doi.org/10.1111/bjh.15277

Atkinson, E. G., Artomov, M., Karczewski, K. J., Loboda, A. A., Rehm, H. L., MacArthur, D. G., Neale, B. M., & Daly, M. J. (2022). Discordant genotype calls across technology platforms elucidate variants with systematic errors in next-generation sequencing. *bioRxiv*. https://doi.org/10.1101/2022.03.24.485707

Awidi, A. S. (1992). A study of von Willebrand's disease in Jordan. *Annals of hematology*, *64*(6), 299-302. https://doi.org/10.1007/BF01695475

Azhdari, M., Baghaban-Eslaminejad, M., Baharvand, H., & Aghdami, N. (2013). Therapeutic potential of human-induced pluripotent stem cell-derived endothelial cells in a bleomycin-induced scleroderma mouse model. *Stem cell research*, *10*(3), 288-300. https://doi.org/10.1016/j.scr.2012.12.004

Aznar, J. A., Lucía, F., Abad−Franch, L., Jimenez−Yuste, V., Pérez, R., Batlle, J., Balda, I., Parra, R., & Cortina, V. R. (2009). Haemophilia in spain. *Haemophilia*, *15*(3), 665-675. https://doi.org/10.1111/j.1365-2516.2009.02001.x

Babushok, D., & Cuker, A. (2012). VWF sequence variants: innocent until proven guilty. *Blood*, *119*(9), 1959-1960. https://doi.org/10.1182/blood-2012-01-403634

Bachman, J. (2013). Site-directed mutagenesis. *Methods Enzymol*, *529*, 241-248.

Badagabettu, S., Nayak, D. M., Kurien, A., Kamath, V. G., Kamath, A., & George, A. (2018). Effectiveness of a comprehensive educational programme for Accredited Social Health Activists (ASHAs) to identify individuals in the Udupi district with bleeding disorders: A community−based survey. *Haemophilia*, *24*(5), 741-746. https://doi.org/10.1111/hae.13567

Badirou, I., Kurdi, M., Rayes, J., Legendre, P., Christophe, O. D., Lenting, P. J., & Denis, C. V. (2010). von Willebrand factor clearance does not involve proteolysis by ADAMTS−13. *Journal of Thrombosis and Haemostasis*, *8*(10), 2338-2340. https://doi.org/10.1111/j.1538-7836.2010.04012.x

Bagwell, C. B., & Adams, E. G. (1993). Fluorescence spectral overlap compensation for any number of flow cytometry parameters. *Annals of the New York Academy of Sciences*, *677*(1), 167-184.

Bahnak, R., Lavergne, J.-M., Rothschild, C., & Meyer, D. (1991). A stop codon in a patient with severe type III von Willebrand disease. *Blood*, *78*(4), 1148-1149. https://doi.org/10.1182/blood.V78.4.1148.1148

Baker, M. (2012). One-stop shop for disease genes. *Nature*, *491*(7423), 171. https://doi.org/10.1038/491171a

Balduini, C. L., Noris, P., Belletti, S., Spedini, P., & Gamba, G. (1999). In vitro and in vivo effects of desmopressin on platelet function. *Haematologica*, *84*, 891-896.

Barnes, M. R. (2002). SNP and mutation data on the Web–hidden treasures for uncovering. *Comparative and functional genomics*, *3*(1), 67-74. https://doi.org/10.1002/cfg.131

Bar-Nur, O., Russ, H. A., Efrat, S., & Benvenisty, N. (2011). Epigenetic memory and preferential lineage-specific differentiation in induced pluripotent stem cells derived from human pancreatic islet beta cells. *Cell Stem Cell*, *9*(1), 17-23. https://doi.org/10.1016/j.stem.2011.06.007

Baronciani, L., Cozzi, G., Canciani, M. T., Peyvandi, F., Srivastava, A., Federici, A. B., & Mannucci, P. M. (2000). Molecular characterization of a multiethnic group of 21 patients with type 3 von Willebrand disease. *Thromb Haemost*, *84*(4), 536-40. https://doi.org/10.1055/s-0037-1614063

Baronciani, L., Cozzi, G., Canciani, M. T., Peyvandi, F., Srivastava, A., Federici, A. B., & Mannucci, P. M. (2000). Molecular characterization of a multiethnic group of 21 patients with type 3 von Willebrand disease. *Thrombosis and haemostasis*, *84*(10), 536-540. https://10.1055/s-0037-1614063

Baronciani, L., Cozzi, G., Canciani, M. T., Peyvandi, F., Srivastava, A., Federici, A. B., & Mannucci, P. M. (2003). Molecular defects in type 3 von Willebrand disease: updated results from 40 multiethnic patients. *Blood Cells, Molecules, and Diseases*, *30*(3), 264-270. https://doi.org/10.1016/S1079-9796(03)00033-0

Baronciani, L., Goodeve, A., & Peyvandi, F. (2017). Molecular diagnosis of von Willebrand disease. *Haemophilia*, *23*(2), 188-197. https://doi.org/10.1111/hae.13175

Baronciani, L., Peake, I., Schneppenheim, R., Goodeve, A., Ahmadinejad, M., Badiee, Z., Baghaipour, M.-R., Benitez, O., Bodo, I., Budde, U., Cairo, A., Castaman, G., Eshghi, P., Goudemand, J.,

Hassenpflug, W., Hoorfar, H., Karimi, M., Keikhaei, B., Lassila, R., … Peyvandi, F. (2021). Genotypes of European and Iranian patients with type 3 von Willebrand disease enrolled in 3WINTERS-IPS. *Blood advances*, *5*(15), 2987-3001. https://doi.org/10.1182/bloodadvances.2020003397

Barrowcliffe, T. W. (2004). Monitoring haemophilia severity and treatment: new or old laboratory tests? *Haemophilia*, *10*, 109-114. https://doi.org/10.1111/j.1365-2516.2004.00985.x

Barrowcliffe, T. W. (2013). Laboratory testing and standardisation. *Haemophilia*, *19*(6), 799-804. https://doi.org/10.1111/hae.12282

Barrowcliffe, T. W., Hubbard, A. R., & Kitchen, S. (2012). Standards and monitoring treatment. *Haemophilia*, *18*, 61-65. https://doi.org/10.1111/j.1365-2516.2012.02831.x

Bartlett, A., Dormandy, K. M., Hawkey, C. M., Stableforth, P., & Voller, A. (1976). Factor-VIII-related antigen: measurement by enzyme immunoassay. *British Medical Journal*, *6016*, 994-996. https://doi.org/10.1136/bmj.1.6016.994

Bastida, J. M., Lozano, M., Benito, R., Janusz, K., Palma-Barqueros, V., Del Rey, M., Hernández-Sánchez, J. M., Riesco, S., Bermejo, N., González-García, H., Rodriguez-Alén, A., Aguilar, C., Sevivas, T., López-Fernández, M. F., Marneth, A. E., van der Reijden, B. A., Morgan, N. V., Watson, S. P., Vicente, V., … González-Porras, J. R. (2018). Introducing high-throughput sequencing into mainstream genetic diagnosis practice in inherited platelet disorders. *Haematologica*, *103*(1), 148-162. https://doi.org/10.3324/haematol.2017.171132

Batlle, J., Pérez-Rodríguez, A., Corrales, I., Borras, N., Pinto, J. C., López-Fernández, M. F., & Vidal, F. (2019). Update on molecular testing in von Willebrand disease. *Seminars in Thrombosis and Hemostasis*, *45*(07), 708-719. https://doi.org/10.1055/s-0039-1679922

Batlle, J., Pérez-Rodríguez, A., Corrales, I., López-Fernández, M. F., Rodríguez-Trillo, A., Lourés,, E., Cid, A. R., Bonanad, S., Cabrera, N., Moret, A., Parra, R., Mingot-Castellano, M. E., Balda, I., Altisent, C., Pérez-Montes, R., Fisac, R. M., Iruín, G., Herrero, S., Soto, I., … Vidal, F. (2016). Molecular and clinical profile of von Willebrand disease in Spain (PCM–EVW–ES): Proposal for a new diagnostic paradigm. *Thrombosis and haemostasis*, *115*(01), 40-50. https://doi.org/10.1160/TH15-04-0282

Batty, G. D., Gale, C. R., Kivimäki, M., Deary, I. J., & Bell, S. (2020). Comparison of risk factor associations in UK Biobank against representative, general population based studies with conventional response rates: prospective cohort study and individual participant meta-analysis. *BMJ*, *368*(m131). http://dx.doi.org/10.1136/bmj.m131

Bauduer, F., & Ducout, L. (2004). Is the assessment of von Willebrand disease prevalence an achievable challenge? The example of the French Basque Country where blood group O and factor XI deficiency are highly prevalent. *Journal of Thrombosis and Haemostasis*, *2*(10), 1724-1726. https://doi.org/10.1111/j.1538-7836.2004.00930.x

Baz, B., Abouelhoda, M., Owaidah, T., Dasouki, M., Monies, D., & Al Tassan, N. (2021). Molecular classification of blood and bleeding disorder genes. *NPJ Genomic Medicine*, *6*(1), 1-6. https://doi.org/10.1038/s41525-021-00228-2

Bazil, V., & Strominger, J. L. (1991). Shedding as a mechanism of down-modulation of CD14 on stimulated human monocytes. *The Journal of Immunology*, *147*(5), 1567-1574.

Bellissimo, D. B., Christopherson, P. A., Flood, V. H., Gill, J. C., Friedman, K. D., Haberichter, S. L., Shapiro, A. D., Abshire, T. C., Leissinger, C., Hoots, W. K., Lusher, J. M., Ragni, M. V., & Montgomery, R. R. (2012). VWF mutations and new sequence variations identified in healthy controls are more frequent in the African-American population. *Blood, The Journal of the American Society of Hematology*, *119*(9), 2135-2140. https://doi.org/10.1182/blood-2011-10-384610

Belorgey, D., Hägglöf, P., Karlsson-Li, S., & Lomas, D. A. (2007). Protein misfolding and the serpinopathies. *Prion*, *1*(1), 15-20. https://doi.org/10.4161/pri.1.1.3974

Bennett, E., & Dormandy, K. (1966). Pool's cryoprecipitate and exhausted plasma in the treatment of von Willebrand's disease and factor-XI deficiency. *The Lancet*, *288*(7466), 731-732. https://doi.org/10.1016/S0140-6736(66)92985-0

Berber, E., James, P. D., Hough, C., & Lillicrap, D. (2009). An assessment of the pathogenic significance of the R924Q von Willebrand factor substitution. *Journal of Thrombosis and Haemostasis*, *7*(10), 1672-1679. https://doi.org/10.1111/j.1538-7836.2009.03551.x

Bernardo, A. S., Faial, T., Gardner, L., Niakan, K. K., Ortmann, D., Senner, C. E., Callery, E. M., Trotter, M. W., Hemberger, M., Smith, J. C., Bardwell, L., Moffett, A., & Pedersen, R. A. (2011). BRACHYURY and CDX2 mediate BMP-induced differentiation of human and mouse pluripotent

stem cells into embryonic and extraembryonic lineages. *Cell stem cell*, *9*(2), 144-155. https://doi.org/10.1016/j.stem.2011.06.015

Bernstein, F. (1924). Ergebnisse einer biostatistischen zusammen- fassenden Betrachtung uber die erblichen Blutstructuren des Menschen. *Klin. Wochenschr.*, *3*, 1495-1497.

Berntorp, E. (2007). Erik von Willebrand. *Thrombosis Research*, *120*, S3–S4. https://doi.org/10.1016/j.thromres.2007.03.010

Berntorp, E., Ågren, A., Aledort, L., Blombäck, M., Cnossen, M. H., Croteau, S. E., von Depka, M., Federici, A. B., Goodeve, A., Goudemand, J., Mannucci, P. M., Mourik, M., Önundarson, P. T., Rodeghiero, F., Szántó, T., & Windyga, J. (2018). Fifth Åland Island conference on von Willebrand disease. *Haemophilia*, *24*, 5-19. https://doi.org/10.1111/hae.13475

Berntorp, E., & Önundarson, P. T. (2005). Prevalence of von Willebrand disease in the Nordic Region. *Hematology Meeting Reports (formerly Haematologica Reports)*, *1*(4).

Bharathan, S. P., Manian, K. V., Aaalam, S. M., Palani, D., Deshpande, P. A., Pratheesh, M. D., Srivastava, A., & Velayudhan, S. R. (2017). Systematic evaluation of markers used for the identification of human induced pluripotent stem cells. *Biology open*, *6*(1), 100-108. https://doi.org/10.1242/bio.022111

Biggs, R., & Douglas, A. S. (1953). The thromboplastin generation test. *Journal of clinical Pathology*, *6*(1), 23. http://dx.doi.org/10.1136/jcp.6.1.23

Biggs, R., Douglas, A. S., & Macfarlane, R. G. (1953). The formation of thromboplastin in human blood. *The Journal of physiology*, *119*(1), 89-101. https://doi.org/10.1113/jphysiol.1953.sp004830

Biggs, R., Eveling, J., & Richards, G. (1955). The assay of antihaemophilic−globulin activity. *British Journal of Haematology*, *1*(1), 20-34. https://doi.org/10.1111/j.1365-2141.1955.tb05484.x

Biguzzi, E., Castelli, F., Lijfering, W. M., Cannegieter, S. C., Eikenboom, J., Rosendaal, F. R., & van Hylckama Vlieg, A. (2021). Rise of levels of von Willebrand factor and factor VIII with age: Role of genetic and acquired risk factors. *Thrombosis Research*, *197*, 172-178. https://doi.org/10.1016/j.thromres.2020.11.016

Biron, C., Mahieu, B., Rochette, A., Capdevila, X., Castex, A., Amiral, J., D'athis, F., & Schved, J.-F. (1999). Preoperative screening for von Willebrand disease type 1: low yield and limited ability to predict

bleeding. *Journal of Laboratory and Clinical Medicine*, *134*(6), 605-609. https://doi.org/10.1016/S0022-2143(99)90100-2

Biss, T. T., Blanchette, V. S., Clark, D. S., Bowman, M., Wakefield, C. D., Silva, M., Lillicrap, D., James, P. D., & Rand, M. L. (2010). Quantitation of bleeding symptoms in children with von Willebrand disease: use of a standardized pediatric bleeding questionnaire. *Journal of thrombosis and haemostasis*, *8*(5), 950-956. https://doi.org/10.1111/j.1538-7836.2010.03796.x

Blair, P., & Flaumenhaft, R. (2009). Platelet α-granules: Basic biology and clinical correlates. *Blood reviews*, *23*(4), 177-189. https://doi.org/10.1016/j.blre.2009.04.001

Blancher, A. (2013). Evolution of the ABO supergene family. *ISBT Science Series*, *8*(1), 201-206. https://doi.org/10.1111/voxs.12044

Blombäck, B., & Blombäck, M. (1956). Purification of Bovine and Human Fibrinogen. *Acta chemica Scandinavica*, *10*, 147-147.

Bloom, A. L., & Giddins, J. C. (1991). HIV infection and AIDS in von Willebrand's disease. An international survey including data on the prevalence of clinical von Willebrand's disease. In J. M. Lusher & C. M. Kessler (Eds.), *Hemophilia and von Willebrand's disease in the 1990s* (pp. 405-411). Amsterdam: Elsevier Science Publishers.

Bloom, A. L., Peake, I. R., & Giddings, J. C. (1973). The presence and reactions of high and lower-molecular-weight procoagulant factor VIII in the plasma of patients with von Willebrand's disease after treatment: significance for a structural hypothesis for factor VIII. *Thrombosis Research*, *3*(4), 389-404. https://doi.org/10.1016/0049-3848(73)90033-9

Blueprint Epigenome. (2016). *BP_KD BP_Monocyte Version: BluePrint Reagents Percoll 1.078 g/ml Percoll 1000 ml 100 ml 10 x PBS 80 ml 8 ml 1 x PBS 720 ml*. Blueprint Epigenome. Retrieved January 30, 2022, from https://www.blueprint-epigenome.eu/UserFiles/File/Protocols/UCAM_BluePrint_Monocyte.pdf

Bodó, I., Eikenboom, J., Montgomery, R., Patzke, J., Schneppenheim, R., & Di Paola, J. (2015). Platelet-dependent von Willebrand factor activity. Nomenclature and methodology: communication from the SSC of the ISTH. *Journal of thrombosis and haemostasis*, *13*(7), 1345. https://doi.org/10.1111/jth.12964

Bodó, I., Katsumi, A., Tuley, E. A., Eikenboom, J. C., Dong, Z., & Sadler, J. E. (2001). Type 1 von Willebrand disease mutation Cys1149Arg causes intracellular retention and degradation of heterodimers: a possible general mechanism for dominant mutations of oligomeric proteins. *Blood*, *98*(10), 2973-2979. https://doi.org/10.1182/blood.V98.10.2973

Bolignano, D., & Pisano, A. (2016). Good-quality research in rare diseases: trials and tribulations. *Pediatric Nephrology*, *31*(11), 2017-2023. https://doi.org/10.1007/s00467-016-3323-7

Bolton-Maggs, P. H., Favaloro, E. J., Hillcarp, A., Jennings, I., & Kohler, H. P. (2012). Difficulties and pitfalls in the laboratory diagnosis of bleeding disorders. *Haemophilia*, *18*(66-72). https://doi.org/10.1111/j.1365-2516.2012.02830.x

Bombeli, T., Jutzi, M., De Conno, E., Seifert, B., & Fehr, J. (2002). In patients with deep-vein thrombosis elevated levels of factor VIII correlate only with von Willebrand factor but not other endothelial cell-derived coagulation and fibrinolysis proteins. *Blood coagulation & fibrinolysis*, *13*(7), 577-581.

Bonazza, K., Rottensteiner, H., Schrenk, G., Frank, J., Allmaier, G., Turecek, P. L., Scheiflinger, F., & Friedbacher, G. (2015). Shear-dependent interactions of von Willebrand factor with factor VIII and protease ADAMTS 13 demonstrated at a single molecule level by atomic force microscopy. *Analytical chemistry*, *87*(20), 10299-10305. https://doi.org/10.1021/acs.analchem.5b02078 A

Bonthron, D., & Orkin, S. H. (1988). The human von Willebrand factor gene: Structure of the 5′ region. *European journal of biochemistry*, *171*(1-2), 51-57. https://doi.org/10.1111/j.1432-1033.1988.tb13757.x

Borel-Derlon, A., Federici, A. B., Roussel-Robert, V., Goudemand, J., Lee, C. A., Scharrer, I., Rothschild, C., Berntorp, E., Henriet, C., Tellier, Z., Bridey, F., & Mannucci, P. M. (2007). Treatment of severe von Willebrand disease with a high−purity von Willebrand factor concentrate (Wilfactin®): a prospective study of 50 patients. *Journal of thrombosis and haemostasis*, *5*(6), 1115-1124. https://doi.org/10.1111/j.1538-7836.2007.02562.x

Borghi, M., Guglielmini, G., Mezzasoma, A. M., Falcinelli, E., Bury, L., Malvestiti, M., & Gresele, P. (2017). Increase of von Willebrand factor with aging in type 1 von Willebrand disease: fact or fiction? *Haematologica*, *102*(11), e431. https://doi.org/10.3324/haematol.2017.168013

Borhany, M., Shamsi, T., Naz, A., Khan, A., Parveen, K., Ansari, S., & Farzana, T. (2011). Congenital bleeding disorders in Karachi, Pakistan. *Clinical and Applied Thrombosis/Hemostasis*, *17*(6), E131-E137. https://doi.org/10.1177/1076029610391650

Born, G. V., & Cross, M. (1963). The aggregation of blood platelets. *The Journal of physiology*, *168*(1), 178-195. https://doi.org/10.1113/jphysiol.1963.sp007185

Borràs, N., Batlle, J., Pérez-Rodríguez, A., López-Fernández, M. F., Rodríguez-Trillo, A., Lourés, E., Cid, A. R., Bonanad, S., Cabrera, N., Moret, A., Parra, R., Mingot-Castellano, M. E., Balda, I., Altisent, C., Pérez-Montes, R., Fisac, R. M., Iruín, G., Herrero, S., Soto, I., … Corrales, I. (2017). Molecular and clinical profile of von Willebrand disease in Spain (PCM-EVW-ES): comprehensive genetic analysis by next-generation sequencing of 480 patients. *Haematologica*, *102*(12), 2005-2014. https://doi.org/10.3324/haematol.2017.168765

Borràs, N., Garcia-Martínez, I., Batlle, J., Pérez-Rodríguez, A., Parra, R., Altisent, C., López-Fernández, M. F., Fernanda, M., Pinto, J. C., Batlle-López, F., Cid, A. R., Bonanad, S., Cabrera, N., Moret, A., Mingot-Castellano, M. E., Navarro, N., Pérez-Montes, R., Marcellini, S., Moreto, A., … Vidal, F. (2020). Unraveling the Influence of Common von Willebrand factor variants on von Willebrand Disease Phenotype: An Exploratory Study on the Molecular and Clinical Profile of von Willebrand Disease in Spain Cohort. *Thrombosis and haemostasis*, *120*(03), 437-448. https://doi.org/10.1055/s-0040-1702227

Borràs, N., Orriols, G., Batlle, J., Pérez-Rodríguez, A., Fidalgo, T., Martinho, P., López-Fernández, M. F., Rodríguez-Trillo, A., Loures, E., Parra, R., Altisent, C., Cid, A. R., Bonanad, S., Cabrera, N., Moret, A., Mingot-Castellano, M. E., Navarro, N., Pérez-Montes, R., Marcellin, S., … Corrales, I. (2019). Unraveling the effect of silent, intronic and missense mutations on VWF splicing: contribution of next generation sequencing in the study of mRNA. *Haematologica*, *104*(3), 587-598. https://doi.org/10.3324/haematol.2018.203166

Bos, E. S., Van der Doelen, A. A., Rooy, N. V., & Schuurs, A. H. (1981). 3, 3′, 5, 5′-Tetramethylbenzidine as an Ames test negative chromogen for horse-radish peroxidase in enzyme-immunoassay. *Journal of Immunoassay and Immunochemistry*, *2*(3-4), 187-204. https://doi.org/10.1080/15321818108056977

Bouchard, B. A., Williams, J. L., Meisler, N. T., Long, M. W., & Tracy, P. B. (2005). Endocytosis of plasma-derived factor V by megakaryocytes occurs via a clathrin-dependent, specific membrane binding event. *J Thromb Haemost*, (3), 541-551.

Bowen, D. J. (2003). An influence of ABO blood group on the rate of proteolysis of von Willebrand factor by ADAMTS13. *Journal of Thrombosis and Haemostasis*, *1*, 33-40. https://doi.org/10.1046/j.1538-7836.2003.00007.x

Bowes, J. B. (1969). Anaesthetic management of haemothorax and haemoptysis due to von Willebrand's disease. *BJA: British Journal of Anaesthesia*, *41*(10), 894-897. https://doi.org/10.1093/bja/41.10.894

Bowie, E. J., Owen Jr, C. A., Zollman, P. E., Thompson Jr, J. H., & Fass, D. N. (1973). Tests of hemostasis in swine: normal values and values in pigs affected with von Willebrand's disease. *Am J Vet Res*, *34*(11), 1405-7.

Bowie, E. J., Solberg, L. A., Fass, D. N., Johnson, C. M., Knutson, G. J., Stewart, M. L., & Zoecklein, L. J. (1986). Transplantation of normal bone marrow into a pig with severe von Willebrand's disease. *The Journal of clinical investigation*, *78*(1), 26-30. https://doi.org/10.1172/JCI112560

Bowman, M., Hopman, W. M., Rapson, D., Lillicrap, D., & James, P. (2010a). The prevalence of symptomatic von Willebrand disease in primary care practice. *Journal of Thrombosis and Haemostasis*, *8*(1), 213-216. https://doi.org/10.1111/j.1538-7836.2009.03661.x

Bowman, M., Hopman, W. M., Rapson, D., Lillicrap, D., Silva, M., & James, P. (2010b). A prospective evaluation of the prevalence of symptomatic von Willebrand disease (VWD) in a pediatric primary care population. *Pediatric blood & cancer*, *55*(1), 171-173. https://doi.org/10.1002/pbc.22429

Bowman, M., Mundell, G., Grabell, J., Hopman, W. M., Rapson, D., Lillicrap, D., & James, P. (2008). Generation and validation of the Condensed MCMDM−1VWD Bleeding Questionnaire for von Willebrand disease. *Journal of thrombosis and haemostasis*, *6*(12), 2062-2066. ttps://onlinelibrary.wiley.com/doi/10.1111/j.1538-7836.2008.03182.x

Bowman, M., Riddel, J., Rand, M. L., Tosetto, A., Silva, M., & James, P. D. (2009). Evaluation of the diagnostic utility for von Willebrand disease of a pediatric bleeding questionnaire. *Journal of thrombosis and haemostasis*, *7*(8), 1418-1421. https://doi.org/10.1111/j.1538-7836.2009.03499.x

Bowman, M., Tuttle, A., Notley, C., Brown, C., Tinlin, S., Deforest, M., Leggo, J., Blanchette, V. S., Lillicrap, D., & Association of Hemophilia Clinic Directors of Canada. (2013). The genetics of Canadian type 3 von Willebrand disease: further evidence for co-dominant inheritance of mutant alleles. *J Thromb Haemost*, *11*(3), 512-520. https://doi.org/10.1111/jth.12130

Bowman, M. L., Pluthero, F. G., Tuttle, A., Casey, L., Li, L., Christensen, H., Robinson, K. S., Lillicrap, D., Kahr, W. H., & James, P. J. (2017). Discrepant platelet and plasma von Willebrand factor in von Willebrand disease patients with p. Pro2808Leufs* 24. . *Journal of Thrombosis and Haemostasis*, *15*(7), 1403-1411. https://doi.org/10.1111/jth.13722

Boyd, A., Cornish, R., Johnsen, L., Simmonds, S., Syddall, H., Westbury, L., Cooper, C., & Macleod, J. (2018). *Understanding Hospital Episode Statistics (HES). London, UK: CLOSER; 2017*. Retrieved March 20, 2022, from https://www.closer.ac.uk/wp-content/uploads/CLOSER-resource-understanding-hospital-episode-statistics-2018.pdf

Boylan, B., Rice, A. S., De Staercke, C., Eyster, M. E., Yaish, H. M., Knoll, C. M., Bean, C. J., Miller, C. H., & HEMOPHILIA INHIBITOR RESEARCH STUDY INVESTIGATORS. (2015). Evaluation of von Willebrand factor phenotypes and genotypes in Hemophilia A patients with and without identified F8 mutations. *Journal of Thrombosis and Haemostasis,*, *13*, 1036-1042. https://doi.org/10.1111/jth.12902

Branchford, B. R., Ng, C. J., Neeves, K. B., & Di Paola, J. (2015). Microfluidic technology as an emerging clinical tool to evaluate thrombosis and hemostasis. *Thrombosis Research*, *136*(1), 13-19. https://doi.org/10.1016/j.thromres.2015.05.012

Branzei, D., & Foiani, M. (2008). Regulation of DNA repair throughout the cell cycle. *Nature reviews Molecular cell biology*, *9*(4), 297-308. https://doi.org/10.1038/nrm2351

Bratt−Leal, A. M., Carpenedo, R. L., & McDevitt, T. C. (2009). Engineering the embryoid body microenvironment to direct embryonic stem cell differentiation. *Biotechnology progress*, *25*(1), 43-51. https://doi.org/10.1002/btpr.139

Brehm, M. A. (2017). Von Willebrand factor processing. *Hämostaseologie*, *37*(01), 59-72. 10.0.21.106/HAMO-16-06-0018

Bresler, M. A., Sheehan, S., Chan, A. H., & Song, Y. S. (2012). Telescoper: de novo assembly of highly repetitive regions. *Bioinformatics*, *28*(18), i311-i317. https://doi.org/10.1093/bioinformatics/bts399

Britton, A., Milne, B., Butler, T., Sanchez-Galvez, A., Shipley, M., Rudd, A., Wolfe, C. D., Bhalla, A., & Brunner, E. J. (2012). Validating self-reported strokes in a longitudinal UK cohort study (Whitehall II): Extracting Information from hospital medical records versus the Hospital Episode Statistics database. *BMC medical research methodology*, *12*(1), 83. https://doi.org/10.1186/1471-2288-12-83

Brogna, S., McLeod, T., & Peric, M. (2016). The meaning of NMD: translate or perish. *Trends in Genetics*, *32*(7), 395-407. https://doi.org/10.1016/j.tig.2016.04.007

Brown, S. A., & Collins, P. W. (2002). Heterogeneous Detection of A-antigen on von Willebrand Factor Derived from Platelets, Endothelial Cells and Plasma. *Thromb Haemost*, *87*, 990-996.

Brunak, S., Engelbrecht, J., & Knudsen, S. (1991). Prediction of human mRNA donor and acceptor sites from the DNA sequence. *Journal of molecular biology*, *220*(1), 49-65. https://doi.org/10.1016/0022-2836(91)90380-O

Burel, J. G., Pomaznoy, M., Lindestam Arlehamn, C. S., Seumois, G., Vijayanand, P., Sette, A., & Peters, B. (2020). The Challenge of Distinguishing Cell–Cell Complexes from Singlet Cells in Non−Imaging Flow Cytometry and Single−Cell Sorting. *Cytometry Part A*, *97*(11), 1127-1135. https://doi.org/10.1002/cyto.a.24027

Burns, J. C., Glode, M. P., Clarke, S. H., Wiggins Jr, J., & Hathaway, W. E. (1984). Coagulopathy and platelet activation in Kawasaki syndrome: identification of patients at high risk for development of coronary artery aneurysms. *The Journal of pediatrics*, *105*(2), 206-211.

Bycroft, C., Freeman, C., Petkova, D., Band, G., Elliott, L. T., Sharp, K., Motyer, A., Vukcevic, D., Delaneau, O., Cortes, A., Welsh, S., Young, A., Effingham, M., McVean, G., Leslie, S., Allen, N., Donnelly, P., & Marchini, J. (2018). The UK Biobank resource with deep phenotyping and genomic data. *Nature*, *562*(7726), 203-209. https://doi.org/10.1038/s41586-018-0579-z

Cabrera, M. E., Artigas, C. G., Paez, E., Monsalve, V., Zolezzi, P., Arauco, G., Espinoza, R., Hevia, C., & Villegas, J. (1989). Von Willebrand's disease in the IX Region of Chile. *Revista medica de Chile*, *117*(4), 423-430.

Caekebeke−Peerlinck, K. M., Koster, T., & Briët, E. (1989). Bleeding time, blood groups and von Willebrand factor. *British journal of haematology*, *73*(2), 217-220. https://doi.org/10.1111/j.1365-2141.1989.tb00255.x

Callum, J. L., Karkouti, K., & Lin, Y. (2009). Cryoprecipitate: the current state of knowledge. *Transfusion medicine reviews*, *23*(3), 177-188. https://doi.org/10.1016/j.tmrv.2009.03.001

Canis, K., McKinnon, T. A., Nowak, A., Haslam, S., Panico, M., Morris, H. R., Laffan, M. A., & Dell, A. (2012). Mapping the N-glycome of human von Willebrand factor. *Biochemical Journal*, *447*(2), 217-228. https://doi.org/10.1042/BJ20120810

Carew, J. A., Browning, P. J., & Lynch, D. C. (1999). Sulfation of von Willebrand factor. *Blood*, *76*(12), 2530-2539. https://doi.org/10.1182/blood.V76.12.2530.2530

Carey, M. F., Peterson, C. L., & Smale, S. T. (2013). PCR-mediated site-directed mutagenesis. *Cold Spring Harbor Protocols*, *2013*(8), 738-742. https://doi.org/10.1101/pdb.prot076505

Caron, C., Mazurier, C., & Goudemand, J. (2002). Large experience with a factor VIII binding assay of plasma von Willebrand factor using commercial reagents. *British journal of haematology*, *117*(3), 716-718. https://doi.org/10.1046/j.1365-2141.2002.03488.x

Cartwright, A., Webster, S. J., de Jong, A., Dirven, R. J., Bloomer, L. D., Al-Buhairan, A. M., Budde, U., Hallden, C., Habart, D., Goudemand, J., Peake, I. R., Eikenboom, J. C., Goodeve, A. C., & Hampshire, D. J. (2020). Characterization of large in-frame von Willebrand factor deletions highlights differing pathogenic mechanisms. *Blood advances*, *4*(13), 2979-2990. https://doi.org/10.1182/bloodadvances.2018027813

Casaña, P., Martinez, F., Haya, S., Lorenzo, J. I., Espinos, C., & Aznar, J. A. (2000). Q1311X: a novel nonsense mutation of putative ancient origin in the von Willebrand factor gene. *British journal of haematology*, *111*(2), 552-555. https://doi.org/10.1111/j.1365-2141.2000.02410.x

Casari, C., Du, V., Wu, Y. P., Kauskot, A., de Groot, P. G., Christophe, O. D., Denis, C. V., de Laat, B., & Lenting, P. J. (2013). Accelerated uptake of VWF/platelet complexes in macrophages contributes to VWD type 2B–associated thrombocytopenia. *Blood, The Journal of the American Society of Hematology*, *122*(16), 2893-2902. https://doi.org/10.1182/blood-2013-03-493312

Casonato, A., Cozzi, M. R., Ferrari, S., Rubin, B., Gianesello, L., De Marco, L., & Daidone, V. (2022). The lesson learned from the new c. 2547-1G> T mutation combined with p. R854Q: when a type 2N

mutation reveals a quantitative von Willebrand factor defect. *Thrombosis and Haemostasis*. https://doi.org/10.1055/a-1777-6881

Casonato, A., Galletta, E., Sarolo, L., & Daidone, V. (2018). Type 2N von Willebrand disease: Characterization and diagnostic difficulties. *Haemophilia*, *24*(1), 134-140. https://doi.org/10.1111/hae.13366

Casonato, A., Gallinaro, L., Cattini, M. G., Pontara, E., Padrini, R., Bertomoro, A., Daidone, V., & Pagnan, A. (2010). Reduced survival of type 2B von Willebrand factor, irrespective of large multimer representation or thrombocytopenia. *Haematologica*, *95*(8), 1366. https://doi.org/10.3324/haematol.2009.019927

Casonato, A., Pontara, E., Sartorello, F., Cattini, M. G., Sartori, M. T., Padrini, R., & Girolami, A. (2002). Reduced von Willebrand factor survival in type Vicenza von Willebrand disease. *Blood*, *99*(1), 180-184. https://doi.org/10.1182/blood.V99.1.180

Casonato, A., Pontara, E., Zerbinati, P., Zucchetto, A., & Girolami, A. (1998). The evaluation of factor VIII binding activity of von Willebrand factor by means of an ELISA method: significance and practical implications. *American journal of clinical pathology*, *109*(3), 347-352. https://doi.org/10.1093/ajcp/109.3.347

Castaman, G. (2020). How I treat von Willebrand disease. *Thrombosis Research*, *196*(2020), 618-625. https://doi.org/10.1016/j.thromres.2020.07.051

Castaman, G., Eikenboom, J. C., Bertina, R. M., & Rodeghiero, F. (1999). Inconsistency of Association between Type 1 von Willebrand Disease Phenotype and Genotype in Families Identified in an Epidemiological Investigation. *Thromb Haemost*, *82*(03), 1065-1070. https://doi.org/10.1055/s-0037-1614330

Castaman, G., Federici, A. B., Tosetto, A., La Marca, S., Stufano, F., Mannucci, P. M., & Rodeghiero, F. (2012). Different bleeding risk in type 2A and 2M von Willebrand disease: a 2−year prospective study in 107 patients. *Journal of Thrombosis and Haemostasis*, *10*(4), 632-638. https://doi.org/10.1111/j.1538-7836.2012.04661.x

Castaman, G., Lethagan, S., Federici, A. B., Tosetto, A., Goodeve, A., Budde, U., Batlle, J., Meyer, D., Mazurier, C., Fressinaud, E., Goudemand, J., Eikenboom, J., Schneppenheim, R., Ingerslev, J., Vorlova, Z., Habart, D., Holmberg, L., Pasi, J., Hill, F., … Rodeghiero, F. (2008). Response to

desmopressin is influenced by the genotype and phenotype in type 1 von Willebrand disease (VWD): results from the European Study MCMDM-1VWD. *Blood*, *111*(7), 3531-3539. https://doi.org/10.1182/blood-2007-08-109231

Castaman, G., Oliovecchio, E., & Federici, A. B. (2018). Diagnosis and management of von Willebrand disease in Italy. *Ann Blood*, *3*(28). https://doi.org/10.21037/aob.2018.03.03

Castaman, G., Plate, M., Giacomelli, S., Rodeghiero, F., & Duga, S. (2010). Alterations of mRNA processing and stability as a pathogenic mechanism in von Willebrand factor quantitative deficiencies. *Journal of Thrombosis and Haemostasis*, *8*(12), 2736-2742. https://doi.org/10.1111/j.1538-7836.2010.04060.x

Castaman, G., & Rodeghiero, F. (1996). Desmopressin and type II B von Willebrand disease. *Haemophilia*, *2*(2), 73-77. https://doi.org/10.1111/j.1365-2516.1996.tb00018.x

Castaman, G., Rodeghiero, F., Tosetto, A., Cappelletti, A., Baudo, F., Eikenboom, J. C., Federici, A. B., Lethagen, S., Linari, S., Lusher, J., Nishino, M., Petrini, P., Srivastava, A., & Ungerstedt, J. S. (2006). Hemorrhagic symptoms and bleeding risk in obligatory carriers of type 3 von Willebrand disease: an international, multicenter study. *Journal of Thrombosis and Haemostasis*, *4*(10), 2164-2169. https://doi.org/10.1111/j.1538-7836.2006.02070.x

Castaman, G., Tosetto, A., Cappelletti, A., Goodeve, A., Federici, A. B., Batlle, J., Meyer, D., Goudemand, J., Eikenboom, J. C., Schneppenheim, R., Budde, U., Ingerslev, J., Lethagen, S., Hill, F., Peake, I. R., & Rodeghiero, F. (2010). Validation of a rapid test (VWF-LIA) for the quantitative determination of von Willebrand factor antigen in type 1 von Willebrand disease diagnosis within the European multicenter study MCMDM-1VWD. *Thrombosis research*, *126*(3), 227-231. https://doi.org/10.1016/j.thromres.2010.06.013

Castaman, G., Tosetto, A., Federici, A. B., & Rodeghiero, F. (2011). Bleeding tendency and efficacy of anti-haemorrhagic treatments in patients with type 1 von Willebrand disease and increased von Willebrand factor clearance. *Thrombosis and haemostasis*, *105*(04), 647-654. https://doi.org/10.1160/TH10-11-0697

Cattaneo, M. (2009). Light transmission aggregometry and ATP release for the diagnostic assessment of platelet function. *Seminars in thrombosis and hemostasis*, *35*(02), 158-167. https://doi.org/10.1055/s-0029-1220324

Cattaneo, M., Cerletti, C., Harrison, P., Hayward, C. P., Kenny, D., Nugent, D., Nurden, P., Rao, A. K., Schmaier, A. H., Watson, S. P., Lussana, F., Pugliano, M. T., & Michelson, A. D. (2013). Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH. *Journal of Thrombosis and Haemostasis*, *11*, 1183–1189. https://onlinelibrary.wiley.com/doi/10.1111/jth.12231

Challet Meylan, L., Patsch, C., & Thoma, E. (2015, August 06). *Endothelial cells differentiation from hPSCs*. Protocol exchange. Retrieved June 03, 2022, from https://doi.org/10.1038/protex.2015.055

Chandler, W. L., Peerschke, E. I., Castellone, D. D., & Meijer, P. (2011). von Willebrand factor assay proficiency testing: the North American Specialized Coagulation Laboratory Association experience. *American journal of clinical pathology*, *135*(6), 862-869. https://doi.org/10.1309/AJCPH5JK4ONENPAE

Chandraharan, E., & Krishna, A. (2017). Diagnosis and management of postpartum haemorrhage. *BMJ*, *358*, j3875. http://dx.doi.org/10.1036/bmj.j3875

Chang, Y. F., Imam, J. S., & Wilkinson, M. F. (2007). The nonsense-mediated decay RNA surveillance pathway. *Annu. Rev. Biochem*, *76*, 51-74. https://doi.org/10.1146/annurev.biochem.76.050106.093909

Charbonneau, H., Tonks, N. K., Walsh, K. A., & Fischer, E. H. (1988). The leukocyte common antigen (CD45): a putative receptor-linked protein tyrosine phosphatase. *Proceedings of the National Academy of Sciences*, *85*(19), 7182-7186. .https://doi.org/10.1073/pnas.85.19.7182

Chen, B., Solis-Villa, C., Hakenberg, J., Qiao, W., Srinivasan, R. R., Yasuda, M., Balwani, M., Doheny, D., Peter, I., Chen, R., & Desnick, R. J. (2016). Acute intermittent porphyria: predicted pathogenicity of HMBS variants indicates extremely low penetrance of the autosomal dominant disease. *Human mutation*, *37*(11), 1215-1222. https://doi.org/10.1002/humu.23067

Chen, F., Dong, M., Ge, M., Zhu, L., Ren, L., Liu, G., & Mu, R. (2013). The history and advances of reversible terminators used in new generations of sequencing technology. *Genomics, Proteomics & Bioinformatics*, *11*(1), 34-40. http://dx.doi.org/10.1016/j.gpb.2013.01.003

Chen, J., Hinckley, J. D., Haberichter, S., Jacobi, P., Montgomery, R., Flood, V. H., Wong, R., Interlandi, G., Chung, D. W., Lopez, J. A., & Di Paola, J. (2015). Variable content of von Willebrand factor mutant

monomer drives the phenotypic variability in a family with von Willebrand disease. *Blood*, *126*(2), 262-269. https://doi.org/10.1182/blood-2014-11-613935

Cheung, C., Bernardo, A. S., Pedersen, R. A., & Sinha, S. (2014). Directed differentiation of embryonic origin–specific vascular smooth muscle subtypes from human pluripotent stem cells. *Nature Protocols*, *9*(4), 929-938. https://doi.org/10.1038/nprot.2014.059

Chion, C. K., Doggen, C. J., Crawley, J. T., Lane, D. A., & Rosendaal, F. R. (2007). ADAMTS13 and von Willebrand factor and the risk of myocardial infarction in men. *Blood*, *109*(5), 1998-2000. https://doi.org/10.1182/blood-2006-07-038166

Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J. C., & Keller, G. (1998). A common precursor for hematopoietic and endothelial cells. *Development*, (125), 4. https://doi.org/10.1242/dev.125.4.725

Choi, K.-D., Yu, J., Smuga-Otto, K., Salvagiotto, G., Rehrauer, W., Vodyanik, M., Thomson, J., & Slukvin, I. (2009). Hematopoietic and Endothelial Differentiation of Human Induced Pluripotent Stem Cells. *Stem Cells*, *27*(3), 559-567. https://doi.org/10.1634/stemcells.2008-0922

Choosakoonkriang, S., Lobo, B. A., Koe, G. S., Koe, J. G., & Middaugh, C. R. (2003). Biophysical characterization of PEI/DNA complexes. *Journal of pharmaceutical sciences*, *92*(8), 1710-1722. https://doi.org/10.1002/jps.10437

Christensen, R. H. (2018, 8 25). *ordinal—Regression Models for Ordinal Data*. The Comprehensive R Archive Network (CRAN). Retrieved 04 04, 2022, from https://cran.r-project.org/web/packages/ordinal/index.html

Christopherson, P. A., Haberichter, S. L., Flood, V. H., Perry, C. L., Sadler, B. E., Bellissimo, D. B., Paola, J. D., Montgomery, R. R., & The Zimmerman Program Investigators. (2022). Molecular pathogenesis and heterogeneity in type 3 VWD families in US Zimmerman Program. *Journal of Thrombosis and Haemostasis*. https://doi.org/10.1111/jth.15713

Christopherson, P. A., Udani, R. A., Perry, C., Bellissimo, D. B., Haberichter, S. L., Flood, V. H., Friedman, K. D., Cox Gill, J., Montgomery, R. R., & Zimmerman Program Investigators. (2016). Identification of copy number variants in type 1 and type 3 VWD by aCGH in the Zimmerman Program. *Blood*, *128*(22), 872. https://doi.org/10.1182/blood.V128.22.872.872

Chudasama, Y. V., Khunti, K., Gillies, C. L., Dhalwini, N. N., Davies, M. J., Yates, T., & Zaccardi, F. (2020). Healthy lifestyle and life expectancy in people with multimorbidity in the UK Biobank: A

longitudinal cohort study. *PLoS medicine*, *17*(9), e1003332. https://doi.org/10.1371/journal.pmed.1003332

Church, D. M., Schneider, V. A., Graves, T., Auger, K., Cunningham, F., Bouk, N., Chen, H.-C., Agarwala, R., McClaren, W. M., Ritchie, G. R., Albracht, D., Kremitzki, M., Rock, S., Kotkiewicz, H., Kremitzki, C., Wollam, A., Trani, L., Fulton, L., Fulton, R., … Hubbard, T. (2011). Modernizing reference genome assemblies. *PLoS biology*, *9*(7), e1001091. https://doi.org/10.1371/journal.pbio.1001091

Cianci, P., & Selicorni, A. (2015). "Gestalt diagnosis" for children with suspected genetic syndromes. *Italian Journal of Pediatrics*, *41*(2), 1-2. https://doi.org/10.1186/1824-7288-41-S2-A16

Claesson-Welsh, L., Dejana, E., & McDonald, D. M. (2021). Permeability of the endothelial barrier: identifying and reconciling controversies. *Trends in molecular medicine*, *27*(4), 314-331. https://doi.org/10.1016/j.molmed.2020.11.006

Clark, T. G., Bradburn, M. J., Love, S. B., & Altman, D. G. (2003). Survival analysis part I: basic concepts and first analyses. *British journal of cancer*, *89*(2), 232-238. https://doi.org/10.1038/sj.bjc.6601118

Clausen, H., & Hakomori, S. I. (1989). ABH and Related Histo−Blood Group Antigens; Immunochemical Differences in Carrier Isotypes and Their Distribution. *Vox sanguinis*, *56*(1), 1-20. https://doi.org/10.1111/j.1423-0410.1989.tb03040.x

Clemetson, K. J. (2012). Platelets and primary haemostasis. *Thrombosis research*, *129*(3), 220-224. https://doi.org/10.1016/j.thromres.2011.11.036

ClinVar. (2017, Dec 18). *NM_000552.5(VWF):c.3692A>G (p.Asn1231Ser)*. ClinVar. Retrieved Jul 19, 2021, from https://www.ncbi.nlm.nih.gov/clinvar/variation/976752/evidence/

Cohen, G., & Java, R. (1995). Memory for medical history: Accuracy of recall. *Applied Cognitive Psychology*, *9*(4), 273-288. https://doi.org/10.1002/acp.2350090402

Colling, M. E., Friedman, K. D., & Dzik, W. H. (2019). In Vitro Assessment of von Willebrand Factor in Cryoprecipitate, Antihemophilic Factor/VWF Complex (Human), and Recombinant von Willebrand Factor. *Clinical and Applied Thrombosis/Hemostasis*, *25*, 1-6. https://doi.org/10.1177/1076029619873976

Collins, P. W., Cumming, A. M., Goodeve, A. C., & Lillicrap, D. (2008). Type 1 von Willebrand disease: application of emerging data to clinical practice. *Haemophilia*, *14*(4), 685-696. https://doi.org/10.1111/j.1365-2516.2008.01757.x

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P. D., Wu, X., Jiang, W., Marraffini, L., & Zhang, F. (2013). Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science*, *339*(6121), 819-823. https://doi.org/10.1126/science.1231143

Conlan, M. G., Folsom, A. R., Finch, A., Davis, C. E., Sorlie, P., Marcucci, G., Wu, K. K., & ARIC Study Investigators. (1993). Associations of factor VIII and von Willebrand factor with age, race, sex, and risk factors for atherosclerosis. *Thrombosis and haemostasis*, *70*(09), 380-385. https://doi.org/10.1055/s-0038-1649589

Connell, N. T., James, P. D., Brignardello-Petersen, R., Abdul-Kadir, R., Ameer, B., Arapshian, A., Couper, S., Di Paola, J., Eikenboom, J., Giraud, N., Grow, J. M., Haberichter, S., Jacobs-Pratt, V., Konkle, B. A., Kouides, P., Laffan, M., Lavin, M., Leebeek, F. W., McLintock, C., … Flood, V. H. (2021). von Willebrand disease: proposing definitions for future research. *Blood advances*, *5*(2), 565-569. https://doi.org/10.1182/bloodadvances.2020003620

Connolly, S. J., Ezekowitz, M. D., Yusuf, S., Eikelboom, J., Oldgren, J., Parekh, A., Pogue, J., Reilly, P. A., Themeles, E., Varrone, J., Wang, S., Alings, M., Xavier, D., Zhu, J., Diaz, R., Lewis, B. S., Darius, H., Diener, H.-C., Joyner, C. D., … RE-LY Steering Committee and Investigators. (2009). Dabigatran versus warfarin in patients with atrial fibrillation. *New England journal of medicine*, *361*(12), 1139-1151. https://doi.org/10.1056/NEJMoa0905561

Cooper, D. N., & Krawczak, M. (1993). *Human gene mutation*. BIOS Scientific Publishers.

Cooper, G. M., Stone, E. A., Asimenos, G., NISC Comparative Sequencing Program,, Green, E. D., Batzoglou, S., & Sidow, A. (2005). Distribution and intensity of constraint in mammalian genomic sequence. *Genome Res*, *15*(15), 901-913. https://doi.org/10.1101/gr.3577405

Corada, M., Mariotti, M., Thurston, G., Smith, K., Kunkel, R., Brockhaus, M., Lampugnani, M. G., Martin-Padura, I., Stoppacciaro, A., Rocco, L., McDonald, D. M., Ward, P. A., & Dejana, E. (1999). Vascular endothelial–cadherin is an important determinant of microvascular integrity in vivo. *Proceedings of the National Academy of Sciences*, *96*(7), 9815-9820. https://doi.org/10.1073/pnas.96.17.981

Corrales, I. (2022). New approaches to the genetic study of bleeding diathesis in our center: from sanger to next-generation sequencing. *Blood Coagulation & Fibrinolysis*, *33(Supplement 1)*, S19-S21. https://doi.org/10.1097/MBC.0000000000001107

Corrales, I., Ramírez, L., Altisent, C., Parra, R., & Vidal, F. (2009). Rapid molecular diagnosis of von Willebrand disease by direct sequencing. Detection of 12 novel putative mutations in VWF gene. *Thrombosis and haemostasis*, *101*(03), 570-576. https://doi.org/10.1160/th08-08-0500

Corrales, I., Ramirez, L., Altisent, C., Parra, R., & Vidal, F. (2011). The study of the effect of splicing mutations in von Willebrand factor using RNA isolated from patients' platelets and leukocytes. *Journal of Thrombosis and Haemostasis*, *9*(4), 679-688. https://doi.org/10.1111/j.1538-7836.2011.04204.x

Corrales, I., Ramírez, L., Ayats, J., Altisent, C., Parra, R., & Vidal, F. (2010). Integration of molecular and clinical data of 40 unrelated von Willebrand Disease families in a Spanish locus-specific mutation database: first release including 58 mutations. *Haematologica*, *95*(11), 1982-1984. https://doi.org/10.3324/haematol.2010.028977

Corvin, A., Craddock, N., & Sullivan, P. F. (2010). Genome-wide association studies: a primer. *Psychological medicine*, *40*(7), 1063-1077. https://doi.org/10.1017/S0033291709991723

Cossarizza, A., Chang, H. D., Radbruch, A., Akdis, M., Andrä, I., Annunziato, F., Bacher, P., Barnaba, V., Battistini, L., Bauer, W. M., Baumgart, S., Becher, B., Beisker, W., Berek, C., Blanco, A., Borsellino, G., Boulais, P. E., Brinkman, R. R., Buscher, M., … Petriz, J. (2017). Guidelines for the use of flow cytometry and cell sorting in immunological studies. *European journal of immunology*, *47*(10), 1584-1797. https://doi.org/10.1002/eji.201646632

Counts, R. B., Paskell, S. L., & Elgee, S. K. (1978). Disulfide bonds and the quaternary structure of factor VIII/von Willebrand factor. *The Journal of clinical investigation*, *62*(3), 702-709. https://doi.org/10.1172/JCI109178

Cox, D. R. (1972). Regression Models and Life-Tables. *Journal of the Royal Statistical Society*, *34*(2), 187-220. https://doi.org/10.1111/j.2517-6161.1972.tb00899.x

Cramer, E., Meyer, D., Meen, R. l., & Breton-Gorius, J. (1985). Eccentric Localization of von Willebrand Factor in an Internal Structure of Platelet α-Granule Resembling That of Weibel-Palade Bodies. *Blood*, *66*, 710-713.

Cruz, M. A., Yuan, H., Lee, J. R., Wise, R. J., & Handin, R. I. (1995). Interaction of the von Willebrand Factor (vWF) with Collagen: Localization of the primary collagen-binding site by analysis of recombinant vwf a domain polypeptides. *Journal of Biological Chemistry*, *270*(18), 10822-10827. https://doi.org/10.1074/jbc.270.18.10822

Culbertson, M. R. (1999). RNA surveillance: unforeseen consequences for gene expression, inherited genetic disorders and cancer. *Trends in Genetics*, *15*(2), 74-80. https://doi.org/10.1016/S0168-9525(98)01658-8

Cumming, A., Grundy, P., Keeney, S., Lester, W., Enayat, S., Guilliatt, A., Bowen, D., Pasi, J., Keeling, D., Hill, F., Bolton-Maggs, P. H., Hay, C., Collins, P., & UK Haemophilia Centre Doctors' Organisation. (2006). An investigation of the von Willebrand factor genotype in UK patients diagnosed to have type 1 von Willebrand disease. *Thromb Haemost*, *96*(5), 630-641. https://doi.org/10.1160/TH06-07-0383

Dalby, A., Ballester-Beltrán, J., Lincetto, C., Mueller, A., Foad, N., Evans, A., Baye, J., Turro, E., Moreau, T., Tijssen, M. R., & Ghevaert, C. (2018). Transcription factor levels after forward programming of human pluripotent stem cells with GATA1, FLI1, and TAL1 determine megakaryocyte versus erythroid cell fate decision. *Stem cell reports*, *11*(6), 1462-1478. https://doi.org/10.1016/j.stemcr.2018.11.001

Danecek, P., Auton, A., Abecasis, G., Albers, C. A., Banks, E., Pristo, M. A., Banks, E., DePristo, M. A., Handsaker, R. E., Lunter, G., Marth, G. T., Sherry, S. T., McVean, G., Durbin, R., & 1000 Genomes Project Analysis Group. (2011). The variant call format and VCFtools. *Bioinformatics*, *27*(15), 2156-2158. https://doi.org/10.1093/bioinformatics/btr330

Daniel, M., Jaberoo, M. C., Stead, R. E., Reddy, V. M., & Moir, A. A. (2006). Is admission for epistaxis more common in Caucasian than in Asian people? A preliminary study. *Clinical Otolaryngology*, *31*(5), 386-389. https://doi.org/10.1111/j.1749-4486.2006.01274.x

Davis, K. A., Bashford, O., Jewell, A., Shetty, H., Stewart, R. J., Sudlow, C. L., & Hotopf, M. H. (2018). Using data linkage to electronic patient records to assess the validity of selected mental health diagnoses in English Hospital Episode Statistics (HES). *PLoS One*, *13*(3), e0195002. https://doi.org/10.1371/journal.pone.0195002

de Andrade, K. C., Mirabello, L., Stewart, D. R., Karlins, E., Koster, R., Wang, M., Gapstur, S. M., Gaudet, M. M., Freedman, N. D., Landi, M. T., Lemonnier, N., Hainaut, P., Savage, S. A., & Achatz, M. I. (2017). Higher−than−expected population prevalence of potentially pathogenic germline TP53 variants in individuals unselected for cancer history. *Human mutation*, *38*(12), 1723-1730. https://doi.org/10.1002/humu.23320

de Boer, S., & Eikenboom, J. (2019). Von Willebrand Disease: From In Vivo to In Vitro Disease Models. *Hemasphere*, *3*(5). https://doi.org/10.1097/HS9.0000000000000297

Decaris, M. L., Lee, C. I., Yoder, M. C., Tarantal, A. F., & Leach, J. K. (2009). Influence of the oxygen microenvironment on the proangiogenic potential of human endothelial colony forming cells. *Angiogenesis*, *12*(4), 303-311. https://doi.org/10.1007/s10456-009-9152-6

De Ceunynck, K., De Meyer, S., & Vanhoorelbeke, K. (2013). Unwinding the von Willebrand factor strings puzzle. *Blood*, *121*(2), 270-277. https://doi.org/10.1182/blood-2012-07-442285

De Groot, P. G., Federici, A. B., De Boer, H. C., d'Alessio, P., Mannucci, P. M., & Sixma, J. J. (1989). von Willebrand factor synthesized by endothelial cells from a patient with type IIB von Willebrand disease supports platelet adhesion normally but has an increased affinity for platelets. *Proceedings of the National Academy of Sciences*, *86*(10), 3793-3797. https://doi.org/10.1073/pnas.86.10.3793

de Jong, A., Dirven, R. J., Boender, J., Atiq, F., Anvar, S. Y., Leebeek, F. W., van Vlijmen, B. J., & Eikenboom, J. (2020). Ex vivo improvement of a von Willebrand disease type 2A phenotype using an allele-specific small-interfering RNA. *Thrombosis and haemostasis*, *120*(11), 1569-1579. https://doi.org/10.1055/s-0040-1715442

de Jong, A., & Eikenboom, J. (2017). Von Willebrand disease mutation spectrum and associated mutation mechanisms. *Thrombosis research*, *159*, 65-75. https://doi.org/10.1016/j.thromres.2017.09.025

de Jong, A., Weijers, E., Driven, R., de Boer, S., Streur, J., & Eikenboom, J. (2019). Variability of von Willebrand factor−related parameters in endothelial colony forming cells. *Journal of Thrombosis and Haemostasis*, *17*(9), 1544-1554. https://doi.org/10.1111/jth.14558

de la Fuente, B., Kasper, C. K., Rickles, F. R., & Hoyer, L. W. (1985). Response of patients with mild and moderate hemophilia A and von Willebrand's disease to treatment with desmopressin. *Annals of Internal Medicine*, *103*(1), 6-14. https://doi.org/10.7326/0003-4819-103-1-6

Denaxas, S. C., George, J., Herrett, E., Shah, A. D., Kalra, D., Hingorani, A. D., Kivimaki, M., Timmis, A. D., Smeeth, L., & Hemingway, H. (2012). Data resource profile: cardiovascular disease research using linked bespoke studies and electronic health records (CALIBER). *International journal of epidemiology*, *41*(6), 1625-1638. https://doi.org/10.1093/ije/dys188

den Dunnen, J. T., Dalgleish, R., Maglott, D. R., Hart, R. K., Greenblatt, M. S., McGowan−Jordan, J., Roux, A.-F., Smith, T., Antonarakis, S. E., & Taschner, P. E. (2016). HGVS recommendations for the description of sequence variants: 2016 update. *Human mutation*, *37*(6), 564-569. https://doi.org/10.1002/humu.22981

Denis, C., Methia, N., Frenette, P. S., Rayburn, H., Ullman-Culleré, M., Hynes, R. O., & Wagner, D. D. (1998). A mouse model of severe von Willebrand disease: defects in hemostasis and thrombosis. *Proceedings of the National Academy of Sciences*, *95*(16), 9524-9529. https://doi.org/10.1073/pnas.95.16.9524

Denis, C. V. (2002). Molecular and cellular biology of von Willebrand factor. *International journal of hematology*, *75*(1), 3-8. https://doi.org/10.1007/BF02981972

Denis, C. V., André, P., Saffaripour, S., & Wagner, D. D. (2001). Defect in regulated secretion of P-selectin affects leukocyte recruitment in von Willebrand factor-deficient mice. *Proceedings of the National Academy of Sciences*, *98*(7), 4072-4077. https://doi.org/10.1073/pnas.061307098

Denis, C. V., & Wagner, D. D. (1999). Insights from von Willebrand disease animal models. *Cellular and Molecular Life Sciences*, *56*(11), 977-990. https://doi.org/10.1007/s000180050487

Denoeud, F., Kapranov, P., Ucla, C., Frankish, A., Castelo, R., Drenkow, J., Lagarde, J., Alioto, T., Manzano, C., Chrast, J., Dike, S., Wyss, C., Henrichsen, C., Holroyd, N., Dickson, M., Taylor, R., Hance, Z., Foissac, S., Myers, R. M., … Reymond, A. (2007). Prominent use of distal 5′ transcription start sites and discovery of a large number of additional exons in ENCODE regions. *Genome research*, *17*(6), 746-759. https://doi.org/10.1101/gr.5660607

Dent, J. A., Berkowitz, S. D., Ware, J., Kasper, C. K., & Ruggeri, Z. M. (1990). Identification of a cleavage site directing the immunochemical detection of molecular abnormalities in type IIA von Willebrand factor. *Proceedings of the National Academy of Sciences*, *87*(16), 6306-6310. https://doi.org/10.1073/pnas.87.16.6306

Dent, J. A., Galbusera, M., & Ruggeri, Z. M. (1991). Heterogeneity of plasma von Willebrand factor multimers resulting from proteolysis of the constituent subunit. *The Journal of clinical investigation*, *88*(3), 774-782. https://doi.org/10.1172/JCI115376

Desch, K. C., Ozel, A. B., Siemieniak, D., Kalish, Y., Shavita, J. A., Thornburge, C. D., Sharathkumar, A. A., McHugh, C. P., Laurie, C. C., Crenshaw, A., Mirel, D. B., Kim, Y., Cropp, C. D., Molloy, A. M., Kirke, P. N., Bailey-Wilson, J. E., Wilson, A. F., Mills, J. L., Scott, J. M., … Ginsburg, D. (2013). Linkage analysis identifies a locus for plasma von Willebrand factor undetected by genome-wide association. *Proceedings of the National Academy of Sciences*, *110*(2), 588-593. https://doi.org/10.1073/pnas.1219885110

Desmet, F. O., Hamroun, D., Lalande, M., Collod-Beroud, G., Claustres, M., & Béroud, C. (2009). Human Splicing Finder: an online bioinformatics tool to predict splicing signals. *Nucleic acids research*, *37*(9), e67. https://doi.org/10.1093/nar/gkp215

Desprez, D., Drillaud, N., Flaujac, C., Volot, F., Pan-Petesch, B., Beurrier, P., Cussac, V., Feugeas, O., Pailler, Y., Callegarin, A., Trossaërt, M., & de Raucourt, E. (2021). Efficacy and safety of a recombinant Von Willebrand Factor treatment in patients with inherited Von Willebrand Disease requiring surgical procedures. *Haemophilia*, *27*(2), 270-276. https://doi.org/10.1111/hae.14242

Devkota, S. (2018). The road less traveled: strategies to enhance the frequency of homology-directed repair (HDR) for increased efficiency of CRISPR/Cas-mediated transgenesis. *BMB reports*, *51*(9), 437-443. https://doi.org/10.5483/BMBRep.2018.51.9.187

Dhanesha, N., Prakash, P., Doddapattar, P., Khanna, I., Pollpeter, M. J., Nayak, M. K., Staber, J. M., & Chauhan, A. K. (2016). Endothelial Cell–Derived von Willebrand Factor Is the Major Determinant That Mediates von Willebrand Factor–Dependent Acute Ischemic Stroke by Promoting Postischemic Thrombo-Inflammation. *Arterioscler Thromb Vasc Biol*, *36*, 1829-1837.

Diez-Ewald, M., Vizcaíno, G., Arteaga-Vizcaíno, M., Fernández, N., Weir-Medina, J., & Gómez, O. (1991). Epidemiology of von Willebrand's disease in the state of Zulia, Venezuela. *Investigacion clinica*, *32*(4), 187-199.

Dilley, A., Drews, C., Miller, C., Lally, C., Austin, H., Ramaswamy, D., Lurye, D., & Evatt, B. (2001). von Willebrand disease and other inherited bleeding disorders in women with diagnosed menorrhagia. *Obstetrics & Gynecology*, *97*(4), 630-636. https://doi.org/10.1016/S0029-7844(00)01224-2

Djambas Khayat, C., Samaha, H., Noun, P., Bakhos Asmar, J. D., Taher, A., Adib, S., Inati, A., & Sakr, S. (2014). Detection of bleeding disorders in Lebanon: outcomes of a pilot programme. *Haemophilia*, *20*(2), 196-199. https://doi.org/10.1111/hae.12281

Dong, J. F., Noake, J. L., Nolasco, L., Bernardo, A., Arceneaux, W., Shrimpton, C. N., Schade, A. J., McIntire, L. V., Fukikawa, K., & López, J. (2002). ADAMTS-13 rapidly cleaves newly secreted ultralarge von Willebrand factor multimers on the endothelial surface under flowing conditions. *Blood*, *100*(12), 4033-4039. https://doi.org/10.1182/blood-2002-05-1401

Dorgalaleh, A., Tabiban, S., Shams, M., Ala, F., Bahoush, G., Jazebi, M., Manafi, R., Tavasoli, B., & Baghaipour, M. R. (2018). Von Willebrand disease in Iran: diagnosis and management. *Ann blood*, *3*(1). https://doi.org/10.21037/aob.2018.01.01

Doruelo, A. L., Haberichter, S. L., Christopherson, P. A., Boggio, L. N., Gupta, S., Lentz, S. R., Shapiro, A. D., Montgomery, R. R., & Flood, V. H. (2017). Clinical and laboratory phenotype variability in type 2M von Willebrand disease. *Journal of Thrombosis and Haemostasis*, *15*(8), 1559-1566. https://doi.org/10.1111/jth.13742

Downes, K., Megy, K., Duarte, D., Vries, M., Gebhart, J., Hofer, S., Shamardina, O., Deevi, S. V., Stephens, J., Mupeta, R., Tuna, S., Al Hasso, N., Besser, M. W., Cooper, N., Daugherty, L., Gleadall, N., Greene, D., Haimel, M., Martin, H., … Freson, K. (2019). Diagnostic high-throughput sequencing of 2396 patients with bleeding, thrombotic, and platelet disorders. *Blood*, *134*(23), 2082-2091. https://doi.org/10.1182/blood.2018891192

Drews, C. D., Dilley, A. B., Lilly, C., Beckman, M. G., & Evatt, B. (2002). Screening questions to identify women with von Willebrand disease. Journal of the American Medical Women's Association. *J Am Med Womens Assoc*, *57*, 217-18.

Dudley, J. T., Kim, Y., Liu, L., Markov, G. J., Gerold, K., Chen, R., Butte, A. J., & Kumar, S. (2012). Human genomic disease variants: a neutral evolutionary explanation. *Genome Research*, *22*(8), 1383-1394. https://doi.org/10.1101/gr.133702.111

Duemling, W. W., & Norman, C. W. (1961). Mucocutaneous Changes: Manifestations of Blood Dyscrasias and Hemorrhagic Disturbances. *Archives of Dermatology*, *84*(2), 273-289. https://doi.org/10.1001/archderm.1961.01580140099013

Duke, W. W. (1910). The relation of blood platelets to hemorrhagic disease: Description of a method for determining the bleeding time and coagulation time and report of three cases of hemorrhagic disease relieved by transfusion. *Journal of the American Medical Association*, *55*(14), 1185-1192. https://doi.org/10.1001/jama.1910.04330140029009

Dunkley, S., Baker, R. I., Pidcock, M., Price, J., Seldon, M., Smith, M., Street, A., Maher, D., Barrese, G., Stone, C., & Lloyd, J. (2010). Clinical efficacy and safety of the factor VIII/von Willebrand factor concentrate BIOSTATE® in patients with von Willebrand's disease: a prospective multi−centre study. *Haemophilia*, *16*(4), 615-624. https://doi.org/10.1111/j.1365-2516.2010.02206.x

Duray, P. H., Marcal Jr, J. M., Livolsi, V. A., Fisher, R., Scholhamer, C., & Brand, M. H. (1984). Gastrointestinal angiodysplasia: a possible component of von Willebrand's disease. *Human pathology*, *15*(6), 539-544. https://doi.org/10.1016/S0046-8177(84)80007-6

Dushpanova, A., Agostini, S., Ciofini, E., Cabiati, M., Casieri, V., Matteucci, M., Del Ry, S., Clerico, A., Berti, S., & Lionetti, V. (2016). Gene silencing of endothelial von Willebrand Factor attenuates angiotensin II-induced endothelin-1 expression in porcine aortic endothelial cells. *Scientific reports*, *6*(1), 1-12. https://doi.org/10.1038/srep30048 (2016).

Early, P., Rogers, J., Davis, M., Calame, K., Bond, M., Wall, R., & Hood, L. (1980). Two mRNAs can be produced from a single immunoglobulin μ gene by alternative RNA processing pathways. *Cell*, *20*(2), 313-319. https://doi.org/10.1016/0092-8674(80)90617-0

Eastwood, S. V., Mathur, R., Atkinson, M., Brophy, S., Sudlow, C., Flaig, R., Sudlow, C., Flaig, R., de Lusignan, S., Allen, N., & Chaturvedi, N. (2016). Algorithms for the capture and adjudication of prevalent and incident diabetes in UK Biobank. *PloS one*, *11*(9), e0162388. https://doi.org/10.1371/journal.pone.0162388

Eikenboom, J. C. (2001). Congenital von Willebrand disease type 3: clinical manifestations, pathophysiology and molecular biology. *Best Practice & Research Clinical Haematology*, *14*(2), 365-379. https://doi.org/10.1053/beha.2001.0139

Eikenboom, J. C., Castaman, G., Vos, H. L., Bertina, R. M., & Rodeghiero, F. (1998). Characterization of the genetic defects in recessive type 1 and type 3 von Willebrand disease patients of Italian origin. *Thrombosis and haemostasis*, *79*(04), 709-717. https://doi.org/10.1055/s-0037-1615050

Eikenboom, J. C., van Amstel, H. K., Reitsma, P. H., & Briët, E. (1992). Mutations in Severe, Type III von Willebrand's Disease in the Dutch Population: Candidate Missense and Nonsense Mutations Associated with Reduced Levels of von Willebrand Factor Messenger RNA. *Thromb Haemost*, *68*(04), 448-454. https://doi.org/10.1055/s-0038-1646295

Elayaperumal, S., Fouzia, N. A., Biswas, A., Nair, S. C., Viswabandya, A., George, B., Abraham, A., Oldenburg, J., Edison, E. S., & Srivastava, A. (2018). Type−3 von Willebrand disease in India—Clinical spectrum and molecular profile. *Haemophilia*, *24*(6), 930-940. https://doi.org/10.1111/hae.13542

Elbatarny, M., Mollah, S., Grabell, J., Bae, S., Deforest, M., Tuttle, A., Hopman, W., Clark, D. S., Mauer, A. C., Bowman, M., Riddel, J., Christopherson, P. A., Montgomery, R. R., Zimmerman Program Investigators, Rand, M. L., Coller, B., & James, P. D. (2014). Normal range of bleeding scores for the ISTH−BAT: adult and pediatric data from the merging project. *Haemophilia*, *20*(6), 831-835. https://doi.org/10.1111/hae.12503

Elbaz, C., & Sholzberg, M. (2020). An illustrated review of bleeding assessment tools and common coagulation tests. *Research and Practice in Thrombosis and Haemostasis*, *4*(5), 761-773. https://doi.org/10.1002/rth2.12339

Ellgaard, L., & Frickel, E. M. (2003). Calnexin, calreticulin, and ERp57. *Cell biochemistry and biophysics*, *39*(3), 223-247. https://doi.org/10.1385/CBB:39:3:223

Ellies, L. G., Ditto, D., Levy, G. G., Wahrenbrock, M., Ginsburg, D., Varki, A., Le, D. T., & Marth, J. D. (2002). Sialyltransferase ST3Gal-IV operates as a dominant modifier of hemostasis by concealing asialoglycoprotein receptor ligands. *Proceedings of the National Academy of Sciences*, *99*(15), 10042-10047. https://doi.org/10.1073/pnas.142005099

EMBL-EBI. (2016, January 27). *CHEBI:15750 - dolichyl diphosphate*. Chemical Entities of Biological Interest (ChEBI). Retrieved April 05, 2021, from https://www.ebi.ac.uk/chebi/searchId.do?chebiId=CHEBI:15750

Emdin, C. A., Khera, A. V., Chaffin, M., Klarin, D., Natarajan, P., Aragam, K., Haas, M., Bick, A., Zekavat, S. M., Nomura, A., Ardissino, D., Wilson, J. G., Schunkert, H., McPherson, R., Watkins, H., Elosua, R., Brown, M. J., Samani, N. J., Baber, U., … Kathiresan, S. (2018). Analysis of predicted

loss-of-function variants in UK Biobank identifies variants protective for disease. *Nature communications*, *9*(1), 1-8. https://doi.org/10.1038/s41467-018-03911-8

Enayat, M. S., Guillatt, A. M., Surdhar, G. K., Jenkins, P. V., Pasi, K. J., Toh, C. H., Williams, M. D., & Hill, F. G. (2001). Aberrant dimerization of von Willebrand factor as the result of mutations in the carboxy-terminal region: identification of 3 mutations in members of 3 different families with type 2A (phenotype IID) von Willebrand disease. *Blood*, *98*(3), 674-680. https://doi.org/10.1182/blood.V98.3.674

Ensembl. (2021, Feb). *Assembly Converter*. Ensembl. Retrieved Jan 07, 2021, from http://www.ensembl.org/Homo_sapiens/Tools/AssemblyConverter

Ensembl. (2021, February). *Gene: VWF ENSG00000110799*. e!Ensembl. Retrieved April 22, 2021, from http://www.ensembl.org/Homo_sapiens/Gene/Summary?db=core;g=ENSG00000110799;r=12:5948877-6124770

European Bioinformatics Institute (EMBL-EBI). (2021, May). Ensembl Variation - Calculated variant consequences. Retrieved July 11, 2021, from https://www.ensembl.org/info/genome/variation/prediction/predicted_data.html

Evatt, B. (2005). *World Federation of Hemophilia Guide to Developing a National Patient Registry*. World Federation of Haemophilia. Retrieved 01 26, 2022, from http://www1.wfh.org/publication/files/pdf-1288.pdf

Ewenstein, B. (1997). Von Willebrand's disease. *Annual review of medicine*, *48*(1), 525-542. https://doi.org/10.1146/annurev.med.48.1.525

Ewenstein, B. M., Inbal, A., Pober, J. S., & Handin, R. I. (1990). Molecular studies of von Willebrand disease: reduced von Willebrand factor biosynthesis, storage, and release in endothelial cells derived from patients with type I von Willebrand disease. *Blood*, *76*(9), 1901. https://doi.org/10.1182/blood.V75.7.1466.1466

Eze-Nliam, C., Cain, K., Bond, K., Forlenza, K., Jankowski, R., Magyar-Russell, G., Yenokyan, G., & Ziegelstein, R. C. (2012). Discrepancies between the medical record and the reports of patients with acute coronary syndrome regarding important aspects of the medical history. *BMC health services research*, *12*(1), 1-7. https://doi.org/10.1186/1472-6963-12-78

Fass, D. N., Knutson, G. J., & Bowie, E. J. (1978). Porcine Willebrand factor: a population of multimers. *The Journal of laboratory and clinical medicine*, *91*(2), 307-320.

Fasulo, M. R., Biguzzi, E., Abbattista, M., Stufano, F., Pagliari, M. T., Mancini, I., Gorski, M. M., Cannavo, A., Corgiolu, M., Peyvandi, F., & Rosendaal, F. R. (2018). The ISTH bleeding assessment tool and the risk of future bleeding. *Journal of Thrombosis and Haemostasis*, *16*(1), 125-130. https://doi.org/10.1111/jth.13883

Favaloro, E. J. (2010a). Evaluation of commercial von Willebrand factor collagen binding assays to assist the discrimination of types 1 and 2 von Willebrand disease. *Thrombosis and haemostasis*, *104*(11), 1009-1021. https://doi.org/10.1160/TH10-06-0360

Favaloro, E. J. (2011). von Willebrand Disease: Local Diagnosis and Management of a Globally Distributed Bleeding Disorder. *Semin Thromb Hemost*, *37*(5), 440-455. https://doi.org/10.1055/s-0031-1281028

Favaloro, E. J. (2020). Classification of von Willebrand disease in the context of modern contemporary von Willebrand factor testing methodologies. *Research and practice in thrombosis and haemostasis*, *4*(6), 952. https://doi.org/10.1002/rth2.12392

Favaloro, E. J., (Adcock) Funk, D. M., & Lippi, G. (2012). Pre-analytical Variables in Coagulation Testing Associated With Diagnostic Errors in Hemostasis. *Laboratory Medicine*, *43*(2), 1-10. https://doi.org/10.1309/LM749BQETKYPYPVM

Favaloro, E. J., Dean, M., Grispo, L., Exner, T., & Koutts, J. (1994). Von Willebrand's disease: use of collagen binding assay provides potential improvement to laboratory monitoring of desmopressin (DDAVP) therapy. *American journal of hematology*, *45*(3), 205-211. https://doi.org/10.1002/ajh.2830450303

Favaloro, E. J., & Lippi, G. (2017). Reference ranges in hemostasis testing: necessary but imperfect. *J Lab Precis Med*, *2*(18). https://doi.org/10.21037/jlpm.2017.04.05

Favaloro, E. J., Mohammed, S., Vong, R., Oliver, S., Brennan, Y., Favaloro, J. W., & Curnow, J. (2021). How we diagnose 2M von Willebrand disease (VWD): Use of a strategic algorithmic approach to distinguish 2M VWD from other VWD types. *Haemophilia*, *27*(1), 137-148. https://doi.org/10.1111/hae.14204

Favaloro, E. J., & Oliver, S. (2017). Evaluation of a new commercial von Willebrand factor multimer assay. *Haemophilia*, *23*(4), e373-e377. https://doi.org/10.1111/hae.13261

Favaloro, E. J., Soltani, S., McDonald, J., Grezchnik, E., Easton, L., & Favaloro, J. W. (2005). Reassessment of ABO blood group, sex, and age on laboratory parameters used to diagnose von Willebrand disorder: potential influence on the diagnosis vs the potential association with risk of thrombosis. *Am J Clin Pathol*, *124*, 910-917. https://doi.org/10.1309/W76QF806CE80CL2T

Federici, A. B. (1998). Diagnosis of von Willebrand disease. *Haemophilia*, *4*(4), 654-660. https://doi.org/10.1046/j.1365-2516.1998.440654.x

Federici, A. B. (2004). Clinical diagnosis of von Willebrand disease. *Haemophilia*, *10*, 169-176. https://doi.org/10.1111/j.1365-2516.2004.00991.x

Federici, A. B. (2008b). Clinical and molecular markers of inherited von Willebrand disease type 3: are deletions of the VWF gene associated with alloantibodies to VWF? *Journal of Thrombosis and Haemostasis*, *6*, 1726-1728. https://doi.org/10.1111/j.1538-7836.2008.03146.x

Federici, A. B. (2008a). The use of desmopressin in von Willebrand disease: the experience of the first 30 years (1977–2007). *Haemophilia*, *14*(Suppl. 1), 5-14. https://doi.org/10.1111/j.1365-2516.2007.01610.x

Federici, A. B. (2014). Clinical and laboratory diagnosis of VWD. *Hematology Am Soc Hematol Educ Program 2014*, *1*, 524-530. https://doi.org/10.1182/asheducation-2014.1.524

Federici, A. B. (2016). Current and emerging approaches for assessing von Willebrand disease in 2016. *International journal of laboratory hematology*, *38*, 41-49. https://doi.org/10.1111/ijlh.12540

Federici, A. B., Bader, R., Pagani, S., Colibretti, M. L., De Marco, L., & Mannucci, P. M. (1989). Binding of von Willebrand factor to glycoproteins Ib and IIb/IIIa complex: affinity is related to multimeric size. *Br J Haematol*, *73*, 93-99. https://doi.org/10.1111/j.1365-2141.1989.tb00226.x

Federici, A. B., Baduo, F., Caracciolo, C., Mancuso, G., Mazzucconi, M. G., Musso, R., Schinco, P. C., Targhetta, R., & Mannucci, P. M. (2002b). Clinical efficacy of highly purified, doubly virus‐inactivated factor VIII/von Willebrand factor concentrate (Fanhdi®) in the treatment of von Willebrand disease: a retrospective clinical study. *Haemophilia*, *8*(6), 761-767. https://doi.org/10.1046/j.1365-2516.2002.00688.x

Federici, A. B., Castaman, G., Mannucci, P. M., & Italian Association of Hemophilia Centers (AICE). (2002a). Guidelines for the diagnosis and management of von Willebrand disease in Italy. *Haemophilia*, *8*(5), 607-621. https://doi.org/10.1046/j.1365-2516.2002.00672.x

Federici, A. B., Mannucci, P. M., Castaman, G., Baronciani, L., Bucciarelli, P., Canciani, M. T., Pecci, A., Lenting, P. J., & De Groot, P. G. (2009). Clinical and molecular predictors of thrombocytopenia and risk of bleeding in patients with von Willebrand disease type 2B: a cohort study of 67 patients. *Blood*, *113*(3), 526-534. https://doi.org/10.1182/blood-2008-04-152280

Federici, A. B., Mazurier, C., Berntorp, E., Lee, C. A., Scharrer, I., Goudemand, J., Lethagen, S., Nitu, I., Ludwig, G., Hilbert, L., & Mannucci, P. M. (2004). Biologic response to desmopressin in patients with severe type 1 and type 2 von Willebrand disease: results of a multicenter European study. *Blood*, *103*(6), 2032-2038. https://doi.org/10.1182/blood-2003-06-2072

Federici, A. B., Rand, J. H., Bucciarelli, P., Budde, U., Van Genderen, P. J., Mohri, H., Meyer, D., Rodeghiero, F., & Sadler, J. E. (2000). Acquired von Willebrand syndrome: data from an international registry. *Thrombosis and haemostasis*, *84*(08), 345-349. https://10.1055/s-0037-1614018

Fels, S., Boeckelmann, D., Glonnegger, H., Büchsel, M., & Zieger, B. (2022). Novel Likely Pathogenic Variant in the A3 Domain of von Willebrand Factor Leading to a Collagen-Binding Defect. *Hämostaseologie*. https://doi.org/10.1055/a-1701-2181

Fernandez, M. F., Ginsberg, M. H., Ruggeri, Z. M., Batlle, F. J., & Zimmerman, T. S. (n.d.). Multimeric structure of platelet factor VIII/von Willebrand factor: the presence of larger multimers and their reassociation with thrombin-stimulated platelets. *Blood*, *60*, 1132–1138.

Ferraro, F., Kriston-Vizi, J., Metcalf, D. J., Martin-Martin, B., Freeman, J., Burden, J. J., Westmoreland, D., Dyer, C. E., Knight, A. E., Ketteler, R., & Cutler, D. F. (2014). A two-tier Golgi-based control of organelle size underpins the functional plasticity of endothelial cells. *Developmental cell*, *29*(3), 292-304. https://doi.org/10.1016/j.devcel.2014.03.021

Filon, D., Oron, V., Krichevski, S., Shaag, A., Shaag, Y., Warren, T. C., Goldfarb, A., Shnoer, Y., Koren, A., Aker, M., Abramov, A., Rachmilewitz, E. A., Rund, D., Kazazian, H. H., & Oppenheim, A. (1994). Diversity of β-Globin Mutations in Israeli Ethnic Groups Reflects Recent Historic Events. *Am J Hum Genet*, *54*(5), 836-843.

Flaumenhaft, R., Dilks, J. R., Rozenvayn, N., Monahan-Earley, R. A., Feng, D., & Dvorak, A. M. (2005). The actin cytoskeleton differentially regulates platelet α-granule and dense-granule secretion. *Blood*, *105*(10), 3879-3887.

Fleenor, B. S., Marshall, K. D., Rippe, C., & Seals, D. R. (2012). Replicative aging induces endothelial to mesenchymal transition in human aortic endothelial cells: potential role of inflammation. *Journal of vascular research*, *49*(1), 59-64. https://doi.org/10.1159/000329681

Flood, V. H., Christopherson, P. A., Gill, J. C., Friedman, K. D., Haberichter, S. L., Bellissimo, D. B., Udani, R. A., Dasgupta, M., Hoffmann, R. G., Ragni, M. V., Shapiro, A. D., Lusher, J. M., Lentz, S. R., Abshire, T. C., Leissinger, C., Hoots, W. K., Manco-Johnson, M. J., Gruppo, R. A., Boggio, L. N., … Montgomery, R. R. (2016). Clinical and laboratory variability in a cohort of patients diagnosed with type 1 VWD in the United States. *Blood*, *127*(20), 2481-2488. https://doi.org/10.1182/blood-2015-10-673681

Flood, V. H., Cox Gill, J., Friedman, K. D., Bellissimo, D., Haberichter, S. L., & Montgomery, R. R. (2011). von Willebrand disease in the United States: A Perspective from Wisconsin. *Semin Thromb Hemost.*, *37*(5), 528-534. https://doi.org/10.1055/s-0031-1281039

Flood, V. H., Friedman, K. D., Gill, J. C., Haberichter, S. L., Christopherson, P. A., Branchford, B. R., Hoffmann, R. G., Abshire, T. C., Dunn, A. L., Di Paola, J. A., Hoots, W. K., Brown, D. L., Leissinger, C., Lusher, J. M., Ragni, M. V., Shapiro, A. D., & Montgomery, R. R. (2013b). No increase in bleeding identified in type 1 VWD subjects with D1472H sequence variation. *Blood*, *121*(18), 3742-3744. https://doi.org/10.1182/blood-2012-12-471672

Flood, V. H., Friedman, K. D., Gill, J. C., Morateck, P. A., Wren, J. S., Scott, J. P., & Montgomery, R. R. (2009). Limitations of the ristocetin cofactor assay in measurement of von Willebrand factor function. *J Thromb Haemost*, *7*, 18329. https://doi.org/10.1111/j.1538-7836.2009.03594.x

Flood, V. H., Garcia, J., & Haberichter, S. L. (2019). The role of genetics in the pathogenesis and diagnosis of type 1 Von Willebrand disease. *Current opinion in hematology*, *26*(5), 331. https://doi.org/10.1097/MOH.0000000000000524

Flood, V. H., Gill, J. C., Friedman, K. D., Christopherson, P. A., Jacobi, P. M., Hoffman, R. G., Montgomery, R. R., Haberichter, S. L., & Zimmerman Program Investigators. (2013a). Collagen binding provides a sensitive screen for variant von Willebrand disease. *Clinical chemistry*, *59*(4), 684-691. https://doi.org/10.1373/clinchem.2012.199000

Flood, V. H., Schlauderaff, A. C., Haberichter, S. L., Slobodianuk, T. L., Jacobi, P. M., Bellissimo, D. B., Christopherson, P. A., Friedman, K. D., Cox Gill, J., Hoffmann, R. G., Montgomery, R. R., &

Zimmerman Program Investigators. (2015). Crucial role for the VWF A1 domain in binding to type IV collagen. *Blood*, *125*(14), 2297-2304. https://doi.org/10.1182/blood-2014-11-610824

Fogarty, H., Doherty, D., & O'Donnell, J. S. (2020). New developments in von Willebrand disease. *British journal of haematology*, *191*(3), 329-339. https://doi.org/10.1111/bjh.16681

Fokkema, I. F., & Asscheman, D. (2021). *LOVD 3.0 user manual Build 3.0-27*. LOVD. Retrieved February 26, 2022, from https://databases.lovd.nl/shared/docs/LOVD_manual_3.0.pdf

Fokkema, I. F., den Dunnen, J. T., & Taschner, P. E. (2005). LOVD: easy creation of a locus−specific sequence variation database using an "LSDB−in−a−box" approach. *Human mutation*, *26*(2), 63-68. https://doi.org/10.1002/humu.20201

Fokkema, I. F., Taschner, P. E., Schaafsma, G. C., Celli, J., Laros, J. F., & den Dunnen, J. T. (2011). LOVD v. 2.0: the next generation in gene variant databases. *Human mutation*, *32*(5), 557-563. https://doi.org/10.1002/humu.21438

Folsom, A. R., Rosamond, W. D., Shahar, E., Cooper, L. S., Aleksic, N., Nieto, F. J., Rasmussen, M. L., Wu, K. K., & Atherosclerosis Risk in Communities (ARIC) Study Investigators. (1999). Prospective study of markers of hemostatic function with risk of ischemic stroke. *Circulation*, *100*(7), 736-742. https://doi.org/10.1161/01.CIR.100.7.736

Forbes, C. D., Barr, R. D., Reid, G., Thomson, C., Prentice, C. R., McNicol, G. P., & Douglas, A. S. (1972). Tranexamic acid in control of haemorrhage after dental extraction in haemophilia and Christmas disease. *Br Med J*, *2*(5809), 311-313. https://doi.org/10.1136/bmj.2.5809.311

Foster, P. A., Fulcher, C. A., Marti, T., Titani, K., & Zimmerman, T. S. (1987). A major factor VIII binding domain resides within the amino-terminal 272 amino acid residues of von Willebrand factor. *Journal of Biological Chemistry*, *262*(18), 8443-8446. https://doi.org/10.1016/S0021-9258(18)47430-3

Franchini, M., Seidizadeh, O., & Mannucci, P. M. (2021). Prophylactic management of patients with von Willebrand disease. *Therapeutic Advances in Hematology*, *12*, 1-12. https://doi.org/10.1177/20406207211064064

Francioli, L., & MacArthur, D. (2019, October 16). *gnomAD v3.0*. gnomAD. Retrieved April 24, 2022, from https://gnomad.broadinstitute.org/news/2019-10-gnomad-v3-0/

Freedman, R. B., Klappa, P., & Ruddock, L. W. (2002). Protein disulfide isomerases exploit synergy between catalytic and specific binding domains. *EMBO Reports*, *3*(2), 136-140. https://doi.org/10.1093/embo-reports/kvf035

Freeze, H. H., & Kranz, C. (2008). Endoglycosidase and glycoamidase release of N−linked glycans. *Current protocols in immunology*, *83*(1), 8-15. https://doi.org/10.1002/0471142727.mb1713as89

Freiermuth, J. L., Powell−Castilla, I. J., & Gallicano, G. I. (2018). Toward a CRISPR picture: use of CRISPR/Cas9 to model diseases in human stem cells in vitro. *Journal of cellular biochemistry*, *119*(1), 62-68. https://doi.org/10.1002/jcb.26162

Freson, K., & Turro, E. (2017). High−throughput sequencing approaches for diagnosing hereditary bleeding and platelet disorders. *Journal of Thrombosis and Haemostasis*, *15*(7), 1262-1272. https://doi.org/10.1111/jth.13681

Fressinaud, E., & Meyer, D. (1993). International survey of patients with von Willebrand disease and angiodysplasia. *Thrombosis and haemostasis*, *70*(09), 546. https://doi.org/10.1055/s-0038-1649623

Fressinaud, E., Sakariassen, K. S., Rothschild, C., Baumgartner, H. R., & Meyer, D. (1992). Shear rate-dependent impairment of thrombus growth on collagen in nonanticoagulated blood from patients with von Willebrand disease and hemophilia A. *Blood*, *80*(4), 988-994. https://doi.org/10.1182/blood.V80.4.988.988

Friberg, B., Orno, A. K., Lindgren, A., & Lethagen, S. (2006). Bleeding disorders among young women: a population-based prevalence study. *Acta Obstet Gynecol Scand*, *85*, 200-206.

Friberg, L., Rosenqvist, M., & Lip, G. Y. (2012). Net clinical benefit of warfarin in patients with atrial fibrillation: a report from the Swedish atrial fibrillation cohort study. *Circulation*, *125*(19), 2298-2307. https://doi.org/10.1161/CIRCULATIONAHA.111.055079

Frontroth, J. P., Hepner, M., Sciuccati, G., Torres, A. F., Pieroni, G., & Bonduel, M. (2010). Prospective study of low-dose ristocetin-induced platelet aggregation to identify type 2B von Willebrand disease (VWD) and platelet-type VWD in children. *Thrombosis and haemostasis*, *104*(12), 1158-1165. https://doi.org/10.1160/TH10-04-0213

Fu, J., Naren, A. P., Gao, X., Ahmmed, G. U., & Malik, A. B. (2005). Protease-activated receptor-1 activation of endothelial cells induces protein kinase Cα-dependent phosphorylation of syntaxin 4

and Munc18c: role in signaling P-selectin expression. *Journal of Biological Chemistry*, *280*(5), 3178-3184. https://doi.org/10.1074/jbc.M410044200

Fu, W., O'Connor, T., Jun, G., Kang, H. M., Abecasis, G., Leal, S. M., Gabriel, S., Rieder, M. J., Altshuler, D., Shendure, J., Nickerson, D. A., Bamshad, M. J., NHLBI Exome Sequencing Project, & Akey, J. M. (2013). Analysis of 6,515 exomes reveals the recent origin of most human protein-coding variants. *Nature*, *493*(7431), 216-220. https://doi.org/10.1038/nature11690

Fujikawa, K., Suzuki, H., McMullen, B., & Chung, D. (2001). Purification of human von Willebrand factor–cleaving protease and its identification as a new member of the metalloproteinase family. *Blood*, *98*(6), 1662-1666. https://doi.org/10.1182/blood.V98.6.1662

Fujimura, Y., Titani, K., Holland, L. Z., Russell, S. R., Roberts, J. R., Elder, J. H., Ruggeri, Z. M., & Zimmerman, T. S. (1986). von Willebrand factor. A reduced and alkylated 52/48-kDa fragment beginning at amino acid residue 449 contains the domain interacting with platelet glycoprotein Ib. *Journal of Biological Chemistry*, *261*(1), 381-385. https://doi.org/10.1016/S0021-9258(17)42483-5

Fujiyoshi, K., Bruford, E. A., Mroz, P., Sims, C. L., O'Leary, T. J., Lo, A., Chen, N., Patel, N. R., Pravinchandra Patel, K., Seligero, B., Song, M., Monzon, F. A., Carter, A. B., Gulley, M. L., Mockus, S. M., Phung, T. L., Feilotter, H., Williams, H. E., & Ogino, S. (2021). Standardizing gene product nomenclature—a call to action. *Proceedings of the National Academy of Sciences*, *118*(3), e2025207118. https://doi.org/10.1073/pnas.2025207118

Fukuda, M., & Mikoshiba, K. (2001). Tac2−N, an atypical C−type tandem C2 protein localized in the nucleus. *FEBS letters*, *503*(2-3), 217-218. https://doi.org/10.1016/S0014-5793(01)02738-7

Furlan, M., Robles, R., & Lamie, B. (1996). Partial purification and characterization of a protease from human plasma cleaving von Willebrand factor to fragments produced by in vivo proteolysis. *Blood*, *87*(10), 4223-4234. https://doi.org/10.1182/blood.V87.10.4223.bloodjournal87104223

Gale, A. J. (2011). Current Understanding of Hemostasis. *Toxicol Pathol*, *39*(1), 273-280. https://doi.org/10.1177/0192623310389474

Gallinaro, L., Cattini, M. G., Sztukowska, M., Padrini, R., Sartorello, F., Pontara, E., Bertomoro, A., Daidone, V., Pagnan, A., & Casonato, A. (2008). A shorter von Willebrand factor survival in O blood group subjects explains how ABO determinants influence plasma von Willebrand factor. *Blood*, *111*(7), 3540-3545. https://doi.org/10.1182/blood-2007-11-122945

Ganesan, L. P., Kim, J., Wu, Y., Mohanty, S., Phillips, G. S., Birmingham, D. J., Robinson, J. M., & Anderson, C. L. (2012). FcγRIIb on liver sinusoidal endothelium clears small immune complexes. *The Journal of Immunology*, *189*(10), 4981-4988. https://doi.org/10.4049/jimmunol.1202017

Gao, N., White, P., Doliba, N., Golson, M. L., Matschinsky, F. M., & Kaestner, K. H. (2007). Foxa2 Controls Vesicle Docking and Insulin Secretion in Mature β Cells. *Cell Metabolism*, *6*(4), 267-279. https://doi.org/10.1016/j.cmet.2007.08.015

Gaziano, J. M., Concato, J., Brophy, M., Fiore, L., Pyarajan, S., Breeling, J., Whitbourne, S., Deena, J., Shannon, C., Humphries, D., Guarinoc, P., Aslanc, M., Anderson, D., LaFleur, R., Hammond, T., Schaaf, K., Moser, J., Huang, G., Muralidhar, S., … O'Leary, T. J. (2016). Million Veteran Program: A mega-biobank to study genetic influences on health and disease. *Journal of clinical epidemiology*, *70*, 214-223. https://doi.org/10.1016/j.jclinepi.2015.09.016

Gerritsen, H. E., Robles, R., Lämmle, B., & Furlan, M. (2001). Partial amino acid sequence of purified von Willebrand factor–cleaving protease. *Blood*, *98*(6), 1654-1661. https://doi.org/10.1182/blood.V98.6.1654

Geti, I., Ormiston, M. L., Rouhani, F., Toshner, M., Movassagh, M., Nichols, J., Mansfield, W., Southwood, M., Bradley, A., Rana, A. A., Vallier, L., & Morrell, N. W. (2012). A practical and efficient cellular substrate for the generation of induced pluripotent stem cells from adults: blood-derived endothelial progenitor cells. *Stem cells translational medicine*, *1*(12), 855-865. https://doi.org/10.5966/sctm.2012-0093

Giaever, I., & Keese, C. R. (1991). Micromotion of mammalian cells measured electrically. *Proceedings of the National Academy of Sciences*, *88*(17), 7896-7900. https://doi.org/10.1073/pnas.88.17.789

Giblin, J. P., Hewlett, L. J., & Hannah, M. J. (2008). Basal secretion of von Willebrand factor from human endothelial cells. *Blood*, *112*(4), 957-964. https://doi.org/10.1182/blood-2007-12-130740

Gilbert, W. (1978). Why genes in pieces? *Nature*, *271*(5645), 501.

Gill, J. C., Castaman, G., Windyga, J., Kouides, P., Ragni, M., Leebeek, F. W., Obermann-Slupetzky, O., Chapman, M., Fritsch, S., Pavlova, B. G., Presch, I., & Ewenstein, B. (2015). Hemostatic efficacy, safety, and pharmacokinetics of a recombinant von Willebrand factor in severe von Willebrand disease. *Blood*, *126*(17), 2038-2046. https://doi.org/10.1182/blood-2015-02-629873

Gill, J. C., Endres-Brooks, J., Bauer, P. J., Marks, W. J., & Montgomery, R. R. (1987). The effect of ABO blood group on the diagnosis of von Willebrand disease. *Blood*, *69*(6), 1691-1695. https://doi.org/10.1182/blood.V69.6.1691.1691

Ginsburg, D. (2005). Identifying novel genetic determinants of hemostatic balance. *Journal of Thrombosis and Haemostasis*, *3*, 1561–1568. https://doi.org/10.1111/j.1538-7836.2005.01461.x

Ginsburg, D., Handin, R. I., Bonthron, D. T., Donlon, A. T., Bruns, G. A., Latt, S. A., & Orkin, S. H. (1985). Human von Willebrand factor (vWF): isolation of complementary DNA (cDNA) clones and chromosomal localization. *Science*, *228*(4706), 1401-1406. https://doi.org/10.1126/science.3874428

Givan, A. L. (2011). Flow Cytometry: An Introduction. In *Flow Cytometry Protocols* (3rd ed., Vol. 699, pp. 1-30). Humana Press. https://doi.org/10.1007/978-1-61737-950-5_1

Gleadall, N. S., Veldhuisen, B., Gollub, J., Butterworth, A. S., Ord, J., Penkett, C. J., Timmer, T. C., Sauer, C. M., van der Bolt, N., Brown, C., Brugger, K., Dilthey, A. T., Duarte, D., Grimsley, S., van den Hurk, K., Jongerius, J. M., Luken, J., Megy, K., Miflin, G., … Lane, W. J. (2020). Development and validation of a universal blood donor genotyping platform: a multinational prospective study. *Blood advances*, *4*(15), 3495-3506. https://doi.org/10.1182/bloodadvances.2020001894

Gluzman, Y. (1981). SV40-transformed simian cells support the replication of early SV40 mutants. *Cell*, *23*(1), 175-182. https://doi.org/10.1016/0092-8674(81)90282-8

Godbey, W. T., Wu, K. K., & Mikos, A. G. (1999). Poly (ethylenimine) and its role in gene delivery. *Journal of controlled release*, *60*(2-3), 149-160. https://doi.org/10.1016/S0168-3659(99)00090-5

Goldman, D., & Domschke, K. (2014). Making sense of deep sequencing. *International Journal of Neuropsychopharmacology*, *17*(10), 1717-1725. https://doi.org/10.1017/S1461145714000789

Gomez, K., Laffan, M., Keeney, S., Sutherland, M., Curry, N., & Lunt, P. (2019). Recommendations for the clinical interpretation of genetic variants and presentation of results to patients with inherited bleeding disorders. A UK Haemophilia Centre Doctors' Organisation Good Practice Paper. *Haemophilia*, *25*(1), 116-126. https://doi.org/10.1111/hae.13637

Goodeve, A., Eikenboom, J., Castaman, G., Rodeghiero, F., Federici, A. B., Batlle, J., Meyer, D., Mazurier, C., Goudemand, J., Schneppenheim, R., Budde, U., Ingerslev, J., Habart, D., Vorlova, Z., Holmberg, L., Lethagen, S., Pasi, J., Hill, F., Soteh, M. H., … Peake, I. (2007). Phenotype and genotype of a cohort of families historically diagnosed with type 1 von Willebrand disease in the European study,

Molecular and Clinical Markers for the Diagnosis and Management of Type 1 von Willebrand Disease (MCMDM-1VWD). *Blood*, *109*(1), 112-121. https://doi.org/10.1182/blood-2006-05-020784

Goodeve, A. C. (2010). The genetic basis of von Willebrand disease. *Blood reviews*, *24*(3), 123-134. https://doi.org/10.1016/j.blre.2010.03.003

Goodeve, A. C., Perry, D. J., Cumming, T., Hill, M., Jennings, I., Kitchen, S., Walker, I., Gray, E., Jayandharan, G. R., & Tuddenham, E. (2012). Genetics of haemostasis. *Haemophilia*, *18*(73-80). https://doi.org/ 10.1111/j.1365-2516.2012.02832.x

Goodnough, L. T., Levy, J. H., & Murphy, M. F. (2013). Concepts of blood transfusion in adults. *The Lancet*, *381*(9880), 1845-1854. https://doi.org/10.1016/S0140-6736(13)60650-9

Goshua, G., Pine, A. B., Meizlish, M. L., Chang, C. H., Zhang, H., Bahel, P., Baluha, A., Bar, N., Bona, R. D., Burns, A. J., Dela Cruz, C. S., Dumont, A., Halene, S., Hwa, J., Koff, J., Menninger, H., Neparidze, N., Price, C., Siner, J. M., … Lee, A. I. (2020). Endotheliopathy in COVID-19-associated coagulopathy: evidence from a single-centre, cross-sectional study. *The Lancet Haematology*, *7*(8), e575-e582. https://doi.org/10.1016/S2352-3026(20)30216-7

Gralnick, H. R., & Coller, B. S. (1975). Studies of the human factor VIII/von Willebrand's factor protein. II. Identification and characterization of the von Willebrand protein. *Blood*, *46*(3), 417-430. https://doi.org/10.1182/blood.V46.3.417.417

Gralnick, H. R., Williams, S. B., McKeown, L. P., Magruder, L., Hansmann, K., Vail, M., & Parker, R. I. (1991). Platelet von Willebrand factor. *Mayo Clinic Proceedings*, *66*(6), 634-640.

Grambsch, P. M., & Therneau, T. M. (2000). *Modeling Survival Data: Extending the Cox Model*. Springer.

Grandy, R., Tomaz, R. A., & Vallier, L. (2019). Modeling Disease with Human Inducible Pluripotent Stem Cells. *Annual Review of Pathology: Mechanisms of Disease*, *14*, 449-468. https://doi.org/10.1146/annurev-pathol-020117-043634

Green, L., Bolton-Maggs, P., Beattie, C., Cardigan, R., Kallis, Y., Stanworth, S. J., Thachil, J., & Zahra, S. (2018). British Society of Haematology Guidelines on the spectrum of fresh frozen plasma and cryoprecipitate products: their handling and use in various patient groups in the absence of major bleeding. *Br J Haematol*, *181*(1), 54-67. https://doi.org/10.1111/bjh.15167

Green, M. R., & Sambrook, J. (2018). The Hanahan method for preparation and transformation of competent Escherichia coli: high-efficiency transformation. *Cold Spring Harbor Protocols*, *2018*(3), 183-190. https://doi.org/10.1101/pdb.prot101188

Grimm, D. G., Azencott, C. A., Aicheler, F., Gieraths, U., MacArthur, D. G., Samocha, K. E., Cooper, D. N., Stenson, P. D., Daly, M. J., Smoller, J. W., Duncan, L. E., & Borgwardt, K. M. (2015). The evaluation of tools used to predict the impact of missense variants is hindered by two types of circularity. *Human mutation*, *36*(5), 513-523. https://doi.org/10.1002/humu.2276

Gudmundsson, S., Singer-Berk, M., Watts, N. A., Phu, W., Goodrich, J. K., Solomonson, M., Genome Aggregation Database Consortium, Rehm, H. L., MacArthur, D. G., & O'Donnell−Luria, A. (2022). Variant interpretation using population databases: lessons from gnomAD. *Human mutation*, 1–19. https://doi.org/10.1002/humu.24309

Gupta, V., Zhang, Q. J., & Liu, Y. Y. (2011). Evaluation of anticancer agents using flow cytometry analysis of cancer stem cells. In *Drug design and discovery* (Vol. 716, pp. 179-191). Humana Press. https://doi.org/10.1007/978-1-61779-012-6_11

Haberichter, S. L., Balistreri, M., Christopherson, P., Morateck, P., Gavazova, S., Bellissimo, D. B., Manco-Johnson, M. J., Cox Gill, J., & Montgomery, R. R. (2006). Assay of the von Willebrand factor (VWF) propeptide to identify patients with type 1 von Willebrand disease with decreased VWF survival. *Blood*, *108*(10), 3344-3351. https://doi.org/10.1182/blood-2006-04-015065

Haberichter, S. L., Shi, Q., & Montgomery, R. R. (2006). Regulated release of VWF and FVIII and the biologic implications. *Pediatric blood & cancer*, *46*(5), 547-553.

Hagiwara, T., Inaba, H., Yoshida, S., Nagaizumi, K., Arai, M., Hanabusa, H., & Fukutake, F. (1996). A novel mutation Glyl672→ Arg in type 2A and a homozygous mutation in type 2B von Willebrand disease. *Thrombosis and haemostasis*, *76*(08), 253-257. https://doi.org/10.1055/s-0038-1650564

Halaidych, V., Freund, C., van den Hill, F., Salvatori, D. C., Riminucci, M., Mummery, C. L., & Orlova, V. V. (2018). Inflammatory responses and barrier function of endothelial cells derived from human induced pluripotent stem cells. *Stem Cell Reports*, *10*(5), 1642-1656. https://doi.org/10.1016/j.stemcr.2018.03.012

Hamilton, A., Ozelo, M., Leggo, J., Notley, C., Brown, H., Frontroth, J. P., Angelillo-Scherrer, A., Baghaei, F., Enayat, S. M., Favaloro, E., Lillicrap, D., & Othman, M. (2011). Frequency of Platelet type

versus Type 2B von Willebrand Disease An international registry-based study. *Thrombosis and haemostasis*, *105*(03), 501-508. https://doi.org/10.1160/TH10-08-0523

Hampshire, D. J., Abuzenadah, A. M., Cartwright, A., Al-Shammari, N. S., Coyle, R. E., Eckert, M., Al-Buhairan, A. M., Messenger, S. L., Budde, U., Gürsel, T., Ingerslev, J., Peake, I. R., & Goodeve, A. C. (2013). Identification and characterisation of mutations associated with von Willebrand disease in a Turkish patient cohort. *Thromb Haemost*, *110*(2), 264-274. https://doi.org/10.1160/TH13-02-0135.

Hampshire, D. J., Burghel, G. J., Goudemand, J., Bouvet, L. C., Eikenboom, J. C., Schneppenheim, R., Budde, U., Peake, I. R., Goodeve, A. C., & EU-VWD and ZPMCB-VWD study groups. (2010). Polymorphic variation within the VWF gene contributes to the failure to detect mutations in patients historically diagnosed with type 1 von Willebrand disease from the MCMDM-1VWD cohort. *Haematologica*, *95*(12), 2163. https://doi.org/10.3324/haematol.2010.027177

Hampshire, D. J., & Goodeve, A. C. (2011). The international society on thrombosis and haematosis von Willebrand disease database: an update. *Seminars in thrombosis and hemostasis*, *37*(05), 470-479. https://doi.org/10.1055/s-0031-1281031

Handagama, P. J., Shuman, M. A., & Bainton, D. F. (1989). Incorporation of Intravenously Injected Albumin, Immunoglobulin G, and Fibrinogen in Guinea Pig Megakaryocyte Granules. *J. Clin. Invest*, *84*, 73-82.

Hanebutt, F. L., Rolf, N., Loesel, A., Kuhlisch, E., Siegert, G., & Knoefler, R. (2008). Evaluation of desmopressin effects on haemostasis in children with congenital bleeding disorders. *Haemophilia*, *14*(3), 524-530. https://doi.org/10.1111/j.1365-2516.2008.01672.x

Hansen, M. L., Sørensen, R., Clausen, M., Fog-Petersen, M. L., Raunsø, J., Gadsbøll, N., Gislason, G. H., Folke, F., Andersen, S. S., Schramm, T. K., Abildstrøm, S. Z., Poulsen, H. E., Køber, L., & Torp-Pedersen, C. (2010). Risk of bleeding with single, dual, or triple therapy with warfarin, aspirin, and clopidogrel in patients with atrial fibrillation. *Archives of internal medicine*, *170*(16), 1433-1441. https://doi.org/10.1001/archinternmed.2010.271

Harding, A., Cortez-Toledo, E., Magner, N. L., Beegle, J. R., Coleal-Bergum, D. P., Hao, D., Wang, A., Nolta, J. A., & Zhou, P. (2017). Highly efficient differentiation of endothelial cells from pluripotent

stem cells requires the MAPK and the PI3K pathways. *Stem Cells*, *35*(4), 909-919. http://dx.doi.org/10.1002/stem.2577

Harpak, A., Bhaskar, A., & Pritchard, J. K. (2016). Mutation rate variation is a primary determinant of the distribution of allele frequencies in humans. *PLoS genetics*, *12*(12), e1006489. https://doi.org/10.1371/journal.pgen.1006489

Harrington, P., Kyle, P., Cutler, J., & Madan, B. (2018). Successful management of multiple pregnancies in a family with varying severity of Von Willebrand disease. *Obstetric Medicine*, *11*(4), 192-194. https://doi.org/10.1177/1753495X17754150

Harrison, P., Wilbourn, B., Debili, N., Vainchecker, W., Breton-Gorius, J., Lawrie, A. S., Masse, J.-M., Savidge, G. F., & Cramer, E. M. (1989). Uptake of Plasma Fibrinogen into the Alpha Granules of Human Megakaryocytes and Platelets. *J. Clin. Invest.*, *84*, 1320-1324.

Harshfield, E. L., Sims, M. C., Traylor, M., Ouwehand, W. H., & Markus, H. S. (2020). The role of haematological traits in risk of ischaemic stroke and its subtypes. *Brain*, *143*(1), 210-221. https://doi.org/10.1093/brain/awaa007

Hashemi, S., Tackaberry, E. S., Palmer, D. S., Rock, G., & Ganz, P. R. (1990). DDAVP-induced release of von Willebrand factor from endothelial cells in vitro: the effect of plasma and blood cells. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1052*(1), 63-70.

Hassenpflug, W. A., Budde, U., Obser, T., Angerhaus, D., Drewke, E., Schneppenheim, S., & Schneppenheim, R. (2006). Impact of mutations in the von Willebrand factor A2 domain on ADAMTS13-dependent proteolysis. *Blood*, *107*(6), 2339-2345. https://doi.org/10.1182/blood.V83.6.1551.1551

Haverstick, D. M., Cowan, J. F., Yamada, K. M., & Santoro, S. A. (1985). Inhibition of platelet adhesion to fibronectin, fibrinogen, and von Willebrand factor substrates by a synthetic tetrapeptide derived from the cell-binding domain of fibronectin. *Blood*, *66*(4), 946-952. https://doi.org/10.1182/blood.V66.4.946.946

Haworth, S., Mitchell, R., Corbin, L., Wade, K. H., Dudding, T., Budu-Aggrey, A., Carslake, D., Hemani, G., Paternoster, L., Davey Smith, G., Davies, N., Lawson, D. J., & Timpson, N. J. (2019). Apparent latent structure within the UK Biobank sample has implications for epidemiological analysis. *Nature communications*, *10*(1), 1-9. https://doi.org/10.1038/s41467-018-08219-1

Hayward, C. P. (2005). Diagnosis and Management of Mild Bleeding Disorders. *Hematology Am Soc Hematol Educ Program*, *1*(1), 423-428. https://doi.org/10.1182/asheducation-2005.1.423

Hearn, V. M., Smith, Z. G., & Watkins, W. M. (1968). An α-N-acetyl-D-galactosaminyltransferase associated with the human blood-group A character. *Biochemical Journal*, *109*(2), 315-317. https://doi.org/10.1042/bj1090315

Heather, J. M., & Chain, B. (2016). The sequence of sequencers: The history of sequencing DNA. *Genomics*, *107*, 1-8. http://dx.doi.org/10.1016/j.ygeno.2015.11.003

Heijnen, H., & Van Der Sluijs, P. (2015). Platelet secretory behaviour: as diverse as the granules . . . or not? *J Thromb Haemost*, *13*, 2141-2151. https://doi.org/10.1111/jth.13147

Herbert, D. N., Foellmer, B., & Helenius, A. (1995). Glucose trimming and reglucosylation determine glycoprotein association with calnexin in the endoplasmic reticulum. *Cell*, *81*(3), 425-433. https://doi.org/10.1016/0092-8674(95)90395-X

Herrada, J., Adcock, D. M., Casilias, A., Jobe, J. T., Noble, L. S., & Landeros, M. M. (2008). Combined Coagulation Factor XI and Factor XII Deficiency in An Asymptomatic Female. *Blood*, *112*(11), 4096. http://doi.org/10.1182/blood.V112.11.4096.4096

Herzenberg, L. A., Tung, J., Moore, W. A., Herzenberg, L. A., & Parks, D. R. (2006). Interpreting flow cytometry data: a guide for the perplexed. *Nature immunology*, *7*(7), 681-685. https://doi.org/10.1038/ni0706-681

Hettkamp, H., Legler, G., & Bause, E. (1984). Purification by affinity chromatography of glucosidase I, an endoplasmic reticulum hydrolase involved in the processing of asparagine−linked oligosaccharides. *European journal of biochemistry*, *142*(1), 85-90. https://doi.org/10.1111/j.1432-1033.1984.tb08253.x

HGVS. (2020, May 01). *Sequence Variant Nomenclature*. Retrieved May 05, 2021, from http://varnomen.hgvs.org/

Hickson, N., Hampshire, D., Winship, P., Goudeman, J., Schneppenheim, R., Budde, U., Castaman, G., Rodeghiero, F., Federici, A. B., James, P., Peake, I., Eikenboom, J., Goodeve, A., & MCMDM-1VWD and ZPMCB-VWD STUDY GROUPS. (2010). von Willebrand factor variant p. Arg924Gln marks an allele associated with reduced von Willebrand factor and factor VIII levels.

Journal of Thrombosis and Haemostasis, *8*(9), 1986-1993. https://doi.org/10.1111/j.1538-7836.2010.03927.x

Hielscher, A., Ellis, K., Qiu, C., Porterfield, J., & Gerecht, S. (2016). Fibronectin deposition participates in extracellular matrix assembly and vascular morphogenesis. *PloS one*, *11*(1), e0147600. https://doi.org/10.1371/journal.pone.0147600

Higham, J. M., O'Brien, P. M., & Shaw, R. (1990). Assessment of menstrual blood loss using a pictorial chart. *BJOG: An International Journal of Obstetrics & Gynaecology*, *97*(8), 734-739. https://doi.org/10.1111/j.1471-0528.1990.tb16249.x

Hilbert, L., Fressinaud, E., Ribba, A. S., Meyer, D., Mazurier, C., & INSERM Network on Molecular Abnormalities in von Willebrand Disease. (2002). Identification of a new type 2M von Willebrand disease mutation also at position 1324 of von Willebrand factor. *Thrombosis and haemostasis*, *87*(04), 635-640. https://doi.org/10.1055/s-0037-1613060

HipSci. (2014). *HipSci catalogue*. Human Induced Pluripotent Stem Cell Initiative. Retrieved June 07, 2022, from https://www.hipsci.org/lines/#/lines/HPSI1113i-qolg_3

Hirashima, M. (2009). Regulation of endothelial cell differentiation and arterial specification by VEGF and Notch signaling. *Anatomical science international*, *84*(3), 95-101. https://doi.org/10.1007/s12565-009-0026-1

Hiratsuka, S., Kataoka, Y., Nakao, K., Nakamura, K., Morikawa, S., Tanaka, S., Katsuki, M., Maru, Y., & Shibuya, M. (2005). Vascular endothelial growth factor A (VEGF-A) is involved in guidance of VEGF receptor-positive cells to the anterior portion of early embryos. *Molecular and cellular biology*, *25*(1), 355-363. https://doi.org/10.1128/MCB.25.1.355-363.2005

Ho, D. E., Imai, K., King, G., & Stuart, E. A. (2007). Matching as Nonparametric Preprocessing for Reducing Model Dependence in Parametric Causal Inference. *Political Analysis*, *15*(3), 199-236. https://doi.org/10.1093/pan/mpl013

Ho, D. E., Imai, K., King, G., & Stuart, E. A. (2011). MatchIt: Nonparametric Preprocessing for Parametric Causal Inference. *Journal of Statistical Software*, *42*(8). https://doi.org/10.18637/jss.v042.i08

Hodgkins, A., Farne, A., Perera, S., Grego, T., Parry-Smith, D. J., Skarnes, W. C., & Iyer, V. (2015). WGE: a CRISPR database for genome engineering. *Bioinformatics*, *31*(18), 3078-3080. https://doi.org/10.1093/bioinformatics/btv308

Holborow, E. J., Brown, P. C., Glynn, L. E., Hawes, M. D., Gresham, G. A., O'Brien, T. F., & Coombs, R. R. (1960). The distribution of the blood group A antigen in human tissues. *British journal of experimental pathology*, *41*(4), 430.

Hollander, M., & Wolfe, D. A. (1973). *Nonparametric Statistical Methods*. New York: John Wiley & Sons.

Holm, E., Abshire, T. C., Bowen, J., Álvarez, M. T., Bolton-Maggs, P., Carcao, M., Federici, A. B., Cox Gill, J., Halimeh, S., Kempton, C., Key, N. S., Kouides, P., Lail, A., Landorph, A., Leebeek, F., Makris, M., Mannucci, P. M., Mauser-Bunschoten, E. P., Nugent, D., … Berntorp, E. (2015). Changes in bleeding patterns in von Willebrand disease after institution of long-term replacement therapy: results from the von Willebrand Disease Prophylaxis Network. *Blood coagulation & fibrinolysis*, *26*(4), 383-388. https://doi.org/10.1097/MBC.0000000000000257

Holmberg, L., & Nilsson, I. M. (1972). Genetic variants of von Willebrand's disease. *Br Med J*, *3*(5822), 317-320. https://doi.org/10.1136/bmj.3.5822.317

Holmberg, L., Nilsson, I. M., Borge, L., Gunnarsson, M., & Sjörin, E. (1983). Platelet aggregation induced by I-Desamino-8-D-arginine vasopressin (DDAVP) in type IIB von Willebrand's disease. *New England Journal of Medicine*, *309*(14), 816-821. https://doi.org/10.1056/NEJM198310063091402

Holmes, K., Lantz, L. M., Fowlkes, B. J., Schmid, I., & Giorgi, J. V. (2001). Preparation of cells and reagents for flow cytometry. *Current protocols in immunology*, *44*(1), 5.3.1-5.3.24. https://doi.org/10.1002/0471142735.im0503s44

Hong, W. (2020). *Sequence Variant Nomenclature*. Sequence Variant Nomenclature. Retrieved February 22, 2022, from http://varnomen.hgvs.org/bg-material/consultation/svd-wg009/

Hormia, M., Lehto, V. P., & Virtanen, I. (1984). Intracellular localization of factor VIII-related antigen and fibronectin in cultured human endothelial cells: evidence for divergent routes of intracellular translocation. *European journal of cell biology*, *33*(2), 217-228.

Horvath, B., Hegedus, D., Szapary, L., Marton, Z., Alexy, T., Koltai, K., Czopf, L., Wittmann, I., Juricskay, I., Toth, K., & Kesmarky, G. (2004). Measurement of von Willebrand factor as the marker of endothelial dysfunction in vascular diseases. *Experimental & Clinical Cardiology*, *9*(1), 31-34.

Horvath, P., & Barrangou, R. (2010). CRISPR/Cas, the immune system of bacteria and archaea. *Science*, *327*(5962), 167-170. https://doi.org/10.1126/science.1179555

Houdijk, W. P., Sakariassen, K. S., Nievelstein, P. F., & Sixma, J. J. (1985). Role of factor VIII-von Willebrand factor and fibronectin in the interaction of platelets in flowing blood with monomeric and fibrillar human collagen types I and III. *The Journal of clinical investigation*, *75*(2), 531-540. https://doi.org/10.1172/JCI111729

Howard, M. A., & Firkin, B. G. (1971). Ristocetin-a new tool in the investigation of platelet aggregation. *Thrombosis and Haemostasis*, *26*(05), 362-369. https://doi.org/10.1055/s-0038-1653684

Howard, M. A., Montgomery, D. C., & Hardisty, R. M. (1974). Factor-VIII-related antigen in platelets. *Thrombosis Research*, *4*(5), 617-624. https://doi.org/10.1016/0049-3848(74)90218-7

Howard, M. A., Sawers, R. J., & Firkin, B. G. (1973). Ristocetin: a means of differentiating von Willebrand's disease into two groups. *Blood*, *41*(5), 687-690. https://doi.org/10.1182/blood.V41.5.687.687

Howell, G. J., Herbert, S. P., Smith, J. M., Mittar, S., Ewan, L. C., Mohammed, M., Hunter, A. R., Simpson, N., Turner, A. J., Zachary, I., Walker, J. H., & Ponnambalam, S. (2004). Endothelial cell confluence regulates Weibel-Palade body formation. *Molecular membrane biology*, *21*(6), 413-421. https://doi.org/10.1080u09687860400011571

Hoylaerts, M., Lijnen, H. R., & Collen, D. (1981). Studies on the mechanism of the antifibrinolytic action of tranexamic acid. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *673*, 75-85. https://doi.org/10.1016/0304-4165(81)90312-3

Hoylaerts, M. F. (1997). Platelet-vessel wall interactions in thrombosis and restenosis role of von Willebrand factor. *Verhandelingen-Koninklijke Academie voor Geneeskunde van Belgie*, *59*(3), 161-183.

Huang, J., Roth, R., Heuser, J. E., & Sadler, J. E. (2009). Integrin αvβ3 on human endothelial cells binds von Willebrand factor strings under fluid shear stress. *Blood*, *113*(7), 1589-1597. https://doi.org/10.1182/blood-2008-05-158584

Hubbard, A. R., Hamill, M., Beeharry, M., Bevan, S. A., & Heath, A. B. (2011). on behalf of the SSC Sub-Committees on Factor VIII/Factor IX and von Willebrand factor of ISTH. *Value assignment of the WHO 6th International Standard for blood coagulation factor VIII and von Willebrand factor in plasma (07/316)*, *J Thromb Haemost*(9), 2100-2102. https://doi.org/10.1111/j.1538-7836.2011.04471.x

Hubbard, A. R., & Heath, A. B. (2004). Standardization of factor VIII and von Willebrand factor in plasma: calibration of the WHO 5th International Standard (02/150). *J Thromb Haemost*, *2*, 1380-1384. https://doi.org/10.1111/j.1538-7836.2004.00838.x

Hubbard, S. C., & Robbins, P. W. (1979). Synthesis and processing of protein-linked oligosaccharides in vivo. *The Journal of Biological Chemistry*, *254*(11), 4568-4576.

Huerta-Franco, M. R., Banderas, J. W., & Allsworth, J. E. (2018). Ethnic/racial differences in gastrointestinal symptoms and diagnosis associated with the risk of Helicobacter pylori infection in the US. *Clinical and experimental gastroenterology*, *11*, 39-49. https://doi.org/10.2147/CEG.S144967

Huffman, J. E., De Vries, P. S., Morrison, A. C., Sabater-Lleal, M., Kacprowski, T., Auer, P. L., Brody, J. A., Chasman, D. I., Chen, M.-H., Guo, X., Lin, L.-A., Marioni, R. E., Muller-Nurasyid, M., Yanek, L. R., Pankratz, N., Grove, M. L., de Maat, M. P., Cushman, M., Wiggins, K. L., … Smith, N. L. (2015). Rare and low-frequency variants and their association with plasma levels of fibrinogen, FVII, FVIII, and vWF. *Blood*, *126*(11), e19-e29. https://doi.org/10.1182/blood-2015-02-624551

Hussain, S., Baloch, S., Parvin, A., Najmuddin, A., Musheer, F., Junaid, M., Memon, R. N., Bhanbhro, F., Ullah, H., & Moiz, B. (2018). Inherited Bleeding Disorders—Experience of a Not-for-Profit Organization in Pakistan. *Clinical and Applied Thrombosis/Hemostasis*, *24*(8), 1241-1248. https://doi.org/10.1177/1076029618781033

Iannuzzi, M. C., Nidaka, N., Boehnke, M., Bruck, M. E., Hanna, T. W., Collins, F. S., & Ginsburg, D. (1991). Analysis of the relationship of von Willebrand disease (vWD) and hereditary hemorrhagic telangiectasia and identification of a potential type IIA vWD mutation (IIe865 to Thr). *American journal of human genetics*, *48*(4), 757.

Illumina. (n.d.). *VCF File Annotations*. Illumina. Retrieved April 24, 2022, from https://support.illumina.com/help/BS_App_TS_Amplicon_OLH_15055858/Content/Source/Informatics/VCF-Annotations.htm

Ingram, D. A., Mead, L. E., Tanaka, H., Meade, V., Fenoglio, A., Mortell, K., Pollok, K., Ferkowicz, M. J., Gilley, D., & Yoder, M. C. (2004). Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood*, *104*(9), 2752-2760. https://doi.org/10.1182/blood-2004-04-1396

Ingram, G. I. (1976). The history of haemophilia. *J Clin Pathol*, *29*(6), 469–479. https://doi.org/10.1136/jcp.29.6.469

Interlandi, G., Ling, M., Tu, A. Y., Chung, D. W., & Thomas, W. E. (2012). Structural Basis of Type 2A von Willebrand Disease Investigated by Molecular Dynamics Simulations and Experiments. *PLoS ONE*, *7*(10), e45207. https://doi.org/10.1371/journal.pone.0045207

International HapMap Consortium. (2005). A haplotype map of the human genome. *Nature*, *437*(7063), 1299. https://doi.org/10.1038/nature04226

International Human Genome Sequencing Consortium. (2004). Finishing the euchromatic sequence of the human genome. *Nature*, *431*(7011), 931-945. https://doi.org/10.1038/nature03001

Iorio, A., Oliovecchio, E., Morfini, M., Mannucci, P. M., & Association of Italian Hemophilia Centres Directors. (2008). Italian Registry of Haemophilia and Allied Disorders. Objectives, methodology and data analysis. *Haemophilia*, *14*(3), 444-453. https://doi.org/10.1111/j.1365-2516.2008.01679.x

Ishihara, J., Ishihara, A., Starke, R. D., Peghaire, C. R., Smith, K. E., McKinnon, T. A., Smith, K. E., McKinnon, T. A., Tabata, Y., Sasaki, K., White, M. J., Fukunaga, K., Laffan, M. A., Lutolf, M. P., Randi, A. M., & Hubbell, J. A. (2019). The heparin binding domain of von Willebrand factor binds to growth factors and promotes angiogenesis in wound healing. *Blood*, *133*(24), 2559-2569. https://doi.org/10.1182/blood.2019000510

Itan, Y., Shang, L., Boisson, B., Boisson, B., Ciancanelli, M. J., Markle, J. G., Martinez-Barricarte, R., Scott, E., Shah, I., Stenson, P. D., Gleeson, J., Cooper, D. N., Quintana-Murci, L., Zhang, S.-Y., Abel, L., & Casanova, J.-L. (2016). The mutation significance cutoff: gene-level thresholds for variant predictions. *Nature methods*, *13*(2), 109-110. https://doi.org/10.1038/nmeth.3739

Ivy, A. C., Nelson, D., & Bucher, G. (1941). The standardization of certain factors in the cutaneous "venostasis" bleeding time technique. *The Journal of laboratory and clinical medicine*, *26*(11), 1812-1822. https://doi.org/10.5555/uri:pii:S0022214341901282

Jacobi, P. M., Gill, J. C., Flood, V. H., Jakab, D. A., Friedman, K. D., & Haberichter, S. L. (2012). Intersection of mechanisms of type 2A VWD through defects in VWF multimerization, secretion, ADAMTS-13 susceptibility, and regulated storage. *Blood*, *119*(19), 4543-4553. https://doi.org/10.1182/blood-2011-06-360875

Jaffe, E. A., Hoyer, L. W., & Nachman, R. L. (1973). Synthesis of antihemophilic factor antigen by cultured human endothelial cells. *The Journal of clinical investigation*, *52*(11), 2757-2764. https://doi.org/10.1172/JCI107471

James, P. D., Connell, N. T., Ameer, B., Di Paola, J., Eikenboom, J., Giraud, N., Haberichter, S., Jacobs-Pratt, V., Konkle, B., McLintock, C., McRae, S., Montgomery, R., O'Donnell, J. S., Scappe, N., Sidonio Jr, R., Flood, V. H., Husainat, N., Kalot, M. A., & Mustafa, R. A. (2021). ASH ISTH NHF WFH 2021 guidelines on the diagnosis of von Willebrand disease. *Blood advances*, *5*(1), 280-300. https://doi.org/10.1182/bloodadvances.2020003265

James, P. D., & Goodeve, A. C. (2011). von Willebrand disease. *Genetics in Medicine*, *13*(5), 365-376. https://doi.org/10.1097/GIM.0b013e3182035931

James, P. D., Lillicrap, D., & Mannucci, P. M. (2013). Alloantibodies in von Willebrand disease. *Blood*, *122*(5), 636-640. https://doi.org/10.1182/blood-2012-10-462085

James, P. D., & Lillicrap, D. P. (2011). The Diagnosis and Management of von Willebrand Disease in Canada. *Semin Thromb Hemost*, *37*(5), 522-527. https://doi.org/10.1055/s-0031-1281038

James, P. D., Notley, C., Hegadorn, C., Leggo, J., Tuttle, A., Tinlin, S., Brown, C., Andrews, C., Labelle, A., Chirinian, Y., O'Brien, L., Othman, M., Rivard, G., Rapson, D., Hough, C., Lillicrap, D., & Association of Hemophilia Clinic Directors of Canada. (2007). The mutational spectrum of type 1 von Willebrand disease: results from a Canadian cohort study. *Blood*, *109*(1), 145-154. https://doi.org/10.1182/blood-2006-05-021105

Ji, J., Ng, S. H., Sharma, V., Neculai, D., Hussein, S., Sam, M., Trinh, Q., Church, G. M., Mcpherson, J. D., Nagy, A., & Batada, N. N. (2012). Elevated coding mutation rate during the reprogramming of human somatic cells into induced pluripotent stem cells. *Stem cells*, *30*(3), 435-440. https://doi.org/10.1002/stem.1011

Johnsen, J. M., Auer, P. L., Morrison, A. C., Jiao, S., Wei, P., Haessler, J., Fox, K., McGee, S. R., Smith, J. D., Carlson, C. S., Smith, N., Boerwinkle, E., Kooperberg, C., Nickerson, D. A., Rich, S. S., Green, D., Peters, U., Cushman, M., Reiner, A. P., & on behalf of the NHLBI Exome Sequencing Project. (2013). Common and rare von Willebrand factor (VWF) coding variants, VWF levels, and factor VIII levels in African Americans: the NHLBI Exome Sequencing Project. *122*, *4*, 590-597. https://doi.org/10.1182/blood-2013-02-485094

Jokela, V., Lassila, R., Szanto, T., Joutsi−Korhonen, L., Armstrong, E., Oyen, F., Schneppenheim, S., & Schneppenheim, R. (2013). Phenotypic and genotypic characterization of 10 Finnish patients with von Willebrand disease type 3: discovery of two main mutations. *Haemophilia*, *19*(6), e344-e348. https://doi.org/10.1111/hae.12225

Jones, C., Sattler, S., & Gekle, R. (2021). Traumatic splenic laceration with delayed rupture secondary to coughing in a patient with Von Willebrand disease. *American Journal of Emergency Medicine*, *50*, 812.e5-812.e7. https://doi.org/10.1016/j.ajem.2021.05.064

Just, S. (2017). Laboratory testing for von Willebrand disease: the past, present, and future state of play for von Willebrand factor assays that measure platelet binding activity, with or without ristocetin. *Seminars in thrombosis and hemostasis*, *43*(01), 75-91. https://doi.org/10.1055/s-0036-1592164

Kafkas, Ş., Althubaiti, S., Gkoutos, G. V., Hoehndorf, R., & Schofield, P. N. (2021). Linking common human diseases to their phenotypes; development of a resource for human phenomics. *Journal of biomedical semantics*, *12*(1), 1-15. https://doi.org/10.1186/s13326-021-00249-x

Kalot, M. A., Al-Khatib, M., Connell, N. T., Flood, V., Brignardello−Petersen, R., James, P., Mustafa, R. A., & VWD working group. (2020). An international survey to inform priorities for new guidelines on von Willebrand disease. *Haemophilia*, *26*(1), 106-116. https://doi.org/10.1111/hae.13881

Kanaji, S., Fahs, S. A., Shi, Q., Haberichter, S. L., & Montgomery, R. R. (2012). Contribution of platelet versus endothelial VWF to platelet adhesion and hemostasis. *J Thromb Haemost*, *10*(8), 1646–1652.

Kannicht, C., Fisseau, C., Hofmann, W., Kröning, M., & Fuchs, B. (2015). ADAMTS13 content and VWF multimer and triplet structure in commercially available VWF/FVIII concentrates. *Biologicals*, *43*(2), 117-122. https://doi.org/10.1016/j.biologicals.2014.11.006

Karczewski, K. J., Francioli, L. C., Tiao, G., Cummings, B. B., Alföldi, J., Wang, Q., Collins, R. L., Laricchia, K. M., Ganna, A., Birnbaum, D. P., Gauthier, L. D., Brand, H., Solomonson, M., Watts, N. A., Rhodes, D., Singer-Berk, M., England, E. M., Seaby, E. G., Kosmicki, J. A., … MacArthur, D. G. (2020). The mutational constraint spectrum quantified from variation in 141,456 humans. *Nature*, *581*, 434–443. https://doi.org/10.1038/s41586-020-2308-7

Karki, R., Pandya, D., Elston, R. C., & Ferlini, C. (2015). Defining “mutation” and “polymorphism” in the era of personal genomics. *BMC medical genomics*, *8*(1), 1-7. https://doi.org/10.1186/s12920-015-0115-z

Karkkainen, M. J., Mäkinen, T., & Alitalo, K. (2002). Lymphatic endothelium: a new frontier of metastasis research. *Nature cell biology*, *4*(1), E2-E5. https://doi.org/10.1038/ncb0102-e2

Kasatkar, P., Shetty, S., & Ghosh, K. (2014). Genetic Heterogeneity in a Large Cohort of Indian Type 3 von Willebrand Disease Patients. *PLoS One*, *9*(3), e92575. https://doi.org/10.1371/journal.pone.0092575

Katsumi, A., Tuley, E. A., Bodó, I., & Sadler, J. E. (2000). Localization of disulfide bonds in the cystine knot domain of human von Willebrand factor. *Journal of Biological Chemistry*, *275*(33), 25585-25594. https://doi.org/10.1074/jbc.M002654200

Kaufmann, J. E., Oksche, A., Wollheim, C. B., Günther, G., Rosenthal, W., & Vischer, U. M. (2000). Vasopressin-induced von Willebrand factor secretion from endothelial cells involves V2 receptors and cAMP. *The Journal of clinical investigation*, *106*(1), 107-116. https://doi.org/10.1172/JCI9516

Kaufmann, J. E., & Vischer, U. M. (2003). Cellular mechanisms of the hemostatic effects of desmopressin (DDAVP). *Journal of Thrombosis and Haemostasis*, *1*, 682-689. https://doi.org/10.1046/j.1538-7836.2003.00190.x

Keeling, D., Beavis, J., Marr, R., Sukhu, K., & Bignell, P. (2011). A family with type 2M VWD with normal VWF: RCo but reduced VWF: CB and a M1761K mutation in the A3 domain. *Haemophilia*, *18*(1), e33. https://doi.org/10.1111/j.1365-2516.2011.02676.x

KEGG. (2020, October 23). *Monosaccharide Codes*. KEGG: Kyoto Encyclopedia of Genes and Genomes. Retrieved April 03, 2021, from https://www.genome.jp/kegg/catalog/codes2.html

Keipert, C., Hesse, J., Haschberger, B., Heiden, M., Seitz, R., van den Berg, H. M., Hilger, A., & the ABIRISK Consortium. (2015). The growing number of hemophilia registries: quantity vs. quality. *Clinical Pharmacology & Therapeutics,*, *97*(5), 492-501. https://doi.org/10.1002/cpt.83

Kennedy, S. D., Igarashi, Y., & Kickler, T. S. (1997). Measurement of In Vitro P-Selectin Expression by Flow Cytometry. *American journal of clinical pathology*, *107*(1), 99-104.

Kessler, M. D., Yerges-Armstrong, L., Taub, M. A., Shetty, A. C., Maloney, K., Jeng, L. J., Ruczinski, I., Levin, A. M., Williams, L. K., Beaty, T. H., Mathias, R. A., Barnes, K. C., Consortium on Asthma among African-ancestry Populations in the Americas (CAAPA), & O'Connor, T. D. (2016). Challenges and disparities in the application of personalized genomic medicine to populations with African ancestry. *Nature communications*, *7*(1), 1-8. https://doi.org/10.1038/ncomms12521

Khan, M. K., Khan, S. Q., & Malik, N. A. (2014). Spectrum of Von Willebrand's disease in Punjab: clinical features and types. *Journal of Ayub Medical College, Abbottabad: JAMC*, *26*(4), 470-473.

Kilpinen, H., Goncalves, A., Leha, A., Afzal, V., Alasoo, K., Ashford, S., Bala, S., Bensaddek, D., Casale, F. P., Culley, O. J., Danecek, P., Faulconbridge, A., Harrison, P. W., Kathuria, A., McCarthy, D., McCarthy, S., Meleckyte, R., Memari, Y., Moens, N., … Gaffney, D. J. (2017). Common genetic variation drives molecular heterogeneity in human iPSCs. *Nature*, *546*(7658), 370-375. https://doi.org/10.1038/nature22403

Kimberland, M. L., Hou, W., Aflonso-Pecchio, A., Wilson, S., Rao, Y., Zhang, S., & Lu, Q. (2018). Strategies for controlling CRISPR/Cas9 off-target effects and biological variations in mammalian genome editing experiments. *Journal of biotechnology*, *284*, 91-101. https://doi.org/10.1016/j.jbiotec.2018.08.007

Kimelman, D. (2006). Mesoderm induction: from caps to chips. *Nature Reviews Genetics*, *7*(5), 360-372. https://doi.org/10.1038/nrg1837

Kircher, M., & Kelso, J. (2010). High−throughput DNA sequencing–concepts and limitations. *Bioessays*, *32*(6), 524-536. https://doi.org/10.1002/bies.200900181

Kircher, M., Witten, D. M., O'Roak, B. J., Cooper, G. M., & Shendure, J. (2014). A general framework for estimating the relative pathogenicity of human genetic variants. *Nature genetics*, *46*(3), 310-315. https://doi.org/10.1038/ng.2892

Kiskin, F. N., Chang, C. H., Huang, C. J., Kwieder, B., Cheung, C., Dunmore, B. J., Serrano, F., Sinha, S., Morrell, N. W., & Rana, A. A. (2018). Contributions of BMPR2 mutations and extrinsic factors to cellular phenotypes of pulmonary arterial hypertension revealed by induced pluripotent stem cell modeling. *American journal of respiratory and critical care medicine*, *198*(2), 271-275. https://doi.org/10.1164/rccm.201801-0049LE

Klamroth, R., Gröner, A., & Simon, T. L. (2014). Pathogen inactivation and removal methods for plasma−derived clotting factor concentrates. *Transfusion*, *54*(5), 1406-1417. https://doi.org/10.1111/trf.12423

Klein, H. G. (2005). Pathogen inactivation technology: cleansing the blood supply. *Journal of internal medicine*, *257*(3), 224-237. https://doi.org/10.1111/j.1365-2796.2005.01451.x

Klein, R. J., Zeiss, C., Chew, E. Y., Tsai, J. Y., Sackler, R. S., Haynes, C., Henning, A. K., SanGiovanni, J. P., Mane, S. M., Mayne, S. T., Bracken, M. B., Ferris, F. L., Ott, J., Barnstable, C., & Hoh, J. (2005). Complement factor H polymorphism in age-related macular degeneration. *Science*, *308*(5720), 385-389. https://doi.org/10.1126/science.1109557

Kobata, A., Grollman, E. F., & Ginsburg, V. (1968). An enzymatic basis for blood type B in humans. *Biochemical and biophysical research communications*, *32*(2), 272-277. https://doi.org/10.1016/0006-291X(68)90380-X

Koedam, J. A., Meijers, J. C., Sixma, J. J., & Bouma, B. N. (1988). Inactivation of human factor VIII by activated protein C. Cofactor activity of protein S and protective effect of von Willebrand factor. *The Journal of clinical investigation*, *82*(4), 1236-1243. https://doi.org/10.1172/JCI113721

Komatsu, M., Waguri, S., Ueno, T., Iwata, J., Murata, S., Tanida, I., Ezaki, J., Mizushima, N., Ohsumi, Y., Uchiyama, Y., Kominami, E., Tanaka, K., & Chiba, T. (2005). Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *Journal of Cell Biology*, *169*(3), 425-434. https://doi.org/10.1083/jcb.200412022

König, G., Obser, T., Marggraf, O., Schneppenheim, S., Budde, U., Schneppenheim, R., & Brehm, M. A. (2019). Alteration in GPIIb/IIIa binding of VWD-associated von Willebrand factor variants with C-terminal missense mutations. *Thrombosis and haemostasis*, *119*(07), 1102-1111. https://doi.org/10.1055/s-0039-1687878

Korsten, P., Wallbach, M., & Binder, C. (2020). Type 2A von Willebrand disease and systemic sclerosis: Vonicog alfa reduced gastrointestinal bleeding. *Research and Practice in Thrombosis and Haemostasis*, *4*(7), 1230-1234. https://doi.org/10.1002/rth2.12426

Koscielny, J., von Tempelhoff, G.-F., Ziemer, S., Radtke, H., Schmutzler, M., Sinha, P., Salama, A., Kiesewetter, H., & Latza, R. (2004). A Practical Concept for Preoperative Management of Patients With Impaired Primary Hemostasis. *Clinical and Applied Thrombosis/Hemostasis*, *10*(2), 155-166. https://doi.org/10.1177/107602960401000206

Kovacevic, K. D., Buchtele, N., Schoergenhofer, C., Derhaschnig, U., Gelbenegger, G., Brostjan, C., Zhu, S., Gilbert, J. C., & Jilma, B. (2020). The aptamer BT200 effectively inhibits von Willebrand factor (VWF) dependent platelet function after stimulated VWF release by desmopressin or endotoxin. *Scientific reports*, *10*(1), 1-9. https://doi.org/10.1038/s41598-020-68125-9

Krawczak, M., & Cooper, D. N. (1997). The Human Gene Mutation Database. *Trends Genet*, *13*, 121-122.

Kruse-Jarres, R., & Johnsen, J. M. (2018). How I treat type 2B von Willebrand disease. *Blood*, *131*(12), 1292-1300. https://doi.org/10.1182/blood-2017-06-742692

Kubisz, P., Sokol, J., Simurda, T., Plamenova, I., Dobrotova, M., Holly, P., Skornova, I., & Stasko, J. (2018). Diagnosis and management of von Willebrand disease in Slovakia. *Annals of Blood*, *3*(9). http://dx.doi.org/10.21037/aob.2018.01.03

Kumar, K. R., Cowley, M. J., & Davis, R. L. (2019). Next-generation sequencing and emerging technologies. *Seminars in thrombosis and hemostasis*, *45*(07), 661-673. https://doi.org/10.1055/s-0039-1688446

Kumar, R., Stain, A. M., Hillard, P., & Carcao, M. (2013). Consequences of delayed therapy for sports−related bleeds in patients with mild−to−moderate haemophilia and type 3 von Willebrand's disease not on prophylaxis. *Haemophilia*, *19*(4), e264-e267. https://doi.org/10.1111/hae.12162

Kumar, S., Blangero, J., & Curran, J. E. (2018). Induced Pluripotent Stem Cells in Disease Modeling and Gene Identification. *In: DiStefano, J. (eds) Disease Gene Identification. Methods in Molecular Biology*, *1706*, 17-38. https://doi.org/10.1007/978-1-4939-7471-9_2

Kumar, S., Kishore, R., Gupta, V., Jain, M., & Shukla, J. (2010). Prevalence and spectrum of von Willebrand disease in Eastern Uttar Pradesh. *Indian Journal of Pathology and Microbiology*, *53*(3), 486-489.

Kuo, M. C., Patschan, D., Patschan, S., Cohen-Gould, L., Park, H. C., Ni, J., Addabbo, F., & Goligorsky, M. S. (2008). Ischemia-induced exocytosis of Weibel-Palade bodies mobilizes stem cells. *Journal of the American Society of Nephrology*, *19*(12), 2321-2330. https://doi.org/10.1681/ASN.2007111200

Kuo, S. C., & Lampen, J. O. (1974). Tunicamycin -- an inhibitor of yeast glycoprotein synthesis. *Biochemical and Biophysical Research Communications*, *58*(1), 287-295. https://doi.org/10.1016/0006-291X(74)90925-5

LaBrecque, D., Khan, A., Sarin, S., & Le Mair, A. W. (2014). Esophageal varices. *World Gastroenterol Organ Glob Guidel*, 1-14.

Laffan, M., Brown, S. A., Collins, P. W., Cumming, A. M., Hill, F. G., Keeling, D., Peake, I. R., & Pasi, K. J. (2004). The diagnosis of von Willebrand disease: a guideline from the UK Haemophilia Centre Doctors' Organization. *Haemophilia*, *10*(3), 199-217. https://doi.org/10.1111/j.1365-2516.2004.00894.x

Laffan, M., Sathar, J., & Johnsen, J. M. (2021). von Willebrand disease: Diagnosis and treatment, treatment of women, and genomic approach to diagnosis. *Haemophilia*, *27*, 66-74. https://doi.org/10.1111/hae.14050

Laffan, M. A., Lester, W., O'Donnell, J. S., Will, A., Tait, R. C., Goodeve, A., Millar, C. M., & Keeling, D. M. (2014). The diagnosis and management of von Willebrand disease: A United Kingdom Haemophilia Centre Doctors Organization guideline approved by the British Committee for Standards in Haematology. *Br J Haematol*, *167*(4), 453-465. https://doi.org/10.1111/bjh.13064

LaFramboise, T. (2009). Single nucleotide polymorphism arrays: a decade of biological, computational and technological advances. *Nucleic acids research*, *37*(13), 4181-4193. https://doi.org/10.1093/nar/gkp552

Lak, M., Peyvandi, F., & Mannucci, P. M. (2000). Clinical manifestations and complications of childbirth and replacement therapy in 385 Iranian patients with type 3 von Willebrand disease. *British journal of haematology*, *111*(4), 1236-1239. https://doi.org/10.1111/j.1365-2141.2000.02507.x

Lamotte, M., Annemans, L., Evers, T., & Kubin, M. (2006). A multi-country economic evaluation of low-dose aspirin in the primary prevention of cardiovascular disease. *Pharmacoeconomics*, *24*(2), 155-169. https://doi.org/10.2165/00019053-200624020-00005

Landrum, M. J., & Kattman, B. L. (2018). ClinVar at five years: Delivering on the promise. *Human mutation*, *39*(11), 1623-1630. https://doi.org/10.1002/humu.23641

Landrum, M. J., Lee, J. M., Benson, M., Brown, G. R., Chao, C., Chitipiralla, S., Gu, B., Hart, J., Hoffman, D., Jang, W., Karapetyan, K., Katz, K., Liu, C., Maddipatla, Z., Malheiro, A., McDaniel, K., Ovetsky, M., Riley, G., Zhou, G., … Maglott, D. R. (2018). ClinVar: improving access to variant interpretations and supporting evidence. *Nucleic acids research*, *46*(D1), D1062-D1067. https://doi.org/10.1093/nar/gkx1153

Landrum, M. J., Lee, J. M., Riley, G. R., Jang, W., Rubenstein, W. S., Church, D. M., & Maglott, D. R. (2014). ClinVar: public archive of relationships among sequence variation and human phenotype. *Nucleic acids research*, *42*(D1), D980-D985. https://doi.org/10.1093/nar/gkt1113

Landsteiner, K. (1901). Über Agglutinationserscheinungen normalen menschlichen Blutes. *Wien Klin Wochenschr*, *14*, 1132-1134.

Lane, W. J., Westhoff, C. M., Uy, J. M., Aguad, M., Smeland-Wagman, R., Kaufman, R. M., Rehm, H. L., Green, R. C., Silberstein, L. E., & MedSeq Project. (2016). Comprehensive red blood cell and platelet antigen prediction from whole genome sequencing: proof of principle. *Transfusion*, *56*(3), 743-754. https://doi.org/10.1111/trf.13416

Langdell, R. D., Wagner, R. H., & Brinkhous, K. M. (1953). Effect of antihemophilic factor on one-stage clotting tests: a presumptive test for hemophilia and a simple one-stage antihemophilic factor assay procedure. *The Journal of laboratory and clinical medicine*, *41*(4), 637-647. https://doi.org/10.5555/uri:pii:0022214353903126

Lanktree, M. B., Haghighi, A., Guiard, E., Iliuta, I. A., Song, X., Harris, P. C., Paterson, A. D., & Pei, Y. (2018). Prevalence estimates of polycystic kidney and liver disease by population sequencing. *Journal of the American Society of Nephrology*, *29*(10), 2593-2600. https://doi.org/10.1681/ASN.2018050493

Lapid, C., & Gao, Y. (2006, August 14). *PrimerX*. Bioinformatics.org. Retrieved May 7, 2022, from https://www.bioinformatics.org/primerx/

Larsen, R. D., Ernst, L. K., Nair, R. P., & Lowe, J. B. (1990). Molecular cloning, sequence, and expression of a human GDP-L-fucose: beta-D-galactoside 2-alpha-L-fucosyltransferase cDNA that can form the H blood group antigen. *Proceedings of the National Academy of Sciences*, *87*(17), 6674-6678.

Lavin, M., Aguila, S., Schneppenheim, S., Dalton, N., Jones, K. L., O'Sullivan, J. M., O'Connell, N. M., Ryan, K., White, B., Byrne, M., Rafferty, M., Doyle, M. M., Nolan, M., Preston, R. J., Budde, U., James, P., Di Paola, J., & O'Donnell, J. S. (2017). Novel insights into the clinical phenotype and pathophysiology underlying low VWF levels. *Blood*, *130*(21), 2344-2353. https://doi.org/10.1182/blood-2017-05-786699

Lavin, M., & O'Donnell, J. S. (2016). New treatment approaches to von Willebrand disease. *Hematology Am Soc Hematol Educ Program*, *1*, 683-689. https://doi.org/10.1182/asheducation-2016.1.683

Lawrie, A. S., Mackie, I. J., Machin, S. J., & Peyvandi, F. (2011). Evaluation of an automated platelet–based assay of ristocetin cofactor activity. *Haemophilia*, *17*(2), 252-256. https://doi.org/10.1111/j.1365-2516.2010.02419.x

Le, V. S., Tran, K. T., Bui, H. T., Le, H. T., Nguyen, C. D., Do, D. H., Ly, H. T., Pham, L. T., Dao, L. T., & Nguyen, L. T. (2019). A Vietnamese human genetic variation database. *Human mutation*, *40*(10), 1664-1675. https://doi.org/10.1002/humu.23835

Ledford−Kraemer, M. R. (2010). Analysis of von Willebrand factor structure by multimer analysis. *American journal of hematology*, *85*(7), 510-514. https://doi.org/10.1002/ajh.21739

Lee, G., Papapetrou, E. P., Kim, H., Chambers, S. M., Tomishima, M. J., Fasano, C. A., Ganat, Y. M., Menon, J., Shimizu, F., Viale, A., Tabar, V., Sadelain, M., & Studer, L. (2009). Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs. *Nature*, *461*(7262), 402-406. https://doi.org/10.1038/nature08320

Leebeek, F. W., & de Wee, E. M. (2010). Von Willebrand Disease type 3: an update. *Von Willebrand Disease in the Netherlands*, 31-40.

Leebeek, F. W., & Eikenboom, J. C. (2016). Von Willebrand's disease. *New England Journal of Medicine*, *375*(1), 2067-2080. https://doi.org/10.1056/NEJMra1601561

Lehmann, M., Ashworth, K., Manco-Johnson, M., Di Paola, J., Neeves, K. B., & Ng, C. J. (2018). Evaluation of a microfluidic flow assay to screen for von Willebrand disease and low von Willebrand factor levels. *J Thromb Haemost*, *16*, 104-115. https://doi.org/10.1111/jth.13881

Leissinger, C., Becton, D., Cornell, C., & Cox Gill, J. (2001). High−dose DDAVP intranasal spray (Stimate®) for the prevention and treatment of bleeding in patients with mild haemophilia A, mild or moderate type 1 von Willebrand disease and symptomatic carriers of haemophilia A. *Haemophilia*, *7*(3), 258-266. https://doi.org/10.1046/j.1365-2516.2001.00500.x

Leissinger, C., Carcao, M., Gill, J. C., Journeycake, J., Singleton, T., & Valentino, L. (2014). Desmopressin (DDAVP) in the management of patients with congenital bleeding disorders. *Haemophilia*, *20*(2), 158-167. https://doi.org/10.1111/hae.12254

Lek, M., Karczewski, K. J., Minikell, E. V., Samocha, K. E., Banks, E., Fennell, T., O'Donnell-Luria, A. H., Ware, J. S., Hill, A. J., Cummings, B. B., Tukiainen, T., Birnbaum, D. P., Kosmicki, J. A., Duncan, L. E., Estrada, K., Zhao, F., Zou, J., Pierce-Hoffman, E., Berghout, J., … Exome Aggregation Consortium. (2016). Analysis of protein-coding genetic variation in 60,706 humans. *Nature*, *536*(7616), 285-291. https://doi.org/10.1038/nature19057

Lenk, H., Nilsson, I. M., Holmberg, L., & Weissbach, G. (1988). Frequency of different types of von Willebrand's disease in the GDR. *Acta Medica Scandinavica*, *224*(3), 275-280. https://doi.org/10.1111/j.0954-6820.1988.tb19373.x

Lenting, P. J., Neels, J. G., van den Berg, B. M., Clijsters, P. P., Meijerman, D. W., Pannekoek, H., van Mourik, J. A., Mertens, K., & van Zonneveld, A.-J. (1999). The light chain of factor VIII comprises a binding site for low density lipoprotein receptor-related protein. *Journal of Biological Chemistry*, *274*(34), 23734-23739. https://doi.org/10.1074/jbc.274.34.23734

Lentz, S. R., & Sadler, J. E. (1993). Homocysteine inhibits von Willebrand factor processing and secretion by preventing transport from the endoplasmic reticulum. *Blood*, *81*(3), 683-689. https://doi.org/10.1182/blood.V81.3.683.683

Lethagen, S., Carlson, M., & Hillcarp, A. (2004). A comparative in vitro evaluation of six von Willebrand factor concentrates. *Haemophilia*, *10*(3), 243-249. https://doi.org/10.1111/j.1365-2516.2004.00893.x

Lethagen, S., Isaksson, C., Schaedel, C., & Holmberg, L. (2002). Von Willebrand's disease caused by compound heterozygosity for a substitution mutation (T1156M) in the D3 domain of the von Willebrand factor and a stop mutation (Q2470X). *Thrombosis and haemostasis*, *88*(09), 421-426. https://doi.org/10.1055/s-0037-1613232

Lethagen, S., Kyrle, P. A., Castaman, G., Haertel, S., Mannucci, P. M., & Haemate P® Surgical Study Group. (2007). von Willebrand factor/factor VIII concentrate (Haemate® P) dosing based on pharmacokinetics: a prospective multicenter trial in elective surgery. *Journal of thrombosis and haemostasis*, *5*(7), 1420-1430. https://doi.org/10.1111/j.1538-7836.2007.02588.x

Levi, M., Levy, J. H., Anderson, H. F., & Truloff, D. (2010). Safety of recombinant activated factor VII in randomized clinical trials. *New England Journal of Medicine*, *363*(19), 1791-1800. https://doi.org/10.1056/NEJMoa1006221

Levine, J. D., Harlan, J. M., Harker, L. A., Joseph, M. L., & Counts, R. B. (1982). Thrombin-mediated release of factor VIII antigen from human umbilical vein endothelial cells in culture. *Blood*, *60*(2), 531-534. https://doi.org/10.1182/blood.V60.2.531.531

Levy, G., & Ginsburg, D. (2001). Getting at the Variable Expressivity of Von Willebrand Disease. *Thromb Haemost*, *86*, 144-148. https://doi.org/10.1055/s-0037-1616211

Lewis, J. H., Tagnon, H. J., Davidson, C. S., Minot, G. R., & Taylor, F. H. (1946). The relation of certain fractions of the plasma globulins to the coagulation defect in hemophilia. *Blood*, *1*(2), 166-172. https://doi.org/10.1182/blood.V1.2.166.166

Li, C. H., & Tam, P. K. (1998). An iterative algorithm for minimum cross entropy thresholding. *Pattern Recognition Letters*, *19*(8), 771-776. https://doi.org/10.1016/s0167-8655(98)00057-9

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., & 1000 Genome Project Data Processing Subgroup. (2009). The sequence alignment/map format and SAMtools. *Bioinformatics*, *25*(16), 2078-2079. https://doi.org/10.1093/bioinformatics/btp352

Li, H., Ruan, J., & Durbin, R. (2008). Mapping short DNA sequencing reads and calling variants using mapping quality scores. *Genome research*, *18*(11), 1851-1858. https://doi.org/10.1101/gr.078212.108

Li, Z., Hu, S., Ghosh, Z., Han, Z., & Wu, J. C. (2011). Functional characterization and expression profiling of human induced pluripotent stem cell-and embryonic stem cell-derived endothelial cells. *Stem cells and development*, *20*(10), 1701-1710. https://doi.org/10.1089/scd.2010.0426

Liang, Q., Qin, H., Ding, Q., Xie, X., Wu, R., Wang, H., Wang, H., Hu, Y., & Wang, X. (2017). Molecular and clinical profile of VWD in a large cohort of Chinese population: application of next generation sequencing and CNVplex® technique. *Thrombosis and haemostasis*, *117*(08), 1534-1548. https://doi.org/10.1160/TH16-10-0794

Lillicrap, D. (2009). Genotype/phenotype association in von Willebrand disease: is the glass half full or empty? *Journal of Thrombosis and Haemostasis*, *7*, 65-70. https://doi.org/10.1111/j.1538-7836.2009.03367.x

Lillicrap, D. (2013). von Willebrand disease: advances in pathogenetic understanding, diagnosis, and therapy. *Blood*, *122*(23), 3735-3740. https://doi.org/10.1182/blood-2013-06-498303

Lin, Y., Weisdorf, D. J., Solovey, A., & Hebbel, R. P. (2000). Origins of circulating endothelial cells and endothelial outgrowth from blood. *The Journal of clinical investigation*, *105*(1), 71-77. https://doi.org/10.1172/JCI8071

Lind, S. (1991). The Bleeding Time Does Not Predict Surgical Bleeding. *Blood*, *77*(12), 2547-2552.

Lindblad, D., Blomenkamp, K., & Teckman, J. (2007). Alpha−1−antitrypsin mutant Z protein content in individual hepatocytes correlates with cell death in a mouse model. *Hepatology*, *46*(4), 1228-1235. https://doi.org/10.1002/hep.21822

Linderman, M. D., Paudyal, C., Shakeel, M., Kelley, W., Bashir, A., & Gelb, D. B. (2021). NPSV: A simulation-driven approach to genotyping structural variants in whole-genome sequencing data. *GigaScience*, *10*(7), giab046. https://doi.org/10.1093/gigascience/giab046

Linkert, M., Rueden, C. T., Allan, C., Burel, J.-B., Moore, W., Patterson, A., Loranger, B., Moore, J., Neves, C., MacDonald, D., Tarkowska, A., Sticco, C., Hill, E., Rossner, M., Eliceiri, K. W., & Swedlow, J. R. (2010). Metadata matters: access to image data in the real world. *The Journal of Cell Biology*, *189*(5), 777-782. 10.1083/jcb.201004104

Lip, G. Y., & Blann, A. (1997). von Willebrand factor: a marker of endothelial dysfunction in vascular disorders? *Cardiovascular Research*, *34*(2), 255-265. https://doi.org/10.1016/S0008-6363(97)00039-4

Lippok, S., Kolšek, K., Löf, A., Eggert, D., Vanderlinden, W., Müller, J. P., König, G., Obser, T., Röhrs, K., Schneppenheim, S., Budde, U., Baldauf, C., Aponte-Santamaría, C., Gräter, F., Schneppenheim, R., Rädler, J. O., & Brehm, M. A. (2016). von Willebrand factor is dimerized by protein disulfide isomerase. *Blood, The Journal of the American Society of Hematology*, *127*(9), 1183-1191. https://doi.org/10.1182/blood-2015-04-641902

Liu, G.-H., Suzuki, K., Qu, J., Sancho-Martinez, I., Yi, F., Li, M., Kumar, S., Nivet, E., Kim, J., Soligalla, R. D., Dubova, I., Goebl, A., Plongthongkum, N., Fung, H.-L., Zhang, K., Loring, J. F., Laurent, L. C., & Belmonte, J. C. (2011). Targeted gene correction of laminopathy-associated LMNA mutations in patient-specific iPSCs. *Cell stem cell*, *8*(6), 688-694. https://doi.org/10.1016/j.stem.2011.04.019

Liu, M., Rehman, S., Tang, X., Gu, K., Fan, Q., Chen, D., & Ma, W. (2019). Methodologies for improving HDR efficiency. *Frontiers in genetics*, *9*(691). https://doi.org/10.3389/fgene.2018.00691

Liu, X., Qi, J., Xu, X., Zeisberg, M., Guan, K., & Zeisberg, E. M. (2016). Differentiation of functional endothelial cells from human induced pluripotent stem cells: a novel, highly efficient and cost effective method. *Differentiation*, *92*(4), 225-236. https://doi.org/10.1016/j.diff.2016.05.004

Loesberg, C., Gonsalves, M. D., Zandbergen, J., Willems, C., Van Aken, W. G., Stel, H. V., Van Mourik, J. A., & De Groot, P. G. (1983). The effect of calcium on the secretion of factor VIII-related antigen

by cultured human endothelial cells. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *763*(2), 160-168. https://doi.org/10.1016/0167-4889(83)90039-3

Loh, Y. H., Agarwal, S., Park, I. H., Urbach, A., Huo, H., Heffner, G. C., Kim, K., Miller, J. D., Ng, K., & Daley, G. Q. (2009). Generation of induced pluripotent stem cells from human blood. *Blood*, *113*(22), 5476-5479. https://doi.org/10.1182/blood-2009-02-204800

Lopes da Silva, M., & Cutler, D. F. (2016). von Willebrand factor multimerization and the polarity of secretory pathways in endothelial cells. *Blood*, *128*(2), 277-285. https://doi.org/10.1182/blood-2015-10-677054

Lopes-da-Silva, M., McCormack, J. J., Burden, J. J., Harrison-Lavoie, K. J., Ferraro, F., & Cutler, D. F. (2019). A GBF1-dependent mechanism for environmentally responsive regulation of ER-Golgi transport. *Developmental cell*, *49*(5), 786-801. https://doi.org/10.1016/j.devcel.2019.04.006

Losson, R., & Lacroute, F. (1979). Interference of nonsense mutations with eukaryotic messenger RNA stability. *Proceedings of the National Academy of Sciences*, *76*(10), 5134-5137. https://doi.org/10.1073/pnas.76.10.5134

LOVD - legend. (2017, March 09). Leiden Open Variation Database. Retrieved July 3, 2021, from https://grenada.lumc.nl/LOVD2/VWF/variants_legend.php?select_db=VWF

Lozier, J. N., & Nichols, T. C. (2013). Animal Models of Hemophilia and Related Bleeding Disorders. *Semin Hematol*, *50*(2), 175-184. https://doi.org/10.1053/j.seminhematol.2013.03.023

Luken, B. M. (2008). Extracellular control of VWF multimer size and thiol−disulfide exchange. *Journal of Thrombosis and Haemostasis*, *6*(7), 1131-1134. https://doi.org/10.1111/j.1538-7836.2008.03031.x

Lumangtad, L. A., & Bell, T. W. (2020). The signal peptide as a new target for drug design. *Bioorganic & medicinal chemistry letters*, *30*(10), 127115. https://doi.org/10.1016/j.bmcl.2020.127115

Lyons, S. E., Brock, M. E., Bowie, E. J., & Ginsburg, D. (1992). Impaired intracellular transport produced by a subset of type IIA von Willebrand disease mutations. *Journal of Biological Chemistry*, *267*(7), 4424-4430. https://doi.org/10.1016/S0021-9258(18)42852-9

Lyons, S. E., Cooney, K. A., Bockenstedt, P., & Ginsburg, D. (1994). Characterization of Leu777Pro and Ile865Thr type IIA von Willebrand disease mutations. *Blood*, *83*(6), 1551-1557. https://doi.org/10.1182/blood.V83.6.1551.1551

Maciag, T., Hoover, G. A., Stemerman, M. B., & Weinstein, R. (1981). Serial propagation of human endothelial cells in vitro. *The Journal of cell biology*, *91*(2), 420-426. https://doi.org/10.1083/jcb.91.2.420

Maherali, N., & Hochedlinger, K. (2008). Guidelines and techniques for the generation of induced pluripotent stem cells. *Cell stem cell*, *3*(6), 595-605. https://doi.org/10.1016/j.stem.2008.11.008

Mahony, B. O., Savini, L., Hara, J. O., & Bok, A. (2017). Haemophilia care in Europe−A survey of 37 countries. *Haemophilia*, *23*(4), e259-e266. https://doi.org/10.1111/hae.13263

Makarova, K. S., Haft, D. H., Barrangou, R., Brouns, S. J., Charpentier, E., Horvath, P., Moineau, S., Mojica, F. J., Wolf, Y. I., Yakunin, A. F., van der Oost, J., & Koonin, E. V. (2011). Evolution and classification of the CRISPR–Cas system. *Nature Reviews Microbiology*, *9*(6), 467-477. https://doi.org/10.1038/nrmicro2577

Mali, P., Yang, L., Esvelt, K., Aach, J., Guell, M., DiCarlo, J. E., Norville, J. E., & Church, G. M. (2013). RNA-guided human genome engineering via Cas9. *Science*, *339*(6121), 823-826. https://doi.org/10.1126/science.1232033

Malzer, E., Szajewska-Skuta, M., Dalton, L. E., Thomas, S. E., Hu, N., Skaer, H., Lomas, D. A., Crowther, D. C., & Marciniak, S. J. (2013). Coordinate regulation of eIF2α phosphorylation by PPP1R15 and GCN2 is required during Drosophila development. *Journal of cell science*, *126*(6), 1406-1415. https://doi.org/10.1242/jcs.117614

Mamathambika, B. S., & Bardwell,, J. C. (2008). Disulfide-linked protein folding pathways. *Annual review of cell and developmental biology*, *24*, 211-235. https://doi.org/10.1146/annurev.cellbio.24.110707.175333

Mancuso, D. J., Tuley, E. A., Westfield, L. A., Lester-Mancuso, T. L., Le Beau, M. M., Sorace, J. M., & Sadler, J. E. (1991). Human von Willebrand factor gene and pseudogene: structural analysis and differentiation by polymerase chain reaction. *Biochemistry*, *30*(1), 253-269. https://doi.org/10.1021/bi00215a036

Mancuso, D. J., Tuley, E. A., Westfield, L. A., Worrall, N. K., Shelton-Inloes, B. B., Sorace, J. M., Alevy, Y. G., & Sadler, J. E. (1989). Structure of the gene for human von Willebrand factor. *Journal of Biological Chemistry*, *264*(33), 19514-19527. https://doi.org/10.1016/S0021-9258(19)47144-5

Manderstedt, E., Lind-Halldén, C., Lethagen, S., & Halldén, C. (2018). Genetic variation in the von Willebrand factor gene in Swedish von Willebrand disease patients. *TH Open*, *2*(01), e39-e48. https://doi.org/10.1055/s-0037-1618571

Manisha, M., Ghosh, K., Shetty, S., Nair, S., Khare, A., Kulkarni, B., Pathare, A. V., Baindur, S., & Mohanty, D. (2002). Spectrum of inherited bleeding disorders from Western India. *Haematologia*, *32*(1), 39-48.

Mannucci, P. M. (1977). Spectrum of von Willebrand's Disease: a Study of 100 Cases: Italian Working Group. *British Journal of Haematology*, *35*(1), 101-112. https://doi.org/10.1111/j.1365-2141.1977.tb00566.x

Mannucci, P. M. (1995). Platelet von Willebrand factor in inherited and acquired bleeding disorders. *Proc. Natl. Acad. Sci. USA*, *92*, 2428-2432. https://doi.org/10.1073/pnas.92.7.242

Mannucci, P. M. (1997). Desmopressin (DDAVP) in the treatment of bleeding disorders: the first 20 years. *Blood*, *90*(7), 2515-2521. https://doi.org/10.1182/blood.V90.7.2515

Mannucci, P. M. (2001). How I treat patients with von Willebrand disease. *Blood*, *97*(7), 1915-1919. https://doi.org/10.1182/blood.V97.7.1915

Mannucci, P. M. (2004). Treatment of von Willebrand's Disease. *New England Journal of Medicine*, *351*(7), 683-694. https://doi.org/10.1056/NEJMra040403

Mannucci, P. M. (2019). New therapies for von Willebrand disease. *Hematology Am Soc Hematol Educ Program*, *2019*(1), 590-595. https://doi.org/10.1182/hematology.2019000368

Mannucci, P. M., Chediak, J., Hanna, W., Byrnes, J., Ledford, M., Ewenstein, B. M., Retzios, A. D., Kapelan, B. A., Schwartz, R. S., Kessler, C., & Alphanate Study Group. (2002). Treatment of von Willebrand disease with a high-purity factor VIII/von Willebrand factor concentrate: a prospective, multicenter study. *Blood*, *99*(2), 450-456. https://doi.org/10.1182/blood.V99.2.450

Mannucci, P. M., & Federici, A. B. (1995). Antibodies to von Willebrand factor in von Willebrand disease. *Adv Exp Med Biol*, *386*, 87-92. https://doi.org/10.1007/978-1-4613-0331-2_7

Mannucci, P. M., Kempton, C., Millar, C., Romond, E., Shapiro, A., Birschmann, I., Ragni, M. V., Cox Gill, J., Yee, T. T., Klamroth, R., Wong, W.-Y., Chapman, M., Engl, W., Turecek, P. L., Suiter, T. M., Ewenstein, B. M., & rVWF Ad Hoc Study Group. (2013). Pharmacokinetics and safety of a novel

recombinant human von Willebrand factor manufactured with a plasma-free method: a prospective clinical trial. *Blood*, *122*(5), 648-657. https://doi.org/10.1182/blood-2013-01-479527

Mannucci, P. M., Pareti, F. I., Ruggeri, Z. M., & Capitanio, A. (1977). 1-Deamino-8-D-arginine vasopressin: a new pharmacological approach to the management of haemophilia and von Willebrand's disease. *The Lancet*, *309*(8107), 869-872.

Manrai, A. K., Funke, B. H., Rehm, H. L., Olesen, M. S., Maron, B. A., Szolovits, P., Margulies, D. M., Loscalzo, J., & Kohane, I. S. (2016). Genetic misdiagnoses and the potential for health disparities. *New England Journal of Medicine*, *375*(7), 655-665. https://doi.org/10.1056/NEJMsa1507092

Mansouritorghabeh, H., Manavifar, L., Banihashem, A., Modaresi, A., Shirdel, A., Shahroudian, M., Shoja-e-Razavi, G., Pousti, H., & Esmaily, H. (2013). An investigation of the spectrum of common and rare inherited coagulation disorders in north-eastern Iran. *Blood Transfusion*, *11*(2), 233-240. https://doi.org/10.2450/2012.0023-12

Marguerie, G. A., Thomas-Maison, N., Ginsberg, M. H., & Plow, E. F. (1984). The platelet−fibrinogen interaction: Evidence for proximity of the Aα chain of fibrinogen to platelet membrane glycoproteins IIb/III. *European journal of biochemistry*, *139*(1), 5-11. https://doi.org/10.1111/j.1432-1033.1984.tb07968.x

Marina, O. C., Sanders, C. K., & Mourant, J. R. (2012). Correlating light scattering with internal cellular structures. *Biomedical optics express*, *3*(2), 296-312. https://doi.org/10.1364/BOE.3.000296

Marti, T., Rosselet, S. J., Titani, K., & Walsh, K. A. (1987). Identification of disulfide-bridged substructures within human von Willebrand factor. *Biochemistry*, *26*(25), 8099-8109. https://doi.org/10.1021/bi00399a013

Matsui, T., Shimoyama, T., Matsumoto, M., Fujimura, Y., Takemoto, Y., Sako, M., Hamako, J., & Titani, K. (1999). ABO blood group antigens on human plasma von Willebrand factor after ABO-mismatched bone marrow transplantation. *Blood*, *94*(8), 2895-2900. https://doi.org/10.1182/blood.V94.8.2895.420a03_2895_2900

Matsui, T., Titani, K., & Mizuochi, T. (1992). Structures of the asparagine-linked oligosaccharide chains of human von Willebrand factor. *Journal of Biological Chemistry*, *267*(13), 8723-8731. https://doi.org/10.1016/S0021-9258(19)50338-6

Matteson, K., Abed, H., Wheeler, T. L., Sung, V. W., Rahn, D. D., Schaffer, J. I., & Balk, E. M. (2012). A Systematic Review Comparing Hysterectomy with Less-Invasive Treatments for Abnormal Uterine Bleeding. *J Minim Invasive Gynecol*, *19*(1), 13-28. https://doi.org/10.1016/j.jmig.2011.08.005

Mauer, A. C., Khazanov, N. A., Levenkova, N., Tian, S., Barbour, E. M., Khalid, C., Tobin, J. N., & Coller, B. S. (2011). Impact of sex, age, race, ethnicity and aspirin use on bleeding symptoms in healthy adults. *J Thromb Haemost*, *9*, 100-108. https://doi.org/10.1111/j.1538-7836.2010.04105.x

Mauser Bunschoten, E., Van Houwelingen, J. C., Visser, E. S., Van Dijken, P. J., Kok, A. J., & Sixma, J. J. (1988). Bleeding symptoms in carriers of hemophilia A and B. *Thrombosis and haemostasis*, *59*(03), 349-352. https://doi.org/10.1055/s-0038-1647493

Mazurier, C. (1992). von Willebrand disease masquerading as haemophilia A. *Thrombosis and haemostasis*, *67*(04), 391-396. https://doi.org/10.1055/s-0038-1648458

Mazurier, C., Dieval, J., Jorieux, S., Delobel, J., & Goudemand, M. (1990). A New von Willebrand Factor (vWF) Defect in a Patient With Factor VIII (FVIII) Deficiency But With Normal Levels and Multimeric Patterns of Both Plasma and Platelet vWF. Characterization of Abnormal vWF/FVIII Interaction. *Blood*, *75*(1), 20-26. https://doi.org/10.1182/blood.V75.1.20.20

Mazurier, C., Goudemand, J., Hilbert, L., Caron, C., Fressinaud, E., & Meyer, D. (2001). Type 2N von Willebrand disease: clinical manifestations, pathophysiology, laboratory diagnosis and molecular biology. *Best Practice & Research Clinical Haematology*, *14*(2), 337-347. https://doi.org/10.1053/beha.2001.0138

Mazurier, C., Parquet-Gernez, A., & Goudemand, M. (1980). The assay of Factor VIII related antigen by an immuno-enzymatic method. *Thrombosis and haemostasis*, *43*(01), 71. https://doi.org/10.1055/s-0038-1650019

McBane, R. D. (2015). *Novel Anticoagulants: How to Manage Patients Who Need a Procedure*. How Many Americans are taking Anticoagulants? Retrieved May 21, 2022, from https://ndafp.org/image/cache/1H_2.pdf

McCallum, C. J., Peake, I. R., Newcombe, R. G., & Bloom, A. L. (1983). Factor VIII levels and blood group antigens. *Thrombosis and haemostasis*, *50*(07), 757. https://doi.org/10.1055/s-0038-1665305

McClellan, A. C., Soucie, J. M., & Kulkarni, R. (2006). Using Geographical Information Systems (GIS) To Examine Associations between Characteristics of Males with Hemophilia and Geographic Distance

to Hemophilia Treatment Centers (HTCs). *Blood*, *108*(11), 3301. https://doi.org/10.1182/blood.V108.11.3301.3301

McCormack, J. J., Lopes da Silva, M., Ferraro, F., Patella, F., & Cutler, D. F. (2017). Weibel− Palade bodies at a glance. *Journal of cell science*, *130*(21), 3611-3617. https://doi.org/10.1242/jcs.208033

McDonald, N. Q., & Hendrickson, W. A. (1993). A structural superfamily of growth factors containing a cystine knot motif. *Cell*, *73*(3), 421-424. https://doi.org/10.1016/0092-8674(93)90127-C

McGrath, R. T., McRae, E., Smith, O. P., & O'Donnell, J. S. (2010). Platelet von Willebrand factor – structure, function and biological importance. *British Journal of Haematology*, *148*, 834-843.

McGrath, R. T., van den Biggelaar,, M., Byrne, B., O'Sullivan, J. M., Rawley, O., O'Kennedy, R., Voorberg, J., Preston, R. J., & O'Donnell, J. S. (2013). Altered glycosylation of platelet-derived von Willebrand factor confers resistance to ADAMTS13 proteolysis. *Blood*, *122*(25), 4107-4110. https://doi.org/10.1182/blood-2013-04-496851

McKay, H., Derome, F., Anwar Haq, M., Whittaker, S., Arnold, E., Adam, F., Heddle, N. M., Rivard, G. E., & Hayward, C. P. (2004). Bleeding risks associated with inheritance of the Quebec platelet disorder. *Blood*, *104*(1). https://doi.org/10.1182/blood-2003-11-4077

McKinnon, T. A., Goode, E. C., Birdsey, G. M., Nowak, A. A., Chan, A. C., Lane, D. A., & Laffan, M. A. (2010). Specific N-linked glycosylation sites modulate synthesis and secretion of von Willebrand factor. *Blood*, *116*(4), 640-648. https://doi.org/10.1182/blood-2010-02-267450

Mckinnon, T. A., Nowak, A. A., Cutler, J., Riddell, A. F., Laffan, M. A., & Millar, C. M. (2012). Characterisation of von Willebrand factor A1 domain mutants I1416N and I1416T: correlation of clinical phenotype with flow−based platelet adhesion. *Journal of Thrombosis and Haemostasis*, *10*(7), 1409-1416. https://doi.org/10.1111/j.1538-7836.2012.04760.x

McLaren, W., Gil, L., Hunt, S. E., Riat, H. S., Ritchie, G. R., Thormann, A., Flicek, P., & Cunningham, F. (2016). The Ensembl Variant Effect Predictor. *Genome Biology*, *17*, 122. https://doi.org/10.1186/s13059-016-0974-4

McVey, J. H., Rallapalli, P. M., Kemball−Cook, G., Hampshire, D. J., Giansily−Blaizot, M., Gomez, K., Perkins, S. J., & Ludlam, C. A. (2020). The European Association for Haemophilia and Allied Disorders (EAHAD) coagulation factor variant databases: Important resources for haemostasis clinicians and researchers. *Haemophilia*, *26*(2), 306-313. https://doi.org/10.1111/hae.13947

Medina, R. J., O'Neill, C. L., Sweeney, M., Guduric-Fuchs, J., Gardiner, T. A., Simpson, D. A., & Stitt, W. A. (2010). Molecular analysis of endothelial progenitor cell (EPC) subtypes reveals two distinct cell populations with different identities. *BMC medical genomics*, *3*(1), 1-13. https://doi.org/10.1186/1755-8794-3-18

Megías−Vericat, J., Monte−Boquet, E., Martín−Cerezuela, M., Cuéllar−Monreal, M. J., Tarazona−Casany, M. V., Pérez−Huertas, P., Bonanad, S., & Poveda−Andrés, J. L. (2018). Pilot evaluation of home delivery programme in haemophilia. *Journal of clinical pharmacy and therapeutics*, *43*(6), 822-828. https://doi.org/10.1111/jcpt.12718

Megy, K., Downes, K., Morel-Kopp, M.-C., Bastida, J. M., Brooks, S., Bury, L., Morgan, N. V., Othman, M., Ouwehand, W. H., Botero, J. P., Rivera, J., Schulze, H., Trégouët, D.-A., & Freson, K. (2021). GoldVariants, a resource for sharing rare genetic variants detected in bleeding, thrombotic, and platelet disorders: Communication from the ISTH SSC Subcommittee on Genomics in Thrombosis and Hemostasis. *Journal of Thrombosis and Haemostasis*, *19*(10), 2612-2617. https://doi.org/10.1111/jth.15459

Meiring, M., Coetzee, M., Kelderman, M., & Badenhorst, P. (2011). Laboratory diagnosis and management of von Willebrand disease in South Africa. *Seminars in thrombosis and hemostasis*, *37*(05), 576-580. https://doi.org/10.1055/s-0031-1281045

Melero-Martin, J. M. (2022). Human Endothelial Colony-Forming Cells. *Cold Spring Harbor Perspectives in Medicine*, a041154. https://doi.org/10.1101/cshperspect.a041154

Melo-Nava, B. M., Benítez, H., Palacios, J. J., Nieva, B., Arenas, D., Jaloma-Cruz, A. R., Navarete, C., Salamenca, F., & Peñaloza, R. (2007). Molecular study of VWF gene from Mexican Mestizo patients with von Willebrand disease, and the finding of three new mutations. *Blood Cells, Molecules, and Diseases*, *39*(3), 361-365. https://doi.org/10.1016/j.bcmd.2007.06.007

Meriane, F., Sultan, Y., Arabi, H., Chafa, O., & Chellali, T. (1991). Incidence of a low von Willebrand factor activity in a population of Algerian students. *Blood*, *78*(Suppl 1), 48a.

Mertens, I., & Gaal, L. V. (2002). Obesity, haemostasis and the fibrinolytic system. *Obesity reviews*, *3*(2), 85-101. https://doi.org/10.1046/j.1467-789X.2002.00056.x

Mertes, G., Schwaab, R., Brackmann, H. H., & Ludwig, M. (1994). A G+3-to-T donor splice site mutation leads to skipping of exon 50 in von Willebrand factor mRNA. *Genomics*, *24*, 190-191. https://doi.org/10.1006/geno.1994.1602

Metcalf, D. J., Nightingale, T. D., Zenner, H. L., Lui-Roberts, W. W., & Cutler, D. F. (2008). Formation and function of Weibel-Palade bodies. *Journal of cell science*, *121*(1), 19-27.

Meyer, D., Fressinaud, E., Hillbert, L., Ribba, A. S., Lavergne, J. M., & Mazurier, C. (2001). Type 2 von Willebrand disease causing defective von Willebrand factor-dependent platelet function. . *Best Practice & Research Clinical Haematology*, *14*(2), 349-364. https://doi.org/10.1053/beha.2001.0137

Meyer, D., Fressinaud, E., & Mazurier, C. (2011). Clinical, laboratory, and molecular markers of type 2 von Willebrand disease. *Von Willebrand Disease: basic and clinical aspects*, 137-147. https://doi.org/10.1002/9781444329926.ch12

Meyer, D., & Larrieu, M. J. (1970). Von Willebrand factor and platelet adhesiveness. *Journal of clinical pathology*, *23*(3), 228-231. http://dx.doi.org/10.1136/jcp.23.3.228

Meyts, I., Bosch, B., Bolze, A., Boisson, B., Itan, Y., Belkadi, A., Pedergnana, V., Moens, L., Picard, C., Cobat, A., Bossuyt, X., Abel, L., & Casanova, J.-L. (2016). Exome and genome sequencing for inborn errors of immunity. *Journal of Allergy and Clinical Immunology*, *138*(4), 957-969. https://doi.org/10.1016/j.jaci.2016.08.003

Michael, J. M., & Kornfeld, S. (1980). Partial purification and characterization of the glucosidases involved in the processing of asparagine-linked oligosaccharides. *Archives of biochemistry and biophysics*, *199*(1), 249-258. https://doi.org/10.1016/0003-9861(80)90278-7

Michaux, G., Hewlett, L. J., Messenger, S. L., Goodeve, A. C., Peake, I. R., Daly, M. E., & Cutler, D. F. (2003). Analysis of intracellular storage and regulated secretion of 3 von Willebrand disease–causing variants of von Willebrand factor. *Blood*, *102*(7), 2452-2458. https://doi.org/10.1182/blood-2003-02-0599

Miesbach, W., & Berntorp, E. (2017). Von Willebrand disease–the ‘Dos’ and ‘Don'ts’ in surgery. *European journal of haematology*, *98*(2), 121-127. https://doi.org/10.1111/ejh.12809

Miesbach, W., & Berntorp, E. (2021). Translating the success of prophylaxis in haemophilia to von Willebrand disease. *Thrombosis Research*, *199*, 67-74. https://doi.org/10.1016/j.thromres.2020.12.030

Miller, C. H., Graham, J. B., Goldin, L. R., & Elston, R. C. (1979). Genetics of classic von Willebrand's disease. I. Phenotypic variation within families. *Blood*, *54*(1), 117-136. https://doi.org/10.1182/blood.V54.1.117.117

Miller, C. H., Haff, E., Platt, S. J., Rawlins, P., Drews, C. D., Dilley, A. B., & Evatt, B. (2003). Measurement of von Willebrand factor activity: relative effects of ABO blood type and race. *Journal of thrombosis and haemostasis*, *1*(10), 2191-2197. https://doi.org/10.1046/j.1538-7836.2003.00367.x

Miller, C. H., Lenzi, R., & Breen, C. (1987). Prevalence of von Willebrand's disease among US adults. *Blood*, *70*(Suppl 1), 377.

Miller, J. L., & Castella, A. (1982). Platelet-type von Willebrand's disease: characterization of a new bleeding disorder. *Blood*, *60*(3), 790-794. https://doi.org/10.1182/blood.V60.3.790.790

Minot, G. (1928). A familial hemorrhagic condition associated with prolongation of the bleeding time. *Am J Med Sci*, *175*, 301-306.

Misra, U. K., & Pizzo, S. V. (2005). Coordinate regulation of forskolin-induced cellular proliferation in macrophages by protein kinase A/cAMP-response element-binding protein (CREB) and Epac1-Rap1 signaling: effects of silencing CREB gene expression on Akt activation. *Journal of biological chemistry*, *280*(46), 38276-38289. https://doi.org/10.1074/jbc.M507332200

Moake, J. L., Turner, N. A., Stathopoulos, N. A., Nolasco, L. H., & Hellums, J. D. (1986). Involvement of large plasma von Willebrand factor (vWF) multimers and unusually large vWF forms derived from endothelial cells in shear stress-induced platelet aggregation. *Journal of clinical investigation*, *78*(6), 1456-1461. https://doi.org/10.1172/JCI112736

Modrow, S., Wenzel, J. J., Schimanski, S., Schwarzbeck, J., Rothe, U., Oldenburg, J., Jilg, W., & Eis−Hübinger, A. M. (2011). Prevalence of nucleic acid sequences specific for human parvoviruses, hepatitis A and hepatitis E viruses in coagulation factor concentrates. *Vox sanguinis*, *100*(4), 351-358. https://doi.org/10.1111/j.1423-0410.2010.01445.x

Mohammed, S., & Favaloro, E. J. (2017). Laboratory Testing for von Willebrand Factor: Factor VIII Binding (for 2N VWD). *In: Favaloro E., Lippi G. (eds) Hemostasis and Thrombosis. Methods in Molecular Biology*, *1646*, 461-472. https://doi.org/10.1007/978-1-4939-7196-1_34

Mohanty, D., Ghosh, K., Marwaha, N., Kaur, S., Chauhan, A., & Das, K. C. (1984). Major blood group antigens-a determinant of factor VIII levels in blood? *Thrombosis and haemostasis*, *51*(03), 414. https://doi.org/10.1055/s-0038-1661121

Mohl, A., Boda, Z., Jager, R., Losonczy, H., Marosi, A., Masszi, T., Nagy, E., Nemes, L., Obser, T., Oyen, F., Radvanyi, G., Schlammadinger, A., Szelessy, Z., Varkonyi, A., Vezendy, K., Vilimi, B., Schneppenheim, R., & Bodo, I. (2011). Common large partial VWF gene deletion does not cause alloantibody formation in the Hungarian type 3 von Willebrand disease population. *J Thromb Haemost*, *9*, 945-52. https://doi.org/10.1111/j.1538-7836.2011.04250.x

Mohl, A., Marschalek, R., Masszi, T., Nagy, E., Obser, T., Oyen, F., Sallai, K., Bodo, I., & Schneppenheim, R. (2008). An Alu−mediated novel large deletion is the most frequent cause of type 3 von Willebrand disease in Hungary. *Journal of Thrombosis and Haemostasis*, *6*(10), 1729-1735. https://doi.org/0.1111/j.1538-7836.2008.03107.x

Mohlke,, K. L., Nichols, W. C., Rehemtulla, A., Kaufman, R. J., Fagerström, H. M., Ritvanen, K. A., Kekomaki, R., & Ginsburg, D. (1996). A common frameshift mutation in von Willebrand factor does not alter mRNA stability but interferes with normal propeptide processing. *British journal of haematology*, *95*(1), 184-191. https://doi.org/10.1046/j.1365-2141.1996.7572377.x

Mohri, H. (2002). High Dose of Tranexamic Acid for Treatment of Severe Menorrhagia in Patients with von Willebrand Disease. *Journal of Thrombosis and Thrombolysis*, *14*, 255-257. https://doi.org/10.1023/A:1025013213192

Monroe, D. (2017). Basic principles underlying coagulation. In *In N. Key, M. Makris, & D. Lillicrap (Eds.), Practical Haemostasis and Thrombosis* (pp. 1-11). Wiley Blackwell.

Moon, R. T., Bowerman, B., Boutros, M., & Perrimon, N. (2002). The promise and perils of Wnt signaling through β-catenin. *Science*, *296*(5573), 1644-1646. https://doi.org/10.1126/science.10715

Moreau, T., Evans, A. L., Vasquez, L., Tijssen, M. R., Yan, Y., Trotter, M. W., Howard, D., Colzani, M., Arumugam, M., Wu, W. H., Dalby, A., Lampela, R., Bouet, G., Hobbs, C. M., Pask, D. C., Payne, H., Ponomaryov, T., Brill, A., Soranzo, N., … Ghevaert, C. (2016). Large-scale production of

megakaryocytes from human pluripotent stem cells by chemically defined forward programming. *Nature communications*, *7*(1), 1-16. https://doi.org/10.1038/ncomms11208

Morelli, V. M., de Visser, M. C., van Tilburg, N. H., Vos, H. L., Eikenboom, J. C., Rosendaal, F. R., & Bertina, R. M. (2007). ABO blood group genotypes, plasma von Willebrand factor levels and loading of von Willebrand factor with A and B antigens. *Thrombosis and haemostasis*, *97*(04), 534-541. https://doi.org/10.1160/TH06-09-0549

Moser, K. A., & Funk, D. M. (2014). Chromogenic factor VIII activity assay. *American journal of hematology*, *89*(7), 781-784. https://doi.org/10.1002/ajh.23723

Mufti, A. H., Ogiwara, K., Swystun, L. L., Eikenboom, J. C., Budde, U., Hopman, W. M., Hallden, C., Goudemand, J., Peake, I. R., Goodeve, A. C., Lillicrap, D., Hampshire, D. J., & European Group on von Willebrand disease (EU-VWD) and Zimmerman Program for the Molecular and Clinical Biology of vonWillebrand disease (ZPMCB-VWD) Study Groups. (2018). The common VWF single nucleotide variants c. 2365A> G and c. 2385T> C modify VWF biosynthesis and clearance. *Blood Advances*, *2*(13), 1585-1594. https://doi.org/10.1182/bloodadvances.2017011643

Murray, G. P., Post, S. R., & Post, G. R. (2020). ABO blood group is a determinant of von Willebrand factor protein levels in human pulmonary endothelial cells. *Journal of clinical pathology*, *73*(6), 347-349. http://dx.doi.org/10.1136/jclinpath-2019-206182

Nachman, R., Levine, R., & Jaffe, E. A. (1977). Synthesis of factor VIII antigen by cultured guinea pig megakaryocytes. *The Journal of clinical investigation*, *60*(4), 914-921. https://doi.org/10.1172/JCI108846

Nagarajan, V. D., Shenoi, A., Burgess, L., & Radulescu, V. C. (2021). Spontaneous pyohaemothorax in a teenager with von Willebrand disease: a case report and review of literature. *BMJ Case Reports*, *14*(8), e241613. http://dx.doi.org/10.1136/bcr-2021-241613

Nair, P. M., Rendo, M. J., Reddoch-Cardenas, K. M., Burris, J. K., Meledeo, M. A., & Cap, A. P. (2020). Recent advances in use of fresh frozen plasma, cryoprecipitate, immunoglobulins, and clotting factors for transfusion support in patients with hematologic disease. *Seminars in Hematology*, *57*(2), 73-82. https://doi.org/10.1053/j.seminhematol.2020.07.006

Nair, S. C., Viswabandya, A., & Srivastava, A. (2011). Diagnosis and management of von Willebrand disease: a developing country perspective. *Seminars in thrombosis and hemostasis*, *37*(05), 587-594. https://doi.org/10.1055/s-0031-1281047

Nascimento, B., Goodnough, L. T., & Levy, J. H. (2014). Cryoprecipitate therapy. *British journal of anaesthesia*, *113*(6), 922-934. https://doi.org/10.1093/bja/aeu158

National Center for Biotechnology Information. (2009). *GRCh37*. NCBI. Retrieved May 04, 2021, from https://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.13/

National Center for Biotechnology Information. (2019, Feb 28). *GRCh38.p13*. NCBI. Retrieved May 04, 2021, from https://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.39

NCBI. (2020, March 23). *Representation of clinical significance in ClinVar and other variation resources at NCBI*. ClinVar. Retrieved July 03, 2021, from https://www.ncbi.nlm.nih.gov/clinvar/docs/clinsig/

NCBI and EMBL-EBI. (2020, October 26). *Matched Annotation from NCBI and EMBL-EBI (MANE)*. RefSeq: NCBI Reference Sequence Database. Retrieved April 22, 2021, from https://www.ncbi.nlm.nih.gov/refseq/MANE/

NEB. (n.d.). *Competent Cell Selection Guide*. NEB. Retrieved May 7, 2022, from https://international.neb.com/tools-and-resources/selection-charts/competent-cell-selection-guide

Nelson, M., & McClelland, M. (1992). [25] Use of DNA methyltransferase/endonuclease enzyme combinations for megabase mapping of chromosomes. *Methods in enzymology*, *216*, 279-303.

Ness, P. M., & Perkins, H. A. (1979). A simple enzyme-immunoassay (EIA) test for factor VIII-related antigen (VIII:AGN). *Thrombosis and haemostasis*, *42*(08), 848-854. https://doi.org/10.1055/s-0038-1666933

Ng, C., Motto, D. G., & Di Paola, J. (2015). Diagnostic approach to von Willebrand disease. *Blood*, *125*(13), 2029-2037. https://doi.org/10.1182/blood-2014-08-528398

Ng, P. C., & Henikoff, S. (2003). SIFT: predicting amino acid changes that affect protein function. *Nucleic Acids Research*, *31*(13), 1. https://doi.org/10.1093/nar/gkg509

Ngo, K. Y., Glotz, V. T., Koziol, J. A., Lynch, D. C., Gitschier, J., Ranieri, P., Ciavarella, N., Ruggeri, Z. M., & Zimmerman, T. S. (1988). Homozygous and heterozygous deletions of the von Willebrand factor gene in patients and carriers of severe von Willebrand disease. *Proceedings of the National Academy of Sciences*, *85*(8), 2753-2757. https://doi.org/10.1073/pnas.85.8.2753

NHS Blood and Transplant. (2018). *Blood types - NHS Blood Donation*. Give Blood. Retrieved January 28, 2022, from https://www.blood.co.uk/why-give-blood/blood-types/

NHS England. (2020). *Clinical Commissioning Policy: Vonicog alfa for the treatment and prevention of bleeding in adults with von Willebrand disease*. https://www.england.nhs.uk/wp-content/uploads/2020/09/1709-cc-policy-vonicog-alfa-for-von-willebrand-disease.pdf

Ni, H., Denis, C. V., Subbarao, S., Degen, J. L., Sato, T. N., Hynes, R. O., & Wagner, D. D. (2000). Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen. *The Journal of clinical investigation*, *106*(3), 385-392. https://doi.org/10.1172/JCI9896

Nichols, T. C., Samama, C. M., Bellinger, D. A., Roussi, J., Reddick, R. L., Bonneau, M., Read, M. S., Balliart, O., Koch, G. G., Vaiman, M., Sigman, J. L., Pignaud, G. A., Brinkhous, K. M., Griggs, T. R., & Drouet, L. (1995). Function of von Willebrand factor after crossed bone marrow transplantation between normal and von Willebrand disease pigs: effect on arterial thrombosis in chimeras. *Proceedings of the National Academy of Sciences*, *92*(7), 2455-2459. https://doi.org/10.1073/pnas.92.7.2455

Nichols, W. C., Lyons, S. E., Harrison, J. S., Cody, R. L., & Ginsburg, D. (1991). Severe von Willebrand disease due to a defect at the level of von Willebrand factor mRNA expression: detection by exonic PCR-restriction fragment length polymorphism analysis. *Proceedings of the National Academy of Sciences*, *88*(9), 3857-3861. https://doi.org/10.1073/pnas.88.9.3857

Nightingale, T., & Cutler, D. (2013). The secretion of von Willebrand factor from endothelial cells; an increasingly complicated story. *Journal of thrombosis and haemostasis*, *11*, 192-201. https://doi.org/10.1111/jth.12225

Nightingale, T. D., Pattni, K., Hume, A. N., Seabra, M. C., & Cutler, D. F. (2009). Rab27a and MyRIP regulate the amount and multimeric state of VWF released from endothelial cells. *Blood*, *113*(30), 5010-5018. https://doi.org/10.1182/blood-2008-09-181206

Nilsson, I. M. (1984). In Memory of Erik Jorpes: von Willebrand's Disease from 1926–1983. *Scandinavian Journal of Haematology*, *33*(S40), 21-43. https://doi.org/10.1111/j.1600-0609.1984.tb02543.x

Nilsson, I. M. (1999). The history of von Willebrand disease. *Haemophilia*, *5*, 7-11. https://doi.org/10.1046/j.1365-2516.1999.0050s2007.x

Nilsson, I. M., Blombäck, M., & Blombäck, B. (1959). v. Willebrand's disease in Sweden. Its pathogenesis and treatment. *Acta Medica Scandinavica*, *164*(3), 263-278. https://doi.org/10.1111/j.0954-6820.1959.tb00189.x

Nilsson, I. M., Blombäck, M., & von Francken, I. (1957). On an Inherited Autosomal Hemorrhagic Diathesis with Antihemophilic Globulin (AHG) Deficiency and Prolonged Bleeding Times. *Acta Medica Scandinavica*, *159*(1), 35-58. https://doi.org/10.1111/j.0954-6820.1957.tb00532.x

Niroula, A., & Vihinen, M. (2016). Variation interpretation predictors: principles, types, performance, and choice. *Human mutation*, *37*(6), 579-597. https://doi.org/10.1002/humu.22987

Nishino, M., Girma, J. P., Rothschild, C., Fressinaud, E., & Meyer, D. (1989). New variant of von Willebrand disease with defective binding to factor VIII. *Blood*, *74*(5), 1591-1599. https://doi.org/10.1182/blood.V74.5.1591.1591

Nosek-Cenkowska, B., Cheang, M. S., Pizzi, N. J., Israels, E. D., & Gerrard, J. M. (1991). Bleeding/bruising symptomatology in children with and without bleeding disorders. *Thrombosis and haemostasis*, *65*(03), 237-241. https://doi.org/10.1055/s-0038-1647491

Nurden, P., Debili, N., Vainchenker, W., Bobe, R., Bredoux, R., Corvazier, E., Combrie, R., Fressinaud, E., Meyer, D., Nurden, A. T., & Enouf, J. (2006). Impaired megakaryocytopoiesis in type 2B von Willebrand disease with severe thrombocytopenia. *Blood*, *108*(8), 2587-2595. https://doi.org/10.1182/blood-2006-03-009449

Nurden, P., Nurden, A. T., La Marca, S., Punzo, M., Baronciani, L., & Federici, A. B. (2009). Platelet morphological changes in 2 patients with von Willebrand disease type 3 caused by large homozygous deletions of the von Willebrand factor gene. *Haematologica*, *94*(11), 1627-1629. https://doi.org/10.3324/haematol.2009.012658

Nusse, R., & Varmus, H. E. (1992). Wnt genes. *Cell*, *69*(7), 1073-1087. https://doi.org/10.1016/0092-8674(92)90630-U

O'Brien, E., Jorde, L. B., Rönnlöf, B., Fellman, J. O., & Eriksson, A. W. (1988). Founder effect and genetic disease in Sottunga, Finland. *American Journal of Physical Anthropology*, *77*(3), 335-346. https://doi.org/10.1002/ajpa.1330770306

O'Brien, L. A., James, P. D., Othman, M., Berber, E., Cameron, C., Notley, C. R., Hegadorn, C. A., Sutherland, J. J., Hough, C., Rivard, G. E., O'Shaunessey, D., Lillicrap, D., & the Association of Hemophilia Clinic Directors of Canada. (2003). Founder von Willebrand factor haplotype associated with type 1 von Willebrand disease. *Blood*, *102*(2), 549-557. https://doi.org/10.1182/blood-2002-12-3693

O'Brien, S. H. (2012). Bleeding scores: are they really useful? *Hematology 2010, the American Society of Hematology Education Program Book, 2012*, *1*, 152-156. https://doi.org/10.1182/asheducation.V2012.1.152.3798226

O'Donnell, J., Boulton, F. E., Manning, R. A., & Laffan, M. A. (2002). Amount of H antigen expressed on circulating von Willebrand factor is modified by ABO blood group genotype and is a major determinant of plasma von Willebrand factor antigen levels. *Arteriosclerosis, thrombosis, and vascular biology*, *22*(2), 335-341. https://doi.org/10.1161/hq0202.103997

O'Donnell, J., & Laffan, M. A. (2001). The relationship between ABO histo−blood group, factor VIII and von Willebrand factor. *Transfusion Medicine*, *11*(4), 343-351. https://doi.org/10.1046/j.1365-3148.2001.00315.x

O'Donnell, J. S., McKinnon, T. A., Crawley, J. T., Lane, D. A., & Laffan, M. A. (2005). Bombay phenotype is associated with reduced plasma-VWF levels and an increased susceptibility to ADAMTS13 proteolysis. *Blood*, *106*(6), 1988-1991. https://doi.org/10.1182/blood-2005-02-0792

O'Donnell, J. S., & O'Sullivan, J. M. (2019). von Willebrand factor promotes wound healing. *Blood*, *133*(24), 2553-2555. https://doi.org/10.1182/blood.2019001175

Oettel, A., Lorenz, M., Stangl, V., Costa, S. D., Zenclussen, A. C., & Schumacher, A. (2016). Human umbilical vein endothelial cells foster conversion of CD4+ CD25− Foxp3− T cells into CD4+ Foxp3+ regulatory T cells via transforming growth factor-β. *Scientific reports*, *6*(1), 1-8. https://doi.org/10.1038/srep23278

Ogino, S., Gulley, M. L., Den Dunnen, J. T., Wilson, R. B., & Association for Molecular Pathology Training and Education Committee. (2007). Standard mutation nomenclature in molecular diagnostics: practical and educational challenges. *The Journal of molecular diagnostics*, *9*(1), 1-6. https://doi.org/10.2353/jmoldx.2007.060081

Ogiwara, K., Swystun, L. L., Paine, A. S., Kepa, S., Choi, S. J., Rejtö, J., Hopman, W., Pabinger, I., & Lillicrap, D. (2021). Factor VIII pharmacokinetics associates with genetic modifiers of VWF and FVIII clearance in an adult hemophilia A population. *Journal of Thrombosis and Haemostasis*, *19*(3), 654-663. https://doi.org/10.1111/jth.15183

Okamoto, S., Tamura, S., Sanda, N., Odaira, K., Hayakawa, Y., Mukaide, M., Suzuki, A., Kanematsu, T., Hayakawa, F., Katsumi, A., Kiyoi, H., Kojima, T., Matsushita, T., & Suzuki, N. (2022). VWF−Gly2752Ser, a novel non−cysteine substitution variant in the CK domain, exhibits severe secretory impairment by hampering C−terminal dimer formation. *Journal of Thrombosis and Haemostasis*. https://doi.org/10.1111/jth.15746

Oliver, J. D., van der Wal, F. J., Bulleid, N. J., & High, S. (1997). Interaction of the thiol-dependent reductase ERp57 with nascent glycoproteins. *Science*, *275*(5296), 86-88. https://doi.org/10.1126/science.275.5296.86

Ollier, W., Sprosen, T., & Peakman, T. (2005). UK Biobank: from concept to reality. *Pharmacogenomics*, *6*, 639–646. https://doi.org/10.2217/14622416.6.6.639

100,000 Genomes Project Pilot Investigators. (2021). 100,000 genomes pilot on rare-disease diagnosis in health care—Preliminary report. *New England Journal of Medicine*, *385*(20), 1868-1880. https://doi.org/10.1056/NEJMoa2035790

1000 Genomes Project Consortium. (2012). An integrated map of genetic variation from 1,092 human genomes. *Nature*, *491*(7422), 56-65. https://doi.org/10.1038/nature11632

Ong, Y. L., Hull, D. R., & Mayne, E. E. (1998). Menorrhagia in von Willebrand disease successfully treated with single daily dose tranexamic acid. *Haemophilia*, *4*, 63-65. https://doi.org/10.1046/j.1365-2516.1998.00147.x

Open Microscopy Environment. (2021, Mar 23). *Bio-Formats Documentation*. Retrieved June 16, 2021, from https://docs.openmicroscopy.org/bio-formats/6.6.1/

Orlova, V. V., Drabsch, Y., Freund, C., Petrus-Reurer, S., van den Hil, F. E., Muenthaisong, S., ten Dijke, P., & Mummery, C. L. (2014). Functionality of endothelial cells and pericytes from human pluripotent stem cells demonstrated in cultured vascular plexus and zebrafish xenografts. *Arteriosclerosis, thrombosis, and vascular biology*, *34*(1), 177-186. https://doi.org/10.1161/ATVBAHA.113.302598

Ormiston, M. L., Toshner, M. R., Kiskin, F. N., Huang, C. J., Groves, E., Morrell, N. W., & Rana, A. A. (2015). Generation and culture of blood outgrowth endothelial cells from human peripheral blood. *JoVE (Journal of Visualized Experiments)*, *106*, e53384. https://dx.doi.org/10.3791/53384

Orstavik, K. H., Magnus, P., Reisner, H., Berg, K., Graham, J. B., & Nance, W. (1985). Factor VIII and factor IX in a twin population. Evidence for a major effect of ABO locus on factor VIII level. *American journal of human genetics*, *37*(1), 89-101.

Othman, M., Chirinian, Y., Brown, C., Notley, C., Hickson, N., Hampshire, D., Buckley, S., Waddington, S., Parker, A., Baker, A., James, P., & Lillicrap, D. (2010). Functional characterization of a 13-bp deletion (c.-1522_-1510del13) in the promoter of the von Willebrand factor gene in type 1 von Willebrand disease. *Blood*, *116*(18), 3645-3652. https://doi.org/10.1182/blood-2009-12-261131

Othman, M., Kaur, H., Favaloro, E., Lillicrap, D., Di Paola, J., Harrison, P., Gresele, P., & The Subcommittees on Von Willebrand Disease and Platelet Physiology. (2016). Platelet type von Willebrand disease and registry report: communication from the SSC of the ISTH. *Journal of Thrombosis and Haemostasis*, *14*(2), 411-414. https://doi.org/10.1111/jth.13204

Ou, W. J., Cameron, P. H., Thomas, D. Y., & Bergeron, J. J. (1993). Association of folding intermediates of glycoproteins with calnexin during protein maturation. *Nature*, *364*(6440), 771-776. https://doi.org/10.1038/364771a0

Ouyang, W., Yu, Z., Yin, J., Su, J., Yang, C., & Ruan, C. (2014). Two novel mutations identified in a type 3 von Willebrand disease patient. *Blood Coagulation & Fibrinolysis*, *25*(8), 909-911. https://doi.org/10.1097/MBC.0000000000000157

Ozaki, K., Ohnishi, Y., Iida, A., Sekine, A., Yamada, R., Tsunoda, T., Sato, H., Sato, H., Hori, M., Nakamura, Y., & Tanaka, T. (2002). Functional SNPs in the lymphotoxin-α gene that are associated with susceptibility to myocardial infarction. *Nature genetics*, *32*(4), 650-654. https://doi.org/10.1038/ng1047

Pacheco, L. D., Costantine, M. M., Saade, G. R., Mucowski, S., Hankins, G. D., & Sciscione, A. C. (2010). von Willebrand disease and pregnancy: a practical approach for the diagnosis and treatment. *American journal of obstetrics and gynecology*, *203*(3), 194-200. https://doi.org/10.1016/j.ajog.2010.02.036

Padilla, A., Moake, J. L., Bernardo, A., Ball, C., Wang, Y., Arya, M., Nolasco, L., Turner, N., Berndt, M. C., Anvari, B., López, J., & Dong, J.-F. (2004). P-selectin anchors newly released ultralarge von Willebrand factor multimers to the endothelial cell surface. *Blood*, *103*(6), 2150-2156. https://doi.org/10.1182/blood-2003-08-2956

Pagliari, M. T., Baronciani, L., Cordiglieri, C., Colpani, P., Cozzi, G., Siboni, S. M., & Peyvandi, F. (2022). The dominant p. Thr274Pro mutation in the von Willebrand factor propeptide causes the von Willebrand disease type 1 phenotype in two unrelated patients. *Haemophilia*. https://doi.org/10.1111/hae.14494

Pamies, D., Bal-Price, A., Simeonov, A., Tagle, D., Allen, D., Gerhold, D., Yin, D., Pistollato, F., Inutsuka, T., Sullivan, K., Stacey, G., Salem, H., Leist, M., Daneshian, M., Vemuri, M. C., McFarland, R., Cooke, S., Fitzpatrick, S. C., Lakshmipathy, U., … Hartung, T. (2017). Good cell culture practice for stem cells and stem-cell-derived models. *Alternatives to Animal Experimentation: ALTEX*, *34*(1), 95-132. https://doi.org/10.14573/altex.1607121

Pareti, F. I., Niiya, K., McPherson, J. M., & Ruggeri, Z. M. (1987). Isolation and characterization of two domains of human von Willebrand factor that interact with fibrillar collagen types I and III. *Journal of Biological Chemistry*, *262*(28), 13835-13841. https://doi.org/10.1016/S0021-9258(19)76501-6

Paschalaki, K. E., & Randi, A. M. (2018). Recent advances in endothelial colony forming cells toward their use in clinical translation. *Frontiers in medicine*, *5*, 295. https://doi.org/10.3389/fmed.2018.00295

Pasi, K. J., Collins, P. W., Keeling, D. M., Brown, S. A., Cumming, A. M., Dolan, G. C., Hay, C. R., Hill, F. G., Laffan, M., & Peake, I. R. (2004). Management of von Willebrand disease: a guideline from theUK Haemophilia Centre Doctors' Organization. *Haemophilia*, *10*, 218-231. https://doi.org/10.1111/j.1365-2516.2004.00886.x

Patel, M. R., Mahaffey, K. W., Garg, J., Pan, G., Singer, D. E., Hacke, W., Breithardt, G., Halperin, J. L., Hankey, G. J., Piccini, J. P., Becker, R. C., Nessel, C. C., Paolini, J. F., Berkowitz, S. D., Fox, K. A., Califf, R. M., & ROCKET AF Steering Committee, for the ROCKET AF Investigators. (2011). Rivaroxaban versus warfarin in nonvalvular atrial fibrillation. *New England Journal of Medicine*, *365*(10), 883-891. https://doi.org/(2011). Rivaroxaban versus warfarin in nonvalvular atrial fibrillation. New England Journal of Medicine, 365(10), 883-89110.1056/NEJMoa1009638

Patel, R. Y., Shah, N., Jackson, A. R., Ghosh, R., Pawliczek, P., Paithankar, S., Baker, A., Riehle, K., Chen, H., Milosavljevic, S., Bizon, C., Rynearson, S., Nelson, T., Jarvik, G. P., Rehm, H. L., Harrison, S. M., Azzariti, D., Powell, B., Babb, L., … on behalf of the ClinGen Resource. (2017). ClinGen Pathogenicity Calculator: a configurable system for assessing pathogenicity of genetic variants. *Genome Medicine*, *9*(1), 1-9. https://doi.org/10.1186/s13073-016-0391-z

Patsch, C., Challet-Meylan, L., Thoma, E. C., Urich, E., Heckel, T., O'Sullivan, J. F., Grainger, S. J., Kapp, F. G., Sun, L., Christensen, K., Xia, Y., Florido, M. H., He, W., Pan, W., Prummer, M., Warren, C. R., Jakob-Roetne, R., Certa, U., Jagasia, R., … Cowan, C. A. (2016). Generation of vascular endothelial and smooth muscle cells from human pluripotent stem cells. *Nature Cell Biology*, *17*(8), 994-1003. https://doi.org/10.1038/ncb3205

Patzke, J., Budde, U., Huber, A., Méndez, A., Muth, H., Obser, T., Peerschke, E., Wilkens, M., & Schneppenheim, R. (2014). Performance evaluation and multicentre study of a von Willebrand factor activity assay based on GPIb binding in the absence of ristocetin. *Blood Coagulation & Fibrinolysis*, *25*(8), 860-870. https://doi.org/10.1097/MBC.0000000000000169

Pawan, P. K., Mahima, Y., Vijai, T., & Manjula, L. (2021). Clinicopathological features of hemophilia in a tertiary care centre of India. *Journal of Family Medicine and Primary Care*, *10*(1), 295-299. https://doi.org/10.4103/jfmpc.jfmpc_1564_20

Pearson, W. R., & Lipman, D. J. (1988). Improved tools for biological sequence comparison. *Proceedings of the National Academy of Sciences*, *85*(8), 2444-2448. https://doi.org/10.1073/pnas.85.8.2444

Pedersen, B. S., Bhetariya, P. J., Brown, J., Kravitz, S. N., Marth, G., Jensen, R. L., Bronner, M. P., Underhill, H. R., & Quinlan, A. R. (2020). Somalier: rapid relatedness estimation for cancer and germline studies using efficient genome sketches. *Genome medicine*, *12*(1), 1-9. https://doi.org/10.1186/s13073-020-00761-2

Peng, H. M., Wang, L. C., Zhai, J. L., Weng, X. S., Fen, B., & Wang, W. (2020). Transfusion-transmitted infections in hemophilia patients who underwent surgical treatment: a study from a single center in north China. *Archives of medical science: AMS*, *16*(2), 308-312. https://doi.org/10.5114/aoms.2020.92892

Pera, M. F. (2011). The dark side of induced pluripotency. *Nature*, *471*(7336), 46-47. https://doi.org/10.1038/471046a

Perez, O. D., Krutzik, P. O., & Nolan, G. P. (2004). Flow cytometric analysis of kinase signaling cascades. In *Flow Cytometry Protocols* (Second ed., Vol. 263, pp. 67-94). Humana Press. https://doi.org/10.1385/1-59259-773-4:067

Pérez-Palma, E., Gramm, M., Nürnberg, P., May, P., & Lal, D. (2019). Simple ClinVar: an interactive web server to explore and retrieve gene and disease variants aggregated in ClinVar database. *Nucleic acids research*, *47*(W1), W99-W105. https://doi.org/10.1093/nar/gkz411

Persyn, M., Athanase, N., Trossaërt, M., Sigaud, M., Ternisien, C., Béné, M. C., & Fouassier, M. (2022). Effect of DDAVP on Platelet Activation and Platelet-Derived Microparticle Generation. *Hamostaseologie*, *42*(03), 185-192.

Pertea, M., Lin, X., & Salzberg, S. (2001). GeneSplicer: a new computational method for splice site prediction. *Nucleic acids research*, *29*(5), 1185-1190. https://doi.org/10.1093/nar/29.5.1185

Petersen, R., Lambourne, J. J., Javierre, B. M., Grassi, L., Kreuzhuber, R., Ruklisa, D., Rosa, I. M., Tome, A. R., Elding, H., van Geffen, J. P., Jiang, T., Farrow, S., Cairns, J., Al-Subaie, A. M., Ashford, S., Attwood, A., Batista, J., Bouman, H., Burden, F., … Frontini, M. (2017). Platelet function is modified by common sequence variation in megakaryocyte super enhancers. *Nature Communications*, *8*(1), 1-12. https://doi.org/10.1038/ncomms16058

Peterson, J. R., Ora, A., Van, P. N., & Helenius, A. (1995). Transient, lectin-like association of calreticulin with folding intermediates of cellular and viral glycoproteins. *Molecular biology of the cell*, *6*(9), 1173-1184. https://doi.org/10.1091/mbc.6.9.1173

Peto, R., Pike, M., Armitage, P., Breslow, N. E., Cox, D. R., Howard, S. V., Mantel, N., McPherson, K., Peto, J., & Smith, P. G. (1977). Design and analysis of randomized clinical trials requiring prolonged observation of each patient. II. analysis and examples. *British journal of cancer*, *35*(1), 1-39. https://doi.org/10.1038/bjc.1977.1

Petrovski, S., & Goldstein, D. B. (2016). Unequal representation of genetic variation across ancestry groups creates healthcare inequality in the application of precision medicine. *Genome Biol*, *17*(157). https://doi.org/10.1186/s13059-016-1016-y

Peyvandi, F., Cannavo, A., Garagiola, I., Palla, R., Mannucci, P. M., Rosendaal, F. R., & the SIPPET Study Group. (2018). Timing and severity of inhibitor development in recombinant versus plasma-derived

factor VIII concentrates: a SIPPET analysis. *J Thromb Haemost*, *16*, 39-43. https://doi.org/10.1111/jth.13888

Peyvandi, F., Garagiola, I., & Baronciani, L. (2011). Role of von Willebrand factor in the haemostasis. *Blood Transfusion*, *9*((Suppl 2)), s3. https://doi.org/10.2450/2011.002S

Peyvandi, F., Kouides, P., Turecek, P. L., Dow, E., & Berntorp, E. (2019a). Evolution of replacement therapy for von Willebrand disease: From plasma fraction to recombinant von Willebrand factor. *Blood reviews*, *38*, 100572. https://doi.org/10.1016/j.blre.2019.04.001

Peyvandi, F., Mamaev, A., Wang, J.-D., Stasyshyn, O., Timofeeva, M., Curry, N., Cid, A. R., Yee, T. T., Kavakli, K., Castaman, G., & Sytkowski, A. (2019b). Phase 3 study of recombinant von Willebrand factor in patients with severe von Willebrand disease who are undergoing elective surgery. *J Thromb Haemost*, *17*, 52-62. https://doi.org/10.1111/jth.14313

Peyvandi, F., Mannucci, P. M., Valsecchi, C., Pontiggia, S., Farina, C., & Retzios, A. D. (2013). ADAMTS13 content in plasma−derived factor VIII/von Willebrand factor concentrates. *American journal of hematology*, *88*(10), 895-898. https://doi.org/10.1002/ajh.23527

Peyvandi, F., Oldenburg, J., & Friedman, K. D. (2016). A critical appraisal of one-stage and chromogenic assays of factor VIII activity. *J Thromb Haemost*, *14*, 248-261. https://doi.org/10.1111/jth.13215

Piera-Velazquez, S., & Jimenez, S. A. (2019). Endothelial to mesenchymal transition: role in physiology and in the pathogenesis of human diseases. *Physiological reviews*, *99*(2), 1281-1324. i:10.1152/physrev.00021.201812810031-9333/19 Copyright © 2019 theAmerican Physiological Society

Pipe, S. W., Montgomery, R. R., Pratt, K. P., Lenting, P. J., & Lillicrap, D. (2016). Life in the shadow of a dominant partner: the FVIII-VWF association and its clinical implications for hemophilia A. *Blood*, *128*(16), 2007-2016. https://doi.org/10.1182/blood-2016-04-713289

Platè, M., Duga, S., Baronciani, L., La Marca, S., Rubini, V., Mannucci, P. M., Federici, A. B., & Asselta, R. (2010). Premature termination codon mutations in the von Willebrand factor gene are associated with allele-specific and position-dependent mRNA decay. *Haematologica*, *95*(1), 172–174. https://doi.org/10.3324/haematol.2009.012336

Plow, E. F., Pierschbacher, M. D., Ruoslahti, E., Marguerie, G. A., & Ginsberg, M. H. (1985). The effect of Arg-Gly-Asp-containing peptides on fibrinogen and von Willebrand factor binding to platelets. *PNAS*, *82*(23), 8057-8061. https://doi.org/10.1073/pnas.82.23.8057

Plug, I., Mauser-Bunschoten, E. P., Bröcker-Vriends, A. H., Kristian Ploos van Amstel, H., van der Bom, J. G., van Diemen-Homan, J. E., Willemse, J., & Rosendaal, F. R. (2006). Bleeding in carriers of hemophilia. *Blood*, *108*(1), 52-56. https://doi.org/10.1182/blood-2005-09-3879

Pockley, A. G., Foulds, G. A., Oughton, J. A., Kerkvliet, N. I., & Multhoff, G. (2015). Immune cell phenotyping using flow cytometry. *Current Protocols in Toxicology*, *66*(1). https://doi.org/10.1002/0471140856.tx1808s66

Polo, J. M., Liu, S., Figueroa, M. E., Kulalert, W., Eminli, S., Tan, K. Y., Apostolou, E., Stadtfeld, M., Li, Y., Shioda, T., Natesan, S., Wagers, A. J., Melnick, A., Evans, T., & Hochedlinger, K. (2010). Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. *Nature biotechnology*, *28*(8), 848-855. https://doi.org/10.1038/nbt.1667

Poole, T. J., Finkelstein, E. B., & Cox, C. M. (2001). The role of FGF and VEGF in angioblast induction and migration during vascular development. *Developmental Dynamics*, *220*(1), 1-17. https://doi.org/10.1002/1097-0177(2000)9999:9999<::AID-DVDY1087>3.0.CO;2-2

Popa, M., Hecker, M., & Wagner, A. H. (2022). Inverse Regulation of Confluence-Dependent ADAMTS13 and von Willebrand Factor Expression in Human Endothelial Cells. *Thrombosis and Haemostasis*, *122*(04), 611-622. https://doi.org/ 10.1055/s-0041-1733800

Popejoy, A., & Fullerton, S. (2016). Genomics is failing on diversity. *Nature*, *538*, 161-164. https://doi.org/10.1038/538161a

Pottinger, B. E., Read, R. C., Paleolog, E. M., Higgins, P. G., & Pearson, J. D. (1989). von Willebrand factor is an acute phase reactant in man. *Thrombosis research*, *53*(4), 387-394. https://doi.org/10.1016/0049-3848(89)90317-4

Power, C., & Thomas, C. (2011). Changes in BMI, Duration of Overweight and Obesity, and Glucose Metabolism: 45 Years of Follow-up of a Birth Cohort. *Diabetes Care*, *34*(9), 1986-1991. https://doi.org/10.2337/dc10-1482

Preston, R. J., Rawley, O., Gleeson, E. M., & O'Donnell, J. S. (2013). Elucidating the role of carbohydrate determinants in regulating hemostasis: insights and opportunities. *Blood*, *121*(19), 3801-3810. https://doi.org/10.1182/blood-2012-10- 415000

Price, V. E., Hawes, S. A., Bouchard, A., Vaughan, A., Jarock, C., & Kuhle, S. (2015). Unmeasured costs of haemophilia: the economic burden on families with children with haemophilia. *Haemophilia*, *21*(4), e294-e299. https://doi.org/10.1111/hae.12715

Proctor, R. R., & Rapaport, S. I. (1961). The partial thromboplastin time with kaolin. *Am J Clin Path*, *36*, 212-219. https://doi.org/10.1093/ajcp/36.3.212

Purcell, S., Neale, B., Todd-Brown, K., Todd-Brown, K., Thomas, L., Ferreira, M. A., Bender, D., Maller, J., Sklar, P., de Bakker, P. I., Daly, M. J., & Sham, P. C. (2007). PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am. J. Hum. Genet.*, *81*, 559-575. https://doi.org/10.1086/519795

Purkey, M. R., Seeskin, Z., & Chandra, R. (2014). Seasonal variation and predictors of epistaxis. *The laryngoscope*, *124*(9), 2028-2033. https://doi.org/10.1002/lary.24679

Purvis, A. R., & Sadler, J. E. (2004). A covalent oxidoreductase intermediate in propeptide-dependent von Willebrand factor multimerization. *Journal of Biological Chemistry*, *279*(48), 49982-49988. https://doi.org/10.1074/jbc.M408727200

Qin, H. H., Wang, X. F., Ding, L. Q., Xu, G., Zhang, L. W., Dai, J., Lu, Y.-L., Xi, X., & Wang, H. (2011). Phenotype and genotype analysis of threeChinese pedigrees with von Willebrand disease. *Chin J Hematol*, *32*(2), 99-102.

Qin, H. H., Xing, F. Z., Wang, F. X., Ding, L. Q., Xi, X. D., & Wang, H. L. (2014). Similarity in joint and mucous bleeding syndromes in type 2N von Willebrand disease and severe hemophilia A coexisting with type 1 von Willebrand disease in two Chinese pedigrees. *Blood Cells, Molecules, and Diseases*, *52*(4), 181-185. https://doi.org/10.1016/j.bcmd.2013.11.005

Quick, A. J., Stanley-Brown, M., & Bancroft, F. W. (1935). A study of the coagulation defect in hemophilia and in jaundice. *Am J Med Sci*, *190*, 501-511.

Quintáns, B., Ordóñez-Ugalde, A., Cacheiro, P., Carracedo, A., & Sobrido, M. J. (2014). Medical genomics: The intricate path from genetic variant identification to clinical interpretation. *Applied & translational genomics*, *3*(3), 60-67. https://doi.org/10.1016/j.atg.2014.06.001

Quiroga, T., Goycoolea, M., Panes, O., Aranda, E., Martínez, C., Belmont, S., Muñoz, B., Zúñiga, P., Pereira, J., & Mezzano, D. (2007). High prevalence of bleeders of unknown cause among patients with inherited mucocutaneous bleeding. A prospective study of 280 patients and 299 controls. *Haematologica*, *92*(3), 357-365. https://doi.org/10.3324/haematol.10816

Raab, S., Klingenstein, M., Liebau, S., & Linta, L. (2014). A comparative view on human somatic cell sources for iPSC generation. *Stem Cells International*, *2014*(Article ID 768391), 12 pages. https://doi.org/10.1155/2014/768391

Ragni, M. V., Bontempo, F. A., & Hassett, A. C. (1999). von Willebrand disease and bleeding in women. *Haemophilia*, *5*, 313-317. https://doi.org/10.1046/j.1365-2516.1999.00342.x

Ramensky, V., Bork, P., & Sunyaev, S. (2002). Human non−synonymous SNPs: server and survey. *Nucleic acids research*, *30*(17), 3894-3900. https://doi.org/10.1093/nar/gkf493

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., & Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nature protocols*, *8*(11), 2281-2308. https://doi.org/10.1038/nprot.2013.143

Randi, A. M., Laffan, M. A., & Starke, R. D. (2013). Von Willebrand Factor, Angiodysplasia and Angiogenesis. *Mediterr J Hematol Infect Dis.*, *5*(1), e2013060. https://doi.org/10.4084/MJHID.2013.060

Rangarajan, S. (2011). Von Willebrand factor – two sides and the edge of a coin. *Haemophilia*, *17*, 61-64. https://doi.org/10.1111/j.1365-2516.2010.02323.x

Rapoport, T. A. (2007). Protein translocation across the eukaryotic endoplasmic reticulum and bacterial plasma membranes. *Nature*, *450*(7170), 663-669. https://doi.org/10.1038/nature06384

Rasband, W. S. (1997-2018). *ImageJ*. Retrieved June 16, 2021, from https://imagej.nih.gov/ij/

Rashid, S. T., Corbineau, S., Hannan, N., Marciniak, S. J., Miranda, E., Alexander, G., Huang-Doran, I., Griffin, J., Ahrlund-Richter,, L., Skepper, J., Semple, R., Weber, A., Lomas, D. A., & Vallier, L. (2010). Modeling inherited metabolic disorders of the liver using human induced pluripotent stem cells. *The Journal of clinical investigation*, *120*(9), 3127-3136. https://doi.org/10.1172/JCI43122

Rassoulzadegan, M., Ala, F., Jazebi, M., Enayat, M. S., Tabibian, S., Shams, M., Bahraini, M., & Dorgalaleh, A. (2020). Molecular and clinical profile of type 2 von Willebrand disease in Iran: a thirteen-year

experience. *International Journal of Hematology*, *111*(4), 535-543. https://doi.org/10.1007/s12185-019-02814-8

Rauch, A., Wohner, N., Christophe, O. D., Denis, C. V., Susen, S., & Lenting, P. J. (2013). On the Versatility of von Willebrand Factor. *Mediterr J Hematol Infect Dis.*, *5*(1), e2013046. https://doi.org/10.4084/MJHID.2013.046

Raut, S., & Hubbard, A. R. (2010). International reference standards in coagulation. *Biologicals*, *38*(4), 423-429. https://doi.org/10.1016/j.biologicals.2010.02.009

Rawley, O., & Lillicrap, D. (2021). Functional roles of the von Willebrand factor propeptide. *Hämostaseologie*, *41*(01), 063-068. https://doi.org/10.1055/a-1334-8002

Reese, M. G., Eeckman, F. H., Kulp, D., & Haussler, D. (1997). Improved splice site detection in Genie. *Journal of computational biology*, *4*(3), 311-323. https://doi.org/10.1089/cmb.1997.4.311

Rentzsch, P., Schubach, M., Shendure, J., & Kircher, M. (2021). CADD-Splice—improving genome-wide variant effect prediction using deep learning-derived splice scores. *Genome medicine*, *13*(1), 1-12. https://doi.org/10.1186/s13073-021-00835-9

Rentzsch, P., Witten, D., Cooper, G. M., Shendure, J., & Kircher, M. (2019). CADD: predicting the deleteriousness of variants throughout the human genome. *Nucleic acids research*, *47*(D1), D886-D894. https://doi.org/10.1093/nar/gky1016

Reuter, J. A., Spacek, D. V., & Snyder, M. P. (2015). High-throughput sequencing technologies. *Molecular cell*, *58*(4), 586-597. https://doi.org/10.1016/j.molcel.2015.05.004

Revel-Wilk, S., Schmugge, M., Carcao, M. D., Blanchette, P., Rand, M., & Blanchette, V. S. (2003). Desmopressin (DDAVP) Responsiveness in Children With von Willebrand Disease. *Journal of pediatric hematology/oncology*, *25*(11), 874-879.

Rezende, S. M., Pinheiro, K., Caram, C., Genovez, G., & Barca, D. (2009). Registry of inherited coagulopathies in Brazil: first report. *Haemophilia*, *15*(1), 142-149. https://doi.org/10.1111/j.1365-2516.2008.01907.x

Ribba, A. S., Loisel, I., Lavergne, J. M., Juhan-Vague, I., Obert, B., Cherel, G., Meyer, D., & Girma, J. (2001). Ser968Thr mutation within the A3 domain of von Willebrand factor (VWF) in two related patients leads to a defective binding of VWF to collagen. *Thrombosis and haemostasis*, *86*(09), 848-854. https://doi.org/10.1055/s-0037-1616142

Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., Grody, W., Hegde, M., Lyon, E., Spector, E., Voelkerding, K., Rehm, H. L., & ACMG Laboratory Quality Assurance Committee. (2015). Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genetics in medicine*, *17*(5), 405-423. https://doi.org/10.1038/gim.2015.30

Riepe, T. V., Khan, M., Roosing, S., Cremers, F. P., & 't Hoen, P. A. (2021). Benchmarking deep learning splice prediction tools using functional splice assays. *Human Mutation*. https://doi.org/10.1002/humu.24212

Rimmer, E. K., & Houston, D. S. (2018). Bleeding by the numbers: the utility and the limitations of bleeding scores, bleeding prediction tools, and bleeding case definitions. *Transfusion and Apheresis Science*, *57*(4), 458-462. https://doi.org/10.1016/j.transci.2018.07.004

Ring, D. B., Johnson, K. W., Henriksen, E. J., Nuss, J. M., Goff, D., Kinnick, T. R., Ma, S. T., Reeder, J. W., Samuels, I., Slabiak, T., Wagman, A. S., Hammond, M.-E. W., & Harrison, S. D. (2003). Selective glycogen synthase kinase 3 inhibitors potentiate insulin activation of glucose transport and utilization in vitro and in vivo. *Diabetes*, *52*(3), 588-595. https://doi.org/10.2337/diabetes.52.3.588

Robertson, J. D., Yenson, P. R., Rand, M. L., Blanchette, V. S., Carcao, M. D., Notley, C., Lillicrap, D., & James, P. D. (2011). Expanded phenotype–genotype correlations in a pediatric population with type 1 von Willebrand disease. *Journal of Thrombosis and Haemostasis*, *9*(9), 1752-1760. https://doi.org/10.1111/j.1538-7836.2011.04423.x

Robilliard, L. D., Kho, D. T., Johnson, R. H., Anchan, A., O'Carroll, S. J., & Graham, E. S. (2018). The importance of multifrequency impedance sensing of endothelial barrier formation using ECIS technology for the generation of a strong and durable paracellular barrier. *Biosensors*, *8*(3), 64. https://doi.org/10.3390/bios8030064

Robinson, J. T., Thorvaldsdóttir, H., Wenger, A. M., Zehir, A., & Mesirov, J. P. (2017). Variant review with the integrative genomics viewer. *Cancer research*, *77*(21), e31-e34. https://doi.org/10.1158/0008-5472.CAN-17-0337

Robinson, P. N., Köhler, S., Bauer, S., Seelow, D., Horn, D., & Mundlos, S. (2008). The Human Phenotype Ontology: a tool for annotating and analyzing human hereditary disease. *The American Journal of Human Genetics*, *83*(5), 610-615. .https://doi.org/10.1016/j.ajhg.2008.09.017

Rodeghiero, F., & Castaman, G. (2001). Congenital von Willebrand disease type 1: definition, phenotypes, clinical and laboratory assessment. *Best Practice & Research Clinical Haematology*, *14*(2), 321-335. https://doi.org/10.1053/beha.2001.0136

Rodeghiero, F., Castaman, G., & Dini, E. (1987). Epidemiological investigation of the prevalence of von Willebrand's disease. *Blood*, *69*(2), 454-459. https://doi.org/10.1182/blood.V69.2.454.454

Rodeghiero, F., Castaman, G., Tosetto, A., Batlle, J., Baudo, F., Cappelletti, A., Casana, P., De Bosch, N., Eikenboom, J. C., Federici, A. B., Lethagen, S., Linari, S., & Srivastava, A. (2005). The discriminant power of bleeding history for the diagnosis of type 1 von Willebrand disease: an international, multicenter study. *Journal of thrombosis and haemostasis*, *3*(12), 2619-2626. https://doi.org/10.1111/j.1538-7836.2005.01663.x

Rodeghiero, F., Castaman, G. C., Tosetto, A., Lattuada, A., & Mannucci, P. M. (1990). Platelet von Willebrand factor assay: results using two methods for platelet lysis. *Thrombosis research*, *59*(2), 259-267.

Rodeghiero, F., Tosetto, A., Abshire, T., Arnold, D. M., Coller, B., James, P., Neunert, C., Lillicrap, D., & ISTH/SSC joint VWF and Perinatal/Pediatric Hemostasis Subcommittees Working Group. (2010). ISTH/SSC bleeding assessment tool: a standardized questionnaire and a proposal for a new bleeding score for inherited bleeding disorders. *Journal of Thrombosis and Haemostasis*, *8*(9), 2063-2065. https://doi.org/10.1111/j.1538-7836.2010.03975.x

Rodenburg, R. J. (2018). The functional genomics laboratory: functional validation of genetic variants. *Journal of Inherited Metabolic Disease*, *41*, 297-307. https://doi.org/10.1007/s10545-018-0146-7

Rodgers, S. E., Lloyd, J. V., Mangos, H. M., Duncan, E. M., & McRae, S. J. (2011). Diagnosis and management of adult patients with von Willebrand disease in South Australia. *Seminars in thrombosis and hemostasis*, *37*(05), 535-541. https://doi.org/10.1055/s-0031-1281040

Rodheghiero, F., Castaman, G., & Mannucci, P. M. (1996). Prospective Multicenter Study on Subcutaneous Concentrated Desmopressin for Home Treatment of Patients with von Willebrand Disease and Mild or Moderate Hemophilia A. *Thromb Haemost*, *76*(05), 692-696. https://doi.org/10.1055/s-0038-1650645

Rodriguez, V., Titapiwatanakun, R., Moir, C., Schmidt, K. A., & Pruthi, R. K. (2010). To circumcise or not to circumcise? Circumcision in patients with bleeding disorders. *Haemophilia*, *16*(2), 272-276. https://doi.org/10.1111/j.1365-2516.2009.02119.x

Rogozin, I. B., & Milanesi, L. (1997). Analysis of donor splice sites in different eukaryotic organisms. *Journal of molecular evolution*, *45*(1), 50-59. https://doi.org/10.1007/PL00006200

Rondaij, M. G., Bierings, R., Kragt, A., van Mourik, J. A., & Voorberg, J. (2006, 5). Dynamics and plasticity of Weibel-Palade bodies in endothelial cells. . *Arteriosclerosis, thrombosis, and vascular biology*, *26*(5), 1002-1007. https://doi.org/10.1161/01.ATV.0000209501.56852.6c

Rosen, S. (1984). Assay of factor VIII: C with a chromogenic substrate. *Scandinavian Journal of Haematology*, *33*(S40), 139-145. https://doi.org/10.1111/j.1600-0609.1984.tb02556.x

Rosenbaum, P. R., & Rubin, D. B. (1983). The Central Role of the Propensity Score in Observational Studies for Causal Effects. *Biometrika*, *70*(1), 41-55. https://doi.org/10.1093/biomet/70.1.41

Rosenfeld, M. G., Lin, C. R., Amara, S. G., Stolarsky, L., Roos, B. A., Ong, E. S., & Evans, R. M. (1982). Calcitonin mRNA polymorphism: peptide switching associated with alternative RNA splicing events. *Proceedings of the National Academy of Sciences*, *79*(6), 1717-1721. https://doi.org/10.1073/pnas.79.6.1717

Roussi, J., Drouet, L., Sigman, J., Vaiman, M., Pignaud, G., Bonneau, M., Masse, J.-M., & Cramer, E. M. (1995). Absence of incorporation of plasma von Willebrand factor into porcine platelet a−granules. *British Journal of Haematology*, *90*(3), 661-668. https://doi.org/10.1111/j.1365-2141.1995.tb05598.x

Royston, P. (1995). Remark AS R94: A remark on algorithm AS 181: The W-test for normality. *Journal of the Royal Statistical Society. Series C (Applied Statistics)*, *44*(4), 547-551. https://doi.org/10.2307/2986146

Rubin, D. B. (1973). Matching to Remove Bias in Observational Studies. *Biometrics*, *29*(1), 159. https://doi.org/10.2307/2529684

Rufaihah, A. J., Huang, N. F., Kim, J., Herold, J., Volz, K. S., Park, T. S., Lee, J. C., Zambidis, E. T., Reijo-Pera, R., & Cooke, J. P. (2013). Human induced pluripotent stem cell-derived endothelial cells exhibit functional heterogeneity. *American journal of translational research*, *5*(1), 21-35.

Ruggeri, Z. M. (1997). von Willebrand factor. *The Journal of clinical investigation*, *99*(4), 559-564. https://doi.org/10.1172/JCI119195

Ruggeri, Z. M., De Marco, L., Gatti, L., Bader, R., & Montgomery, R. R. (1983). Platelets have more than one binding site for von Willebrand factor. *The Journal of clinical investigation*, *72*(1), 1-12. https://doi.org/10.1172/JCI110946

Ruggeri, Z. M., Pareti, F. I., Mannucci, P. M., Ciavarella, N., & Zimmerman, T. S. (1980). Heightened interaction between platelets and factor VIII/von Willebrand factor in a new subtype of von Willebrand's disease. *New England Journal of Medicine*, *302*(19), 1047-1051. https://doi.org/10.1056/NEJM198005083021902

Ruggeri, Z. M., & Ware, J. (1993). von Willebrand factor. *The FASEB journal*, *7*(2), 308-316. https://doi.org/10.1096/fasebj.7.2.8440408

Ruggeri, Z. M., & Zimmerman, T. S. (1980). Variant von Willebrand's disease: characterization of two subtypes by analysis of multimeric composition of factor VIII/von Willebrand factor in plasma and platelets. *The Journal of clinical investigation*, *65*(6), 1318-1325.

Ruggeri, Z. M., & Zimmerman, T. S. (1981). The complex multimeric composition of factor VIII/von Willebrand factor. *Blood*, *57*(6), 1140-1143. https://doi.org/10.1182/blood.V57.6.1140.1140

Ruggeri, Z. M., & Zimmerman, T. S. (1987). von Willebrand factor and von Willebrand disease. *Blood*, *70*(4), 895-904. https://doi.org/10.1182/blood.V70.4.895.895

Rydz, N., Grabell, J., Lillicrap, D., & James, P. D. (2015). Changes in von Willebrand factor level and von Willebrand activity with age in type 1 von Willebrand disease. *Haemophilia*, *21*(5), 636-641. https://doi.org/10.1111/hae.12664

Rydz, N., & James, P. D. (2012). The evolution and value of bleeding assessment tools. *Journal of Thrombosis and Haemostasis*, *10*(11), 2223-2229. https://doi.org/10.1111/j.1538-7836.2012.04923.x

Rydz, N., Swystun, L. L., Notley, C., Paterson, A. D., Jacob Riches, J., Sponagle, K., Boonyawat, B., Montgomery, R. R., James, P. D., & Lillicrap, D. (2013). The C-type lectin receptor CLEC4M binds, internalizes, and clears von Willebrand factor and contributes to the variation in plasma von Willebrand factor levels. *Blood*, *121*(26), 5228-5237. https://doi.org/10.1182/blood-2012-10-457507

Sa, S., Gu, M., Chappell, J., Shao, N.-Y., Ameen, M., Elliott, K. A., Li, D., Grubert, F., Li, C. G., Taylor, S., Cao, A., Ma, Y., Fong, R., Nguyen, L., Wu, J. C., Snyder, M. P., & Rabinovitch, M. (2017). Induced

pluripotent stem cell model of pulmonary arterial hypertension reveals novel gene expression and patient specificity. *American journal of respiratory and critical care medicine*, *195*(7), 930-941. https://doi.org/10.1164/rccm.201606-1200OC

Sabater-Lleal, M., Huffman, J. E., De Vries, P. S., Marten, J., Mastrangelo, M. A., Song, C., Pankratz, N., Ward-Caviness, C. K., Yanek, L. R., Trompet, S., Delgado, G. E., Guo, X., Bartz, T. M., Martinez-Perez, A., Germain, M., de Haan, H. G., Ozel, A. B., Polasek, O., Smith, A. V., … Smith, N. L. (2019). Genome-wide association transethnic meta-analyses identifies novel associations regulating coagulation factor VIII and von Willebrand factor plasma levels. *Circulation*, *139*(5), 620-635. https://doi.org/10.1161/CIRCULATIONAHA.118.034532

Sadler, B., Christopherson, P. A., Haller, G., Montgomery, R. R., Di Paola, J., & Zimmerman Program Investigators. (2021). von Willebrand factor antigen levels are associated with burden of rare nonsynonymous variants in the VWF gene. *Blood*, *137*(23), 3277–3283. https://doi.org/10.1182/blood.2020009999

Sadler, B., Minard, C. G., Haller, G., Gurnett, C. A., O'Brien, S. H., Wheeler, A., Jain, S., Sharma, M., Zia, A., Kulkarni, R., Mullins, E., Ragni, M. V., Sidonio, R., Dietrich, J. E., Kouides, P. A., Di Paola, J., & Srivaths, L. (2022). Whole-exome analysis of adolescents with low VWF and heavy menstrual bleeding identifies novel genetic associations. *Blood Advances*, *6*(2), 420-428. https://doi.org/10.1182/bloodadvances.2021005118

Sadler, J. E. (1994). A revised classification of von Willebrand disease. *Thrombosis and haemostasis*, *71*(04), 520-525. https://doi.org/10.1055/s-0038-1642471

Sadler, J. E. (1998). Biochemistry and genetics of von Willebrand factor. *Annual review of biochemistry*, *67*(1), 395-424. https://doi.org/10.1146/annurev.biochem.67.1.395

Sadler, J. E. (2005). New concepts in von Willebrand disease. *Annu. Rev. Med.*, *56*, 173-191. https://doi.org/10.1146/annurev.med.56.082103.104713

Sadler, J. E. (2009). Low von Willebrand factor: sometimes a risk factor and sometimes a disease. *ASH Education Program Book*, *2009*(1), 106-112. https://doi.org/10.1182/asheducation-2009.1.106

Sadler, J. E. (2017). Pathophysiology of thrombotic thrombocytopenic purpura. *Blood*, *130*(10), 1181-1188. https://doi.org/10.1182/blood-2017-04-636431

Sadler, J. E., Budde, U., Eikenboom, J. C., Favaloro, E. J., Hill, F. G., Holmberg, L., Ingerslev, J., Lee, C. A., Lillicrap, D., Mannucci, P. M., Mazurier, C., Meyer, D., Nichols, W. L., Nishino, M., Peake, I. R., Rodeghiero, F., Schneppenheim, R., Ruggeri, Z. M., Srivastava, A., … Working Party on von Willebrand Disease Classification. (2006). Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. *Journal of thrombosis and haemostasis*, *4*(10), 2103-2114. https://doi.org/10.1111/j.1538-7836.2006.02146.x

Sadler, J. E., Mannucci, P. M., Berntorp, E., Bochkov, N., Boulyjenkov, V., Ginsburg, D., Meyer, D., Peake, I., Rodeghiero, F., & Srivastava, A. (2000). Impact, diagnosis and treatment of von Willebrand disease. *Thrombosis and haemostasis*, *84*(08), 160-174. https://doi.org/10.1055/s-0037-1613992

Sagheer, S., Rodgers, S., Yacoub, O., Dauer, R., Mcrae, S., & Duncan, E. (2016). Comparison of von Willebrand factor (VWF) activity levels determined by Hemos IL AcuStar assay and Hemos IL LIA assay with ristocetin cofactor assay by aggregometry. *Haemophilia*, *22*(3), e200-e207. https://doi.org/10.1111/hae.12937

Sahoo, T., Naseem, S., Ahluwalia, J., Marwaha, R. K., Trehan, A., & Bansal, D. (2020). Inherited bleeding disorders in north Indian children: 14 years' experience from a tertiary care center. *Indian Journal of Hematology and Blood Transfusion*, *36*(2), 330-336. https://doi.org/10.1007/s12288-019-01233-3

Sakariassen, K. S., Bolhuis, P. A., & Sixma, J. J. (1979). Human blood platelet adhesion to artery subendothelium is mediated by factor VIII–von Willebrand factor bound to the subendothelium. *Nature*, *279*(5714), 636-638. https://doi.org/10.1038/279636a0

Salem, R. O., & Van Cott, E. M. (2007). A new automated screening assay for the diagnosis of von Willebrand disease. *American journal of clinical pathology*, *127*(5), 730-735. https://doi.org/10.1309/CEPND3LFHQ87XU4D

Salzman, E. W. (1963). Measurement of platelet adhesiveness. A simple in vitro technique demonstrating an abnormality in van Willebrand's disease. *J. Lab. Clin. Med*, *62*, 724-735. :https://doi.org/10.5555/uri:pii:0022214363902257

Salzman, G. C. (1999). Light scatter: detection and usage. *Current protocols in cytometry*, *9*(1), 1-13. https://doi.org/10.1002/0471142956.cy0113s09

Sambrook, J. (1977). Adenovirus amazes at cold spring harbor. *Nature*, *268*(5616), 102-104.

Samsel, L., Dagur, P. K., Raghavachari, N., Seamon, C., Kato, G. J., & McCoy Jr, J. P. (2013). Imaging flow cytometry for morphologic and phenotypic characterization of rare circulating endothelial cells. *Cytometry Part B: Clinical Cytometry*, *84*(6), 379-389. https://doi.org/10.1002/cyto.b.21088

Sanders, Y. V., Giezenaar, M. A., Laros−van Gorkom, B. A., Meijer, K., Van der Bom, J. G., Cnossen, M. H., Nijziel, M. R., Ypma, P. F., Fijnvandraat, K., Eikenoom, J., Mauser-Bunschoten, E. P., Leebeek, F. W., & WiN Study Group. (2014). von Willebrand disease and aging: an evolving phenotype. *Journal of Thrombosis and Haemostasis*, *12*(7), 1066-1075. https://doi.org/10.1111/jth.12586

Şap, F., Kavaklı, T., Kavaklı, K., & Dizdarer, C. (2013). The Prevalence of von Willebrand Disease and Significance of in Vitro Bleeding Time (PFA-100) in von Willebrand Disease Screening in the İzmir Region. *Turk J Haematol*, *30*(1), 40-47. https://doi.org/10.4274/tjh.2011.0020

Sarode, R., Bandarenko, N., Brecher, M. E., Kiss, J. E., Marques, M. B., Szczepiorkowski, Z. M., & Winters, J. L. (2014). Thrombotic thrombocytopenic purpura: 2012 American Society for Apheresis (ASFA) consensus conference on classification, diagnosis, management, and future research. *Journal of clinical apheresis*, *29*(3), 148-167. https://doi.org/10.1002/jca.21302

Sato, H., & Singer, R. H. (2021). Cellular variability of nonsense-mediated mRNA decay. *Nature Communications*, *12*(1), 1-12. https://doi.org/10.1038/s41467-021-27423-0

Savage, B., Sixma, J. J., & Ruggeri, Z. M. (2002). Functional self-association of von Willebrand factor during platelet adhesion under flow. *Proceedings of the National Academy of Sciences*, *99*(1), 425-430. https://doi.org/10.1073/pnas.012459599

Savji, N., Rockman, C. B., Skolnick, A. H., Guo, Y., Adelman, M. A., Riles, T., & Berger, J. S. (2013). Association between advanced age and vascular disease in different arterial territories: a population database of over 3.6 million subjects. *Journal of the American College of Cardiology*, *61*(16), 1736-1743.

Scharberg, E. A., Olsen, C., & Bugert, P. (2016). The H blood group system. *Immunohaematology*, *32*(3), 112-118.

Schillemans, M., Karampini, E., Kat, M., & Bierings, R. (2019). Exocytosis of Weibel–Palade bodies: how to unpack a vascular emergency kit. *Journal of Thrombosis and Haemostasis*, *17*(1), 6-18. https://doi.org/10.1111/jth.14322

Schillemans, M., Karampini, E., van den Eshof, B. L., Gangaev, A., Hofman, M., van Breevoort, D., Meems, H., Janssen, H., Mulder, A. A., Jost, C. R., Escher, J. C., Adam, R., Carter, T., Koster, A. J., van den Biggelaar, M., Voorberg, J., & Bierings, R. (2018). Weibel-Palade body localized syntaxin-3 modulates von Willebrand factor secretion from endothelial cells. *Arteriosclerosis, Thrombosis, and Vascular Biology,*, *38*(7), 1549-1561. https://doi.org/10.1161/ATVBAHA.117.310701

Schillemans, M., Kat, M., Westeneng, J., Gangaev, A., Hofman, M., Nota, B., van Alphen, F. P., de Boer, M., van den Biggelaar, M., Margadant, C., Voorberg, J., & Bierings, R. (2019). Alternative trafficking of Weibel−Palade body proteins in CRISPR/Cas9−engineered von Willebrand factor–deficient blood outgrowth endothelial cells. *Research and practice in thrombosis and haemostasis*, *3*(4), 718-732. https://doi.org/10.1002/rth2.12242

Schlachetzki, F., Zhu, C., & Pardridge, W. M. (2002). Expression of the neonatal Fc receptor (FcRn) at the blood–brain barrier. *Journal of neurochemistry*, *81*(1), 203-206. https://doi.org/10.1046/j.1471-4159.2002.00840.x

Schleef, M., Strobel, E., Dick, A., Frank, J., Schramm, W., & Spannagl, M. (2005). Relationship between ABO and Secretor genotype with plasma levels of factor VIII and von Willebrand factor in thrombosis patients and control individuals. *British journal of haematology*, *128*(1), 100-107. https://doi.org/10.1111/j.1365-2141.2004.05249.x

Schneider, S. W., Nuschele, S., Wixforth, A., Gorzelanny, C., Alexander-Katz, A., Netz, R. R., & Scheider, M. F. (2007). Shear-induced unfolding triggers adhesion of von Willebrand factor fibers. *Proceedings of the National Academy of Science*, *104*(19), 7899-7903. https://doi.org/10.1073/pnas.0608422104

Schneider, V. A., Graves-Lindsay, T., Howe, K., Bouk, N., Chen, H.-C., Kitts, P. A., Murphy, T. D., Pruitt, K. D., Thibaud-Nissen, F., Albracht, D., Fulton, R. S., Kremitzki, M., Magrini, V., Markovic, C., McGrath, S., Meltz Steinberg, K., Auger, K., Chow, W., Collins, J., … Church, D. M. (2017). Evaluation of GRCh38 and de novo haploid genome assemblies demonstrates the enduring quality of the reference assembly. *Genome research*, *27*(5), 849-864. https://doi.org/10.1101/gr.213611.116

Schneppenheim, R., Brassard, J., Krey, S., Budde, U., Kunicki, T. J., Holmberg, L., Ware, J., & Ruggeri, Z. M. (1996). Defective dimerization of von Willebrand factor subunits due to a Cys-> Arg mutation in

type IID von Willebrand disease. *Proceedings of the National Academy of Sciences*, *93*(8), 3581-3586. https://doi.org/10.1073/pnas.93.8.3581

Schneppenheim, R., Budde, U., Krey, S., Drewke, E., Bergmann, F., Lechler, E., Oldenburg, J., & Schwabb, R. (1996). Results of a screening for von Willebrand disease type 2N in patients with suspected haemophilia A or von Willebrand disease type 1. *Thrombosis and haemostasis*, *76*(10), 598-602. https://doi.org/10.1055/s-0038-1650628

Schneppenheim, R., Federici, A. B., Budde, U., Castaman, G., Drewke, E., Krey, S., Mannucci, P. M., Riesen, G., Rodeghiero, F., Zieger, B., & Zimmerman, R. (2000). Von Willebrand disease type 2M "Vicenza" in Italian and German patients: identification of the first candidate mutation (G3864A; R1205H) in 8 families. *Thrombosis and haemostasis*, *83*(01), 136-140. https://doi.org/10.1055/s-0037-1613769

Schneppenheim, R., Krey, S., Bergmann, F., Bock, D., Budde, U., Lange, M., Linde, R., Mittler, U., Meili, E., Mertes, G., Olek, K., Plendl, H., & Simeoni, E. (1994). Genetic heterogeneity of severe von Willebrand disease type III in the German population. *Human genetics*, *94*(6), 640-652. https://doi.org/10.1007/BF00206958

Schneppenheim, R., Michiels, J. J., Obser, T., Oyen, F., Pieconka, A., Schneppenheim, S., Will, K., Zieger, B., & Budde, U. (2010). A cluster of mutations in the D3 domain of von Willebrand factor correlates with a distinct subgroup of von Willebrand disease: type 2A/IIE. *Blood*, *115*(23), 4894-4901. https://doi.org/10.1182/blood-2009-07-226324

Scholes, S., & Mindell, J. S. (2018, December 4). *Health Survey for England 2017 Cardiovascular diseases*. Health Survey for England. Retrieved May 21, 2022, from http://healthsurvey.hscic.gov.uk/media/78646/HSE17-CVD-rep.pdf

Schouten, J. P., McElgunn, C. J., Waaijer, R., Zwijnenburg, D., Diepvens, F., & Pals, G. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Research*, *30*(12), e57. https://doi.org/10.1093/nar/gnf056

Schumpp-Vonach, B., Kresbach, G., Schlaeger, E. J., & Steiner, B. (1995). Stable expression in Chinese hamster ovary cells of a homogeneous recombinant active fragment of human platelet glycoprotein Ibα. *Cytotechnology*, *17*(2), 133-141. https://www.doi.org/10.1007/bf00749401

Seaman, C. D., Bertolet, M., Zhang, J., & Ragni, M. V. (2021). Quality of periprocedural care in patients with von Willebrand disease. *Haemophilia*, *27*(5), 830-836. https://doi.org/10.1111/hae.14389

Selvam, S. N., Casey, L. J., Bowman, M. L., Hawke, L. G., Longmore, A. J., Mewburn, J., Ormiston, M., Archer, S. L., Maurice, D. H., & James, P. D. (2017). Abnormal angiogenesis in blood outgrowth endothelial cells derived from von Willebrand disease patients. . *Blood coagulation & fibrinolysis: an international journal in haemostasis and thrombosis*, *28*(7), 521-533. https://doi.org/10.1097/MBC.0000000000000635

Senapathy, P., Shapiro, M. B., & Harris, N. L. (1990). Splice junctions, branch point sites, and exons: Sequence statistics, identification, and applications to genome project. *Methods Enzymol*, *183*, 252-278.

Seyres, D., Cabassi, A., Lambourne, J. J., Burden, F., Farrow, S., McKinney, H., Batista, J., Kempster, C., Pietzner, M., Slingsby, O., Cao, T. H., Quinn, P. A., Stefanucci, L., Sims, M. C., Rehnstrom, K., Adams, C. L., Frary, A., Ergüener, B., Kreuzhuber, R., … Frontini, M. (2022). Transcriptional, epigenetic and metabolic signatures in cardiometabolic syndrome defined by extreme phenotypes. *Clinical epigenetics*, *14*(1), 1-24. https://https://doi.org/10.1186/s13148-022-01257-z

Shahbazi, S., Baniahmad, F., Zakiani-Roudsari, M., Raigani, M., & Mahdian, R. (2012). Nonsense mediated decay of VWF mRNA subsequent to c. 7674-7675insC mutation in type3 VWD patients. *Blood Cells, Molecules, and Diseases*, *49*(1), 48-52. https://doi.org/10.1016/j.bcmd.2012.03.007

Shahbazi, S., Mahdian, R., Ala, F. A., Lavergne, J. M., Denis, C. V., & Christophe, O. D. (2009). Molecular characterization of Iranian patients with type 3 von Willebrand disease. *Haemophilia*, *15*(5), 1058-1064. https://doi.org/10.1111/j.1365-2516.2009.02046.x

Shang, D., Zheng, X. W., Niiya, M., & Zheng, X. L. (2006). Apical sorting of ADAMTS13 in vascular endothelial cells and Madin-Darby canine kidney cells depends on the CUB domains and their association with lipid rafts. *Blood*, *108*(7), 2207-2215. https://doi.org/10.1182/blood-2006-02-002139

Shapiro, A. L., Viñuela, E., & Maizel Jr, J. V. (1967). Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. *Biochemical and biophysical research communications*, *28*(5), 815-820. https://doi.org/10.1016/0006-291X(67)90391-9

Shattil, S. J., Hoxie, J. A., Cunningham, M., & Brass, L. F. (1985). Changes in the platelet membrane glycoprotein IIb. IIIa complex during platelet activation. *Journal of Biological Chemistry*, *260*(20), 11107-11114. https://doi.org/10.1016/S0021-9258(17)39154-8

Sheidley, B. (2016, March 17). *Retiring the word "mutation" in clinical practice*. Beyond the Ion Channel. Retrieved January 27, 2022, from http://epilepsygenetics.net/2016/03/17/retiring-the-word-mutation-in-clinical-practice/

Shelton-Inloes, B. B., Chehab, F. F., Mannucci, P. M., Federici, A. B., & Sadler, J. E. (1987). Gene deletions correlate with the development of alloantibodies in von Willebrand disease. *The Journal of clinical investigation*, *79*(5), 1459-1465. https://doi.org/10.1172/JCI112974

Shi, Q., & Montgomery, R. R. (2010). Platelets as delivery systems for disease treatments. *Advanced drug delivery reviews*, *62*(12), 1196-1203.

Shima, M., Fujimara, Y., Nishiyama, T., Tsujiuchi, T., Narita, N., Matsui, T., Titani, K., Katayuma, M., Yamamoto, F., & Yoshiokaa, A. (1995). ABO blood group genotype and plasma von Willebrand factor in normal individuals. *Vox sanguinis*, *68*(4), 236-240. https://doi.org/10.1111/j.1423-0410.1995.tb02579.x

Shinmyozu, K., Okadome, T., Maruyama, Y., Maruyama, I., Osame, M., & Tara, M. (1991). A study on the frequency of von Willebrand factor deficiency state. *The Japanese Journal of Clinical Hematology*, *32*(1), 67-68.

Shiono, T., Abe, S., Watabe, T., Noro, M., Tamai, M., Akutsu, Y., Ishikawa, M., Suzuki, S., & Mori, K. (1992). Vitreous, retinal and subretinal hemorrhages associated with von Willebrand's syndrome. *Graefe's archive for clinical and experimental ophthalmology*, *230*(5), 496-497. https://doi.org/10.1007/BF00175940

Shumate, A., Zimin, A. V., Sherman, R. M., Puiu, D., Wagner, J. M., Olson, N. D., Pertea, M., Salit, M. L., Zook, J. M., & Salzberg, S. L. (2020). Assembly and annotation of an Ashkenazi human reference genome. *Genome biology*, *21*(1), 1-18. https://doi.org/10.1186/s13059-020-02047-7

Sidonio Jr, R. F., Gunawardena, S., Shaw, P. H., & Ragni, M. (2012). Predictors of von Willebrand disease in children. *Pediatric blood & cancer*, *58*(5), 736-740. https://doi.org/10.1002/pbc.23411

Sidonio Jr, R. F., Zia, A., & Fallaize, D. (2020). Potential undiagnosed VWD or other mucocutaneous bleeding disorder cases estimated from private medical insurance claims. *Journal of Blood Medicine*, *11*(1), 1-11. https://doi.org/10.2147/JBM.S224683

Sie, P., Caron, C., Azam, J., Goudemond, J., Grandjean, H., Boneu, B., & Fournié, A. (2003). Reassessment of von Willebrand factor (VWF), VWF propeptide, factor VIII: C and plasminogen activator inhibitors 1 and 2 during normal pregnancy. *British journal of haematology*, *121*(6), 897-903. https://doi.org/10.1046/j.1365-2141.2003.04371.x

Siekmann, J., Turecek, P., & Schwarz, H. P. (1998). The determination of von Willebrand factor activity by collagen binding assay. *Haemophilia*, *4*(Suppl.3), 15-24. https://doi.org/10.1046/j.1365-2516.1998.0040s3015.x

Siepel, A., Bejerano, G., Pedersen, J. S., Hinrichs, A. S., Hou, M., Rosenbloom, K., Clawson, H., Spieth, J., Hillier, L. W., Richards, S., Weinstock, G. M., Wilson, R. K., Gibbs, R. A., Kent, W. J., Miller, W., & Haussler, D. (2005). Evolutionarily conserved elements in vertebrate, insect, worm, and yeast genomes. *Genome research*, *15*(8), 1034-1050. https://doi.org/10.1101/gr.3715005

Simeoni, I., Stephens, J. C., Hu, F., Deevi, S. V., Megy, K., Bariana, T. K., Lentaigne, C., Schulman, S., Sivapalaratnam, S., Vries, M. J., Westbury, S. K., Green, D., Papadia, S., Alessi, M.-C., Attwood, A., Ballmaier, M., Baynam, G., Bermejo, E., Bertoli, M., … Turro, E. (2016). A high-throughput sequencing test for diagnosing inherited bleeding, thrombotic, and platelet disorders. *Blood*, *127*(23), 2791-2803. https://doi.org/10.1182/blood-2015-12-688267

Simes, J., Becattini, C., Agnelli, G., Eikelboom, J. W., Kirby, A. C., Mister, R., Prandoni, P., Brighton, T. A., & INSPIRE Study Investigators. (2014). Aspirin for the prevention of recurrent venous thromboembolism: the INSPIRE collaboration. *Circulation*, *130*(13), 1062-1071. https://doi.org/10.1161/CIRCULATIONAHA.114.008828

Simmonds, S. J., Syddall, H. E., Walsh, B., Evandrou, M., Dennison, E., Cooper, C., & Aihie Sayer, A. (2014). Understanding NHS hospital admissions in England: linkage of Hospital episode statistics to the Hertfordshire cohort study. *Age and ageing*, *43*(5), 653-660. https://doi.org/10.1093/ageing/afu020

Sims, M. C., Mayer, L., Collins, J. H., Bariana, T. K., Megy, K., Lavenu-Bombled,, C., Seyres, D., Kollipara, L., Burden, F. S., Greene, D., Lee, D., Rodriguez-Romera, A., Alessi, M.-C., Astle, W. J., Bahou,

W. F., Bury, L., Chalmers, E., Da Silva, R., De Candia, E., … Guerrero, J. A. (2020). Novel manifestations of immune dysregulation and granule defects in gray platelet syndrome. *Blood*, *136*(7), 1956-1967. https://doi.org/10.1182/blood.2019004776

Singer, K., & Ramot, B. (1956). Pseudohemophilia type b: hereditary hemorrhagic diathesis characterized by prolonged bleeding time and decrease in antihemophilic factor. *AMA archives of internal medicine*, *97*(6), 715-725. https://doi.org/10.1001/archinte.1956.00250240067006

Sivasubramaniyan, K., Harichandan, A., Schilbach, K., Mack, A. F., Bedke, J., Stenzl, A., Kanz, L., Niederfellner, G., & Bühring, H.-J. (2015). Expression of stage-specific embryonic antigen-4 (SSEA-4) defines spontaneous loss of epithelial phenotype in human solid tumor cells. *Glycobiology*, *25*(8), 902-917. https://doi.org/10.1093/glycob/cwv032

Smadja, D. M., Melero-Martin, J. M., Eikenboom, J., Bowman, M., Sabatier, F., & Randi, A. M. (2019). Standardization of methods to quantify and culture endothelial colony-forming cells derived from peripheral blood: Position paper from the International Society on Thrombosis and Haemostasis SSC. *Journal of thrombosis and haemostasis: JTH*, *17*(7), 1190-1194. https://doi.org/10.1111/jth.14462

Smith, L. J. (2017). Laboratory diagnosis of von Willebrand disease. *American Society for Clinical Laboratory Science*, *30*(2), 65-74. https://doi.org/10.29074/ascls.30.2.65

Smith, N. L., Chen, M. H., Dehghan, A., Strachan, D. P., Basu, S., Soranzo, N., Hayward, C., Rudan, I., Sabater-Lleal, M., Bis, J., de Maat, M. P., Rumley, A., Kong, X., Yang, Q., Williams, F. M., Vitart, V., Campbell, H., Malarstig, A., Wiggins, K. L., … O'Donnell, C. J. (2010). Novel associations of multiple genetic loci with plasma levels of factor VII, factor VIII, and von Willebrand factor: The CHARGE (Cohorts for Heart and Aging Research in Genome Epidemiology) Consortium. *Circulation*, *121*(12), 1382-1392. https://doi.org/10.1161/CIRCULATIONAHA.109.869156

Smock, K. J., & Moser, K. A. (2021). Cryptogenic oozers and bruisers. *Hematology Am Soc Hematol Educ Program*, *1*(85-91). https://doi.org/10.1182/hematology.2021000236

Snape, K., Wedderburn, S., & Barwell, J. (2019). The new genomic medicine service and implications for patients. *Clinical Medicine*, *19*(4), 273. https://doi.org/10.7861/clinmedicine.19-4-273

Snyder, M. W., Adey, A., Kitzman, J. O., & Shendure, J. (2015). Haplotype-resolved genome sequencing: experimental methods and applications. *Nature Reviews Genetics*, *16*(6), 344-358. https://doi.org/10.1038/nrg3903

Soldà, T., Garbi, N., Hämmerling, G. J., & Molinari, M. (2006). Consequences of ERp57 deletion on oxidative folding of obligate and facultative clients of the calnexin cycle. *Journal of Biological Chemistry*, *281*(10), 6219-6226. https://doi.org/10.1074/jbc.M513595200

Soldner, F., & Jaenisch, R. (2012). iPSC disease modeling. *Science*, *338*(6111), 1155-1156. https://doi.org/10.1126/science.1227682

Soldner, F., Laganiere, J., Cheng, A. W., Hockemeyer, D., Gao, Q., Alagappan, R., Khurana, V., Golbe, L. I., Myers, R. H., Lindquist, S., Zhang, L., Guschin, D., Fong, L. K., Vu, J., Meng, X., Urnov, F. D., Rebar, E. J., Gregory, P. D., Zhang, H. S., & Jaenisch, R. (2011). Generation of isogenic pluripotent stem cells differing exclusively at two early onset Parkinson point mutations. *Cell*, *146*(2), 318-331. https://doi.org/10.1016/j.cell.2011.06.019

Solimando, M., Baronciani, L., La Marca, S., Cozzi, G., Asselta, R., Canciani, M. T., Federici, A. B., & Peyvandi, F. (2012). Molecular characterization, recombinant protein expression, and mRNA analysis of type 3 von Willebrand disease: Studies of an Italian cohort of 10 patients. *American journal of hematology*, *87*(9), 870-874. https://doi.org/10.1002/ajh.23265

Son, J. S., Park, C. Y., Lee, G., Park, J. Y., Kim, H., Kim, G., Chi, K. Y., Woo, D.-H., Han, C., Kim, S. K., Park, H.-J., Kim, D.-W., & Kim, J.-H. (2022). Therapeutic correction of hemophilia A using 2D endothelial cells and multicellular 3D organoids derived from CRISPR/Cas9-engineered patient iPSCs. *Biomaterials*, *283*(121429). https://doi.org/10.1016/j.biomaterials.2022.121429

Sood, S. L., Cuker, A., Wang, C., Metjian, A. D., Chiang, E. Y., Soucie, J. M., Konkle, B. A., & The HTCN Study Investigators. (2013). Similarity in joint function limitation in Type 3von Willebrand's disease and moderate haemophilia A. *Haemophilia*, *19*, 595-601. https://doi.org/10.1111/hae.12119

Soucie, J. M., Miller, C. H., Byams, V. R., Payne, A. B., Abe, K., Sidonio Jr, R. F., & Kouides, P. A. (2021). Occurrence rates of von Willebrand disease among people receiving care in specialized treatment centres in the United States. *Haemophilia*, *27*, 445-453. https://doi.org/10.1111/hae.14263

Sousa, N. C., Anicchino-Bizzacchi, J. M., Locatelli, M. F., Castro, V., & Barjas-Castro, M. L. (2007). The relationship between ABO groups and subgroups, factor VIII and von Willebrand factor. *Haematologica*, *92*(2), 236-239. https://doi.org/10.3324/haematol.10457

Souto, J. C., Almasy, L., Muniz-Diaz, E., Soria, J. M., Borrell, M., Bayén, L., Mateo, J., Madoz, P., Stone, W., Blangero, J., & Foncuberta, J. (2000). Functional effects of the ABO locus polymorphism on plasma levels of von Willebrand factor, factor VIII, and activated partial thromboplastin time. *Arteriosclerosis, thrombosis, and vascular biology*, *20*(8), 2024-2028. https://doi.org/10.1161/01.ATV.20.8.2024

Sperzel, M., & Huetter, J. (2007). Evaluation of aprotinin and tranexamic acid in different in vitro and in vivo models of fibrinolysis, coagulation and thrombus formation. *Journal of Thrombosis and Haemostasis*, *5*, 2113–2118. https://doi.org/10.1111/j.1538-7836.2007.02717.x

Sporn, L. A., Chavin, S., & Marder, V. (1986). Biosynthesis of von Willebrand protein by human megakaryocytes. *Journal of Clinical Investigation*, *76*, 1102-1108.

Spradbrow, J., Letourneau, S., Grabell, J., Liang, Y., Riddel, J., Hopman, W., Blanchette, V. S., Rand, M. L., Coller, B. S., Paterson, A. D., & James, P. D. (2020). Bleeding assessment tools to predict von Willebrand disease: Utility of individual bleeding symptoms. *Research and practice in thrombosis and haemostasis*, *4*(1), 92-99. https://doi.org/10.1002/rth2.12256

Springer, T. A. (1994). Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell*, *76*(2), 301-314. https://doi.org/10.1016/0092-8674(94)90337-9

Springer, T. A. (2014). von Willebrand factor, Jedi knight of the bloodstream. *Blood*, *124*(9), 1412-1425. https://doi.org/10.1182/blood-2014-05-378638

Šrámek, A., Eikenboom, J. C., Briët, E., Vandenbroucke, J. P., & Rosendaal, F. R. (1995). Usefulness of patient interview in bleeding disorders. *Archives of internal medicine*, *155*(13), 1409-1415. https://doi.org/10.1001/archinte.1995.00430130095010

Stadinski, B. D., & Huseby, E. S. (2020). How to prevent yourself from seeing double. *Cytometry. Part A: the journal of the International Society for Analytical Cytology*, *97*(11), 1102-1104. https://doi.org/10.1002/cyto.a.24045

Stadler, M., Gruber, G., Kannicht, C., Biesert, L., Radomski, K. U., Suhartono, H., Pock, K., Neisser-Svae, A., Weinberger, J., Romisch, J., & Svae, T. E. (2006). Characterisation of a novel high-purity,

double virus inactivated von Willebrand Factor and Factor VIII concentrate (Wilate®). *Biologicals*, *34*(4), 281-288. https://doi.org/10.1016/j.biologicals.2005.11.010

Starke, R. D., Paschalaki, K. E., Dyer, C. E., Harrison-Lavoie, K. J., Cutler, J. A., McKinnon, T. A., Millar, C. M., Cutler, D. F., Laffan, M. A., & Randi, A. M. (2013). Cellular and molecular basis of von Willebrand disease: studies on blood outgrowth endothelial cells. *Blood*, *121*(14), 2773-2784. https://doi.org/10.1182/blood-2012-06- 435727

Stenson, P. D., Mort, M., Ball, E. V., Chapman, M., Evans, K., Azevedo, L., Hayden, M., Heywood, S., Millar, D. S., Phillips, A. D., & Cooper, D. N. (2020). The Human Gene Mutation Database (HGMD®): optimizing its use in a clinical diagnostic or research setting. *Human genetics*, *139*(10), 1197-1207. https://doi.org/10.1007/s00439-013-1358-4

Stenson, P. D., Mort, M., Ball, E. V., Evans, K., Hayden, M., Heywood, S., Hussain, M., Phillips, A. D., & Cooper, D. N. (2017, March 27). The Human Gene Mutation Database: towards a comprehensive repository of inherited mutation data for medical research, genetic diagnosis and next-generation sequencing studies. *Human Genetics*, *136*, 665-677. https://doi.org/10.1007/s00439-017-1779-6

Stenson, P. D., Mort, M., Ball, E. V., Shaw, K., Phillips, A. D., & Cooper, D. N. (2014). The Human Gene Mutation Database: building a comprehensive mutation repository for clinical and molecular genetics, diagnostic testing and personalized genomic medicine. *Human genetics*, *133*(1), 1-9. https://doi.org/10.1007/s00439-013-1358-4

Stites, D. P., Herschgold, E. J., Perlman, J. D., & Fudenberg, H. H. (1971). Factor VIII detection by hemagglutination inhibition: hemophilia A and von Willebrand's disease. *Science*, *171*(3967), 196-197. https://www.science.org/doi/10.1126/science.171.3967.196

Stockschlaeder, M., Schneppenheim, R., & Budde, U. (2014). Update on von Willebrand factor multimers: focus on high-molecular-weight multimers and their role in hemostasis. *Blood Coagulation & Fibrinolysis*, *25*(3), 206-214.

Stormorken, H., & Erikssen, J. (1977). Plasma antithrombin III and factor VIII antigen in relation to angiographic findings, angina and blood groups in middle-aged men. *Thrombosis and haemostasis*, *38*(08), 0874-0880. https://doi.org/10.1055/s-0038-1651905

Streeter, I., Harrison, P. W., Faulconbridge, A., HipSci Consortium, Flicek, P., Parkinson, H., & Clarke, L. (2017). The human-induced pluripotent stem cell initiative—data resources for cellular genetics. *Nucleic acids research*, *45*(D1), D691-D697. https://doi.org/10.1093/nar/gkw928

Stufano, F., Lawrie, A. S., La Marca, S., Berbenni, C., Baronciani, L., & Peyvandi, F. (2014). A two−centre comparative evaluation of new automated assays for von Willebrand factor ristocetin cofactor activity and antigen. *Haemophilia*, *20*(1), 147-153. https://doi.org/10.1111/hae.12264

Sucker, C., Senft, B., Scharf, R. E., & Zotz, R. B. (2006). Determination of von Willebrand factor activity: evaluation of the HaemosIL™ assay in comparison with established procedures. *Clinical and applied thrombosis/hemostasis*, *12*(3), 305-310. https://doi.org/10.1177/1076029606291428

Sudlow, C., Gallacher, J., Allen, N., Beral, V., Burton, P., Danesh, J., Downey, P., Elliott, P., Green, J., Landray, M., Liu, B., Matthews, P., Ong, G., Pell, J., Silman, A., Young, A., Sprosen, T., Peakman, T., & Collins, R. (2015). UK biobank: an open access resource for identifying the causes of a wide range of complex diseases of middle and old age. *PLoS medicine*, *12*(3), e1001779. https://doi.org/DOI:10.1371/journal.pmed.1001779

Suehiro, Y., Veljkovic, D. K., Fuller, N., Motomura, Y., Massé, J. M., Cramer, E. M., & Hayward, C. P. (2005). Endocytosis and storage of plasma factor V by human megakaryocytes. *Thromb Haemost*, *94*, 585-592.

Sukhu, K., Martin, P. G., Cross, L., Keeling, D. M., & Giangrande, P. L. (2000). Evaluation of the von Willebrand factor antigen (vWF−Ag) assay using an immuno−turbidimetric method (STA Liatest® vWF) automated on the MDA® 180 coagulometer. *Clinical & Laboratory Haematology*, *22*(1), 29-32. https://doi.org/10.1046/j.1365-2257.2000.00281.x

Sutherland, M. S., Cumming, A. M., Bowman, M., Bolton-Maggs, P. H., Bowen, D. J., Collins, P. W., Hay, C. R., Will, A. M., & Keeney, S. (2009b). A novel deletion mutation is recurrent in von Willebrand disease types 1 and 3. *Blood*, *114*(5), 1091-1098. https://doi.org/10.1182/blood-2008-08-173278

Sutherland, M. S., Keeney, S., Bolton−Maggs, P. H., Hay, C. R., Will, A., & Cumming, A. M. (2009a). The mutation spectrum associated with type 3 von Willebrand disease in a cohort of patients from the north west of England. *Haemophilia*, *15*(5), 1048-1057. https://doi.org/10.1111/j.1365-2516.2009.02059.x

Swank, R. T., & Munkres, K. D. (1971). Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gel with sodium dodecyl sulfate. *Analytical biochemistry*, *39*(2), 462-477. https://doi.org/10.1016/0003-2697(71)90436-2

Swinkels, M., Atiq, F., Bürgisser, P. E., Slotman, J. A., Houtsmuller, A. B., de Heus, C., Klumperman, J., Leebeek, F. W., Voorberg, J., Jansen, A. J., & Bierings, R. (2021). Quantitative 3D microscopy highlights altered von Willebrand factor α-granule storage in patients with von Willebrand disease with distinct pathogenic mechanisms. *Res Pract Thromb Haemost*, *5*, e12595. https://doi.org/10.1002/rth2.12595

Swystun, L. L., & James, P. D. (2017). Genetic diagnosis in hemophilia and von Willebrand disease. *Blood Reviews*, *31*(1), 47-56. https://doi.org/10.1016/j.blre.2016.08.003

Swystun, L. L., Lai, J. D., Notley, C., Georgescu, I., Paine, A. S., Mewburn, J., Nesbitt, K., Schledzewski, K., Géraud, C., Kzhyshkowska, J., Goerdt, S., Hopman, W., Montgomery, R. R., James, P. D., & Lillicrap, D. (2018). The endothelial cell receptor stabilin-2 regulates VWF-FVIII complex half-life and immunogenicity. *The Journal of clinical investigation*, *128*(9), 4057-4073. https://doi.org/10.1172/JCI96400

Swystun, L. L., & Lillicrap, D. (2018). Genetic regulation of plasma von Willebrand factor levels in health and disease. *Journal of Thrombosis and Haemostasis*, *16*(12), 2375-2390. https://doi.org/10.1111/jth.14304

Swystun, L. L., Ogiwara, K., Lai, J. D., Ojala, J. R., Rawley, O., Lassalle, F., Notley, C., Rengby, O., Michels, A., Nesbitt, K., Tryggvason, K., & Lillicrap, D. (2019). The scavenger receptor SCARA 5 is an endocytic receptor for von Willebrand factor expressed by littoral cells in the human spleen. *Journal of Thrombosis and Haemostasis*, *17*(8), 1384-1396. https://doi.org/10.1111/jth.14521

Szulman, A. E. (1962). The histological distribution of the blood group substances in man as disclosed by immunofluorescence: II. The H antigen and its relation to A and B antigens. *The Journal of experimental medicine*, *115*(5), 977-996. https://doi.org/10.1084/jem.115.5.977

Tadaka, S., Hishinuma, E., Komaki, S., Motoike, I. N., Kawashima, J., Saigusa, D., Inoue, J., Takayama, J., Okamura, Y., Aoki, Y., Shirota, M., Otsuki, A., Katsuoka, F., Shimizu, A., Tamiya, G., Koshiba, S., Sasaki, M., Yamamoto, M., & Kinoshita, K. (2021). jMorp updates in 2020: large enhancement of

multi-omics data resources on the general Japanese population. *Nucleic acids research*, *49*(D1), D536-D544. https://doi.org/10.1093/nar/gkaa1034

Takahashi, H. (1980). Studies on the pathophysiology and treatment of von Willebrand's disease. IV. Mechanism of increased ristocetin-induced platelet aggregation in von Willebrand's disease. *Thrombosis research*, *19*(6), 857-867. https://doi.org/10.1016/0049-3848(80)90013-4

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., & Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*, *131*(5), 861-872. https://doi.org/10.1016/j.cell.2007.11.019

Talukder, B., Datta, S. S., Mukherjee, S., & Mukherjee, K. (2014). Prevalence of Bombay Group Blood in Southern Bengal Population. *Prevalence of bombay group blood in southern bengal population*, *30*(3), 149. https://doi.org/10.1007/s12288-013-0292-x

Tang, W., Cushman, M., Green, D., Rich, S. S., Lange, L. A., Yang, Q., Tracy, R. P., Tofler, G. H., Basu, S., Wilson, J. G., Keating, B. J., Weng, L.-C., Taylor, H. A., Jacobs Jr, D. R., Delaney, J. A., Palmer, C. D., Young, T., Pankow, J. S., O'Donnell, C. J., … Folsom, A. R. (2015). Gene−centric approach identifies new and known loci for F VIII activity and VWF antigen levels in E uropean Americans and African Americans. *American journal of hematology*, *90*(6), 534-540. https://doi.org/10.1002/ajh.24005

Tanida, I., Mizushima, N., Kiyooka, M., Ohsumi, M., Ueno, T., Ohsumi, Y., & Kominami, E. (1999). Apg7p/Cvt2p: A novel protein-activating enzyme essential for autophagy. *Molecular biology of the cell*, *10*(5), 1367-1379. https://doi.org/10.1091/mbc.10.5.1367

Tatsumi, R., Suzuki, Y., Sumi, T., Sone, M., Suemori, H., & Nakatsuji, N. (2011). Simple and highly efficient method for production of endothelial cells from human embryonic stem cells. *Cell transplantation*, *20*(9), 1423-1430. https://doi.org/10.3727/096368910X547444

Taura, D., Sone, M., Homma, K., Oyamada, N., Takahashi, K., Tamura, N., Yamanaka, S., & Nakao, K. (2009). Induction and isolation of vascular cells from human induced pluripotent stem cells—brief report. *Arteriosclerosis, thrombosis, and vascular biology*, *29*(7), 1100-1103. https://doi.org/10.1161/ATVBAHA.108.182162

Teixeira, L., Ferreira, C., Santos, B. S., & Saavedra, V. (2012). Web−enabled registry of inherited bleeding disorders in Portugal: conditions and perception of the patients. *Haemophilia*, *18*(1), 56-62. https://doi.org/10.1111/j.1365-2516.2011.02574.x

Tennessen, J. A., Bigham, A. W., O'Connor, T., Fu, W., Kenny, E. E., Gravel, S., McGee, S., Do, R., Liu, X., Jun, G., Kang, H. Y., Jordan, D., Leal, S. M., Gabriel, S., Rieder, M. J., Abecasis, G., Altshuler, D., Nickerson, D. A., Boerwinkle, E., … NHLBI Exome Sequencing Project. (2012). Evolution and functional impact of rare coding variation from deep sequencing of human exomes. *Science*, *337*(6090), 64-69. https://doi.org/10.1126/science.1219240

Tetas, M., Chao, H., & Molnar, J. (1970). Incorporation of carbohydrates into endogenous acceptors of liver microsomal fractions. *Archives of biochemistry and biophysics*, *138*(1), 135-146. https://doi.org/10.1016/0003-9861(70)90292-4

Therneau, T., & Grambsch, P. (2000). The cox model. In *Modeling Survival Data: Extending the Cox Model* (pp. 39-77). Springer New York. DOI https://doi.org/10.1007/978-1-4757-3294-8

Tian, B., Hu, J., Zhang, H., & Lutz, C. S. (2005). A large-scale analysis of mRNA polyadenylation of human and mouse genes. *Nucleic Acids Research*, *33*(1), 201-212. https://doi.org/10.1093/nar/gki158

Tiemeier, G. L., de Koning, R., Wang, G., Kostidis, S., Rietjens, R. G., Sol, W. M., Dumas, S. J., Giera, M., van den Berg, C. W., Eikenboom, J. C., van den Berg, B. M., Carmeliet, P., & Rabelink, T. J. (2020). Lowering the increased intracellular pH of human-induced pluripotent stem cell-derived endothelial cells induces formation of mature Weibel-Palade bodies. *Stem cells translational medicine*, *9*(7), 758-772. https://doi.org/10.1002/sctm.19-0392

Tischer, A., Madde, P., Moon-Tasson, L., & Auton, M. (2014). Misfolding of vWF to pathologically disordered conformations impacts the severity of von Willebrand disease. *Biophysical journal*, *107*(5), 1185-1195. https://doi.org/10.1016/j.bpj.2014.07.026

Titani, K., Kumar, S., Takio, K., Ericsson, L. H., Wade, R. D., Ashida, K., Walsh, K. A., Chopek, M. W., Sadler, J. E., & Fujikawa, K. (1986). Amino acid sequence of human von Willebrand factor. *Biochemistry*, *25*(11), 3171-3184. https://doi.org/10.1021/bi00359a015

Torisu, T., Torisu, K., Lee, H. I., Liu, J., Malide, D., Combs, C. A., Wu, X. S., Rovira, I. I., Fergusson, M. M., Weigert, R., Connelly, P. S., Daniels, M. P., Komatsu, M., Cao, L., & Finkel, T. (2013).

Autophagy regulates endothelial cell processing, maturation and secretion of von Willebrand factor. *Nature medicine*, *19*(10), 1281. https://doi.org/10.1038/nm.3288

Tosetto, A. (2013). The Role of Bleeding History and Clinical Markers for the Correct Diagnosis of VWD. *Mediterr J Hematol Infect Dis*, *5*(1), e2013051. https://doi.org/10.4084/mjhid.2013.051

Tosetto, A., Badiee, Z., Baghaipour, M.-R., Baronciani, L., Battle, J., Berntop, E., Bodó, I., Budde, U., Castaman, G., Eikenboom, J. C., Eshghi, P., Ettore, C., Goodeve, A., Goudemand, J., Hay, C. R., Hoorfar, H., Karimi, M., Keikhaei, B., Lassila, R., … Federici, A. B. (2020). Bleeding symptoms in patients diagnosed as type 3 von Willebrand disease: Results from 3WINTERS-IPS, an international and collaborative cross-sectional study. *Journal of Thrombosis and Haemostasis,*, *18*(9), 2145-2154. https://doi.org/10.1111/jth.14886

Tosetto, A., & Castaman, G. (2015). How I treat type 2 variant forms of von Willebrand disease. *Blood*, *125*(6), 907-914. https://doi.org/10.1182/blood-2014-08-551960

Tosetto, A., Castaman, G., & Rodeghiero, F. (2007). Assessing bleeding in von Willebrand disease with bleeding score. *Blood reviews*, *21*(2), 89-97. https://doi.org/10.1016/j.blre.2006.04.002

Tosetto, A., Castman, G., & Rodeghiero, F. (2008). Bleeding scores in inherited bleeding disorders: clinical or research tools? *Haemophilia*, *14*(3), 415-422. https://doi.org/DOI: 10.1111/j.1365-2516.2007.01648.x

Tosetto, A., Castman, G., & Rodeghiero, F. (2013). Bleeders, bleeding rates, and bleeding score. *Journal of Thrombosis and Haemostasis*, *11*, 142-150. https://doi.org/10.1111/jth.12248

Tosetto, A., Rodeghiero, F., Castaman, G., Goodeve, A., Federici, A. B., Batlle, J., Meyer, D., Fressinaud, E., Mazurier, C., Goudemand, J., Eikenboom, J., Schneppenheim, R., Budde, U., Ingerslev, J., Vorlova, Z., Habart, D., Holmberg, L., Lethagen, S., Pasi, J., … Peake, I. (2006). A quantitative analysis of bleeding symptoms in type 1 von Willebrand disease: results from a multicenter European study (MCMDM−1 VWD). *Journal of Thrombosis and Haemostasis*, *4*, 776-773. https://doi.org/10.1111/j.1538-7836.2006.01847.x

Toupance, S., Simonici, S., Labat, C., Dumoulin, C., Lai, T. P., Lakomy, C., Regnault, V., Lacolley, P., George, F. D., Sabatier, F., Aviv, A., Benetos, A., & TELARTA consortium. (2021). Number and Replating Capacity of Endothelial Colony−Forming Cells are Telomere Length Dependent:

Implication for Human Atherogenesis. *Journal of the American Heart Association*, *10*(10), e020606. https://doi.org/10.1161/JAHA.120.020606

Tous, J., Barry, R. G., Arnout, J., Vanrusselt, M., Pascual, Z., & Serra, J. (2009). New automated chemiluminescent VWF: Ag and VWF: RCo assays: preliminary analytical and clinical performance. *J Thromb Haemost*, *7 (Suppl 2)*(1143).

Tran, T., Arnall, J., Moore, D. C., Ward, L., Palkimas, S., & Man, L. (2020). Vonicog Alfa for the Management of Von Willebrand Disease: A Comprehensive Review and Single-Center Experience. *Journal of thrombosis and thrombolysis*, *49*(3), 431-440. https://doi.org/10.1007/s11239-019-02018-1

Trasi, S., Shetty, S., Ghosh, K., & Mohanty, D. (2005). Prevalence & spectrum of von Willebrand disease from western India. *Indian Journal of Medical Research*, *121*(5), 653-658.

Tsai, H. M. (1996). Physiologic cleavage of von Willebrand factor by a plasma protease is dependent on its conformation and requires calcium ion. *Blood*, *87*(10), 4235-4244. https://doi.org/10.1182/blood.V87.10.4235.bloodjournal87104235

Tung, J. W., Heydari, K., Tirouvanziam, R., Sahaf, B., Parks, D. R., Herzenberg, L. A., & Herzenberg, L. A. (2007). Modern flow cytometry: a practical approach. *Clinics in laboratory medicine*, *27*(3), 453-468. https://doi.org/10.1016/j.cll.2007.05.001

Turecek, P. L., Mitterer, A., Matthiessen, H. P., Gritsch, H., Varadi, K., Siekmann, J., Schnecker, K., Plaimauer, B., Kaliwoda, M., Purtscher, M., Woehrer, W., Mundt, W., Muchitsch, E.-M., Suiter, T., Ewenstein, B. M., Ehrlich, H. J., & Schwarz, H. P. (2009). Development of a plasma-and albumin-free recombinant von Willebrand factor. *Hämostaseologie*, *29*(S01), S32-S38. https://doi.org/10.1055/s-0037-1617202

Turecek, P. L., Romeder-Finger, S., Apostol, C., Bauer, A., Crocker−Buque, A., Burger, D. A., Schall, R., & Gritsch, H. (2016). A world−wide survey and field study in clinical haemostasis laboratories to evaluate FVIII: C activity assay variability of ADYNOVATE and OBIZUR in comparison with ADVATE. *Haemophilia*, *22*(6), 957-965. https://doi.org/10.1111/hae.13001

Turner, N. A., & Moake, J. L. (2015). Factor VIII is synthesized in human endothelial cells, packaged in Weibel-Palade bodies and secreted bound to ULVWF strings. *PloS one*, *10*(10), e0140740.

Turro, E., Astle, W. J., Megy, K., Gräf, S., Greene, D., Shamardina, O., Lango Allen, H., Sanchis-Juan, A., Frontini, M., Thys, C., Stephens, J., Mapeta, R., Burren, O. S., Downes, K., Haimel, M., Tuna, S., Deevi, S. V., Aitman, T. J., Bennett, D. L., … Ouwehand, W. H. (2020). Whole-genome sequencing of patients with rare diseases in a national health system. *Nature*, *583*(7814), 96-102. https://doi.org/10.1038/s41586-020-2434-2

The UK10K Consortium. (2015). The UK10K project identifies rare variants in health and disease. *Nature*, *526*(7571), 82-89. https://doi.org/10.1038/nature14962

UK Biobank. (2007, March 21). *UK Biobank: Protocol for a large-scale prospective epidemiological resource*. https://www.ukbiobank.ac.uk. Retrieved Nov 01, 2021, from https://www.ukbiobank.ac.uk/media/gnkeyh2q/study-rationale.pdf

UK Biobank. (2020a, July). *Category 2000 Hospital inpatient - Health-related outcomes*. Showcase. Retrieved October 26, 2021, from https://biobank.ndph.ox.ac.uk/showcase/label.cgi?id=2000

UK Biobank. (2020b, August). *Hospital inpatient data*. Hospital inpatient data. Retrieved October 26, 2021, from https://biobank.ndph.ox.ac.uk/showcase/ukb/docs/HospitalEpisodeStatistics.pdf

UK Biobank. (2021, Oct 26). *Location of UK Biobank assessment centres throughout the United Kingdom*. https://biobank.ndph.ox.ac.uk/. Retrieved Nov 01, 2021, from https://biobank.ndph.ox.ac.uk/ukb/exinfo.cgi?src=UKB_centres_map

UK NEQAS. (2017). *U.K. National External Quality Assessment Scheme for Blood Coagulation Report on Survey 227*. UK NEQAS.

UniProt. (2011, Jan 11). *VWF_HUMAN von Willebrand factor*. Retrieved May 26, 2020, from https://www.uniprot.org/uniprot/P04275.fasta

UniProt. (2021, April 07). *UniProtKB - O95352 (ATG7_HUMAN)*. https://www.uniprot.org. Retrieved April 15, 2021, from https://www.uniprot.org/uniprot/O95352

United Kingdom Haemophilia Centre Doctors' Organisation 2021. (2021, October). *UKHCDO Annual Report 2020 & Bleeding Disorder Statistics for 2020/202*. United Kingdom Haemophilia Centre Doctors' Organisation. http://www.ukhcdo.org/wp-content/uploads/2021/12/2021-UKHCDO-Annual-Report-2020-21-Data.pdf

van Breevoort, D., Snijders, A. P., Hellen, N., Weckhuysen, S., van Hooren, K. W., Eikenboom, J., Valentijn, K., Fernandez-Borja, M., Ceulemans, B., De Jonghe, P., Voorberg, J., Hannah, M., Carter, T., & Bierings, R. (2014). STXBP1 promotes Weibel-Palade body exocytosis through its interaction with the Rab27A effector Slp4-a. *Blood*, *123*(20), 3185-3194. https://doi.org/10.1182/blood-2013-10-535831

Van Craenenbroeck, E. M., Conraads, V. M., Van Bockstaele, D. R., Haine, S. E., Vermeulen, K., Van Tendeloo, V. F., Vrints, C. J., & Hoymans, V. Y. (2008). Quantification of circulating endothelial progenitor cells: a methodological comparison of six flow cytometric approaches. *Journal of Immunological Methods*, *332*(1-2), 31-40. https://doi.org/10.1016/j.jim.2007.12.006

Van De Berg, T., Todaro, A., van Beers, J., Wichapong, K., Heubel-Moenen, F., Castoldi, E., Henskens, Y. M., & Beckers, E. A. (2021). A Novel Quali-Quantitative Defect of VWF. *Blood*, *138*, 1032. https://doi.org/10.1182/blood-2021-147899

Vandenbroucke, E., Mehta, D., Minshall, R., & Malik, A. B. (2008). Regulation of endothelial junctional permeability. *Annals of the new York Academy of Sciences*, *1123*(1), 134-145. https://doi.org/10.1196/annals.1420.016

Vanderperre, B., Lucier, J. F., Bissonnette, C., Motard, J., Tremblay, G., Vanderperre, S., Wisztorski, M., Salzet, M., Boisvert, F.-M., & Roucou, X. (2013). Direct detection of alternative open reading frames translation products in human significantly expands the proteome. *PloS one*, *8*(8), e70698. https://doi.org/10.1371/journal.pone.0070698

van der Velde, K. J., Kuiper, J., Thompson, B. A., Plazzer, J. P., van Valkenhoef, G., de Haan, M., Jongbloed, J. D., Wijmenga, C., de Koning, T. J., Abbott, K. M., Sinke, R., Spurdle, A. B., Macrae, F., Genuardi, M., Sijmons, R. H., A, M., & InSiGHT Group. (2015). Evaluation of CADD scores in curated mismatch repair gene variants yields a model for clinical validation and prioritization. *Human mutation*, *36*(7), 712-719. https://doi.org/10.1002/humu.22798

van Galen, K. P., Engelen, E. T., Mauser-Bunschoten, E. P., van Es, R. J., & Schutgens, R. E. (2019). Antifibrinolytic therapy for preventing oral bleeding in patients with haemophilia or Von Willebrand disease undergoing minor oral surgery or dental extractions. *Cochrane Database of Systematic Reviews*, (4), Art. No.: CD011385. https://doi.org/10.1002/14651858.CD011385.pub3

van Galen, K. P., Sanders, Y. V., Vojinovic, U., Eikenboom, J., Cnossen, M. H., Schutgens, R. E., van der Bom, J. G., Fijnvandraat, K., Laros-Van Gorkom, B. A., Meijer, K., Leebeek, F. W., Mauser-Bunschoten, E. P., & WiN Study Group. (2015). Joint bleeds in von Willebrand disease patients have significant impact on quality of life and joint integrity: a cross−sectional study. *Haemophilia*, *21*(3), e185-e192. https://doi.org/10.1111/hae.12670

van Geffen, M., & van Heerde, W. L. (2012). Global haemostasis assays, from bench to bedside. *Thrombosis research*, *129*(6), 681-687. https://doi.org/10.1016/j.thromres.2011.12.006

Vangenechten, I., & Gadisseur, A. (2020). Improving diagnosis of von Willebrand disease: Reference ranges for von Willebrand factor multimer distribution. *Research and practice in thrombosis and haemostasis*, *4*(6), 1024-1034. https://doi.org/10.1002/rth2.12408

Vangenechten, I., Mayger, K., Smejkal, P., Zapletal, O., Michiels, J. J., Moore, G. W., & Gadisseur, A. (2018). A comparative analysis of different automated von Willebrand factor glycoprotein Ib−binding activity assays in well typed von Willebrand disease patients. *Journal of Thrombosis and Haemostasis*, *16*(7), 1268-1277. https://doi.org/10.1111/jth.14145

Vangenechten, I., Smejkal, P., Zapletal, O., Michiels, J. J., Berneman, Z., Zavrelova, J., Blatný, J., Penka, M., & Gadisseur, A. (2019). Analysis of von Willebrand disease in the South Moravian population (Czech Republic): results from the BRNO-VWD study. *Thrombosis and haemostasis*, *119*(04), 594-605. https://doi.org/10.1055/s-0039-1678528

Vanhoorelbeke, K., Cauwenberghs, N., Vauterin, S., Schlammadinger, A., Mazurier, C., & Deckmyn, H. (2000). A reliable and reproducible ELISA method to measure ristocetin cofactor activity of von Willebrand factor. *Thrombosis and haemostasis*, *83*(01), 107-113. https://doi.org/10.1055/s-0037-1613765

Van Hout, C. V., Tachmazidou, I., Backman, J. D., Hoffman, J. D., Liu, D., Pandey, A. K., Gonzaga-Jauregui, C., Khalid, S., Ye, B., Banerjee, N., Li, A. H., O'Dushlaine, C., Marcketta, A., Staples, J., Schurmann, C., Hawes, A., Maxwell, E., Barnard, L., Lopez, A., … Baras, A. (2020). Exome sequencing and characterization of 49,960 individuals in the UK Biobank. *Nature*, *586*(7831), 749-756. https://doi.org/10.1038/s41586-020-2853-0

van IJzendoorn, D. G., Salvatori, D. C., Cao, X., van den Hil, F., Briaire-de Bruijn, H. I., de Jong, D., Mei, H., Mummery, C. L., Szuhai, K., Bove, J. V., & Orlova, V. V. (2020). Vascular Tumor Recapitulated

in Endothelial Cells from hiPSCs Engineered to Express the SERPINE1-FOSB Translocation. *Cell Reports Medicine*, *1*(9), 100153. https://doi.org/10.1016/j.xcrm.2020.100153

van Loon, J., Dehghan, A., Weihong, T., Trompet, S., McArdle, W. L., Asselbergs, F. W., Folkert, W., Chen, M.-H., Lopez, L. M., Huffman, J. E., Leebeek, F. W., Basu, S., Stott, D. J., Rumley, A., Gansevoort, R. T., Davies, G., Wilson, J. J., Witteman, J. C., Cao, X., … O'Donnell, C. (2016). Genome-wide association studies identify genetic loci for low von Willebrand factor levels. *European Journal of Human Genetics*, *24*(7), 1035-1040. https://doi.org/10.1038/ejhg.2015.222

van Loon, J. E., de Jaegere, P. P., van Vliet, H. H., de Maat, M. P., de Groot, P. G., Simoons, M. L., & Leebeek, F. W. (2011). The in vitro effect of the new antithrombotic drug candidate ALX-0081 on blood samples of patients undergoing percutaneous coronary intervention. *Thrombosis and haemostasis*, *106*(07), 165-171. https://doi.org/10.1160/TH10-12-0804

Vassilakos, A., Michalak, M., Lehrman, M. A., & Williams, D. B. (1998). Oligosaccharide binding characteristics of the molecular chaperones calnexin and calreticulin. *Biochemistry*, *37*(10), 3480-3490. https://doi.org/10.1021/bi972465g

Vehar, G. A., & Davie, E. W. (1980). Preparation and properties of bovine factor VIII (antihemophilic factor). *Biochemistry*, *19*(3), 401-410. https://doi.org/10.1021/bi00544a001

Verfaillie, C. J., Witte, E. D., & Devreese, K. M. (2013). Validation of a new panel of automated chemiluminescence assays for von Willebrand factor antigen and activity in the screening for von Willebrand disease. *International journal of laboratory hematology*, *35*(5), 555-565. https://doi.org/10.1111/ijlh.12087

Verhoef, J. J., Barendrecht, A. D., Nickel, K. F., Dijkxhoorn, K., Kenne, E., Labberton, L., McCarty, O. J., Schiffelers, R., Heijnen, H. F., Hendrickx, A. P., Schellekens, H., Fens, M. H., de Maat, S., Renne, T., & Maas, C. (2017). Polyphosphate nanoparticles on the platelet surface trigger contact system activation. *Blood*, *129*(12), 1707-1717. https://doi.org/10.1182/blood-2016-08-734988

Verweij, C. L., de Vries, C. J., Distel, B., van Zonneveld, J. A., van Kessel, A. G., van Mourik, J. A., & Pannekoek, H. (1985). Construction of cDNA coding for human von Willebrand factor using antibody probes for colony-screening and mapping of the chromosomal gene. *Nucleic Acids Research*, *13*(13), 4699-4717. https://doi.org/10.1093/nar/13.13.4699

Verweij, C. L., Diergaarde, P. J., Hart, M., & Pannekoek, H. (1986). Full−length von Willebrand factor (vWF) cDNA encodes a highly repetitive protein considerably larger than the mature vWF subunit. *The EMBO journal*, *5*(8), 1839-1847. https://doi.org/10.1002/j.1460-2075.1986.tb04435.x

Veyradier, A., Boisseau, P., Fressinaud, E., Caron, C., Ternisien, C., Giraud, M., Zawadzki, C., Trossaert, M., Itzhar-Baikian, N., Dreyfus, M., d'Oiron, R., Borel-Derlon, A., Susen, S., Bezieau, S., Denis, C. V., & Goudemand, J. (2016). A laboratory phenotype/genotype correlation of 1167 French patients from 670 families with von Willebrand disease: a new epidemiologic picture. *Medicine*, *95*(11), e3038. https://doi.org/10.1097/MD.0000000000003038

Veyradier, A., Caron, C., Ternisien, C., Wolf, M., Trossaert, M., Fressinaud, E., & Goudemand, J. (2011). Validation of the first commercial ELISA for type 2N von Willebrand's disease diagnosis. *Haemophilia*, *17*(6), 944-951. https://doi.org/10.1111/j.1365-2516.2011.02499.x

Veyradier, A., Fressinaud, E., Sigaud, M., Wolf, M., & Meyer, D. (1999). A New Automated Method for von Willebrand Factor Antigen Measurement Using Latex Particles. *Thromb Haemost*, *81*(02), 320-321. https://doi.org/10.1055/s-0037-1614468

Villa, P., Iborra, J., Serra, J., Gilsanz, A., Casaña, P., Aznar, J. A., & Aznar, J. (2001). Evaluation of an automated method for the quantification of von Willebrand factor antigen. *Haematologica*, *86*(11), 1180-1185.

Vischer, U. M., & Wagner, D. D. (1994). von Willebrand factor proteolytic processing and multimerization precede the formation of Weibel-Palade bodies. *Blood*, *83*(12), 3536-3544. https://doi.org/10.1182/blood.V83.12.3536.3536

Vlahos, C. J., Matter, W. F., Hui, K. Y., & Brown, R. F. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *Journal of Biological Chemistry*, *269*(7), 5241-5248. https://doi.org/10.1016/S0021-9258(17)37680-9

Vodyanik, M. A., & Slukvin, I. I. (2007). Hematoendothelial differentiation of human embryonic stem cells. *Current protocols in cell biology*, *36*(1), 23-26. https://doi.org/10.1002/0471143030.cb2306s36

Voelkerding, K. V., Dames, S., & Durtschi, J. D. (2010). Next generation sequencing for clinical diagnostics-principles and application to targeted resequencing for hypertrophic cardiomyopathy: a paper from the 2009 William Beaumont Hospital Symposium on Molecular Pathology. *The Journal of molecular diagnostics*, *12*(5), 539-551. https://doi.org/10.2353/jmoldx.2010.100043

von Heijne, G. (1990). The signal peptide. *The Journal of membrane biology*, *115*(3), 195-201.

von Willebrand, E. A. (1926). Hereditär pseudohemofili. *Fin Läkaresällsk Handl*, *68*, 87-112.

Voorberg, J., Fontijn, R., Calafat, J., Janssen, H., Van Mourik, J. A., & Pannekoek, H. (1991). Assembly and routing of von Willebrand factor variants: the requirements for disulfide-linked dimerization reside within the carboxy-terminal 151 amino acids. *The Journal of cell biology*, *113*(1), 195-205. https://doi.org/10.1083/jcb.113.1.195

Waechter, C. J., & Lennarz, W. J. (1976). The role of polyprenol-linked sugars in glycoprotein synthesis. *Annual review of biochemistry*, *45*(1), 95-112. https://doi.org/10.1146/annurev.bi.45.070176.000523

Wagner, D. D. (1990). Cell biology of von Willebrand factor. *Annual review of cell biology*, *6*(1), 217-242.

Wagner, D. D., Fay, P. J., Sporn, L. A., Sinha, S., Lawrence, S. O., & Marder, V. J. (1987). Divergent fates of von Willebrand factor and its propolypeptide (von Willebrand antigen II) after secretion from endothelial cells. . *Proceedings of the National Academy of Sciences*, *84*(7), 1955-1959. https://doi.org/10.1073/pnas.84.7.1955

Wagner, D. D., Mayadas, T., & Marder, V. J. (1986). Initial Glycosylation and Acidic pH in the Golgi Apparatus are Required for Multimerization of von Willebrand Factor. *The Journal of Cell Biology*, *102*(4), 1320-1324. https://doi.org/10.1083/jcb.102.4.1320

Wagner, D. D., Olmsted, J. B., & Marder, V. J. (1982). Immunolocalization of von Willebrand protein in Weibel-Palade bodies of human endothelial cells. *The Journal of cell biology*, *95*(1), 355-360. https://doi.org/10.1083/jcb.95.1.355

Wagner, D. D., Saffaripour, S., Bonfanti, R., Sadler, J. E., Cramer, E. M., Chapman, B., & Mayadas, T. N. (1991). Induction of specific storage organelles by von Willebrand factor propolypeptide. *Cell*, *64*(2), 403-413. https://doi.org/10.1016/0092-8674(91)90648-I

Wahlberg, T., Blombäck, M., Hall, P., & Axelsson, G. (1980). Application of indicators, predictors and diagnostic indices in coagulation disorders. *Methods of information in medicine*, *19*(04), 194-200. https://doi.org/10.1055/s-0038-1635279

Wahlberg, T. B. (1984). A method for the evaluation of clinical information, exemplified for bleeding symptoms in non-severe von Willebrand's disease type I. *Methods of information in medicine*, *23*(03), 143-146. https://doi.org/10.1055/s-0038-1635333

Walsh, R., Thomson, K. L., Ware, J. S., Funke, B. H., Woodley, J., McGuire, K. J., Mazzarotto, F., Blair, E., Seller, A., Taylor, J. C., Minkel, E. V., Exome Aggregation Consortium, MacArthur, D. G., Farrall, M., Cook, S. A., & Watkins, H. (2017). Reassessment of Mendelian gene pathogenicity using 7,855 cardiomyopathy cases and 60,706 reference samples. *Genetics in Medicine*, *19*(2), 192-203. https://doi.org/10.1038/gim.2016.90

Wang, D., Eraslan, B., Wieland, T., Hallström, B., Hopf, T., Zolg, D. P., Zecha, J., Asplund, A., Li, L., Meng, C., Frejno, M., Schmidt, T., Schnatbaum, K., Wilhelm, M., Ponten, F., Uhlen, M., Gagneur, J., Hahne, H., & Kuster, B. (2019). A deep proteome and transcriptome abundance atlas of 29 healthy human tissues. *Molecular systems biology*, *15*(2), e8503. https://doi.org/10.15252/msb.20188503

Wang, J., & Milner, R. (2006). Fibronectin promotes brain capillary endothelial cell survival and proliferation through α5β1 and αvβ3 integrins via MAP kinase signalling. *Journal of Neurochemistry*, *96*(1), 148-159. https://doi.org/10.1111/j.1471-4159.2005.03521.x

Wang, J.-W., Bouwens, E. A., Pintao, M. C., Voorberg, J., Safdar, H., Valentijn, K. M., de Boer, H. C., Mertens, K., Rietsma, P. H., & Eikenboom, J. (2013b). Analysis of the storage and secretion of von Willebrand factor in blood outgrowth endothelial cells derived from patients with von Willebrand disease. *Blood*, *121*(14), 2762-2772. https://doi.org/10.1182/blood-2012-06-434373

Wang, J. W., Valentijn, K. M., de Boer, H. C., Dirven, R. J., van Zonneveld, A. J., Koster, A. J., Voorberg, J., Reitsma, P. H., & Eikenboom, J. (2011). Intracellular storage and regulated secretion of von Willebrand factor in quantitative von Willebrand disease. *Journal of Biological Chemistry*, *286*(27), 24180-24188. https://doi.org/10.1074/jbc.M110.215194

Wang, Q. Y., Song, J., Gibbs, R. A., Boerwinkle, E., Dong, J. F., & Yu, F. L. (2013). Characterizing polymorphisms and allelic diversity of von Willebrand factor gene in the 1000 Genomes. *Journal of thrombosis and haemostasis*, *11*(2), 261-269. https://doi.org/10.1111/jth.12093

Wang, Y., Özen, G., Mellgård, B., Marier, J. F., Barriere, O., Vasilinin, G., & Bhattacharya, I. (2021). Exposure-Response Relationship between Von Willebrand Factor (VWF) Activity and Bleeding Events Following Prophylaxis with Recombinant VWF (rVWF, vonicog alfa) in Patients with Severe Von Willebrand Disease (VWD). *Blood*, *138*(Supplement 1), 2104. https://doi.org/10.1182/blood-2021-146153

Ward, S., O'Sullivan, J. M., & O'Donnell, J. S. (2019). von Willebrand factor sialylation—A critical regulator of biological function. *Journal of Thrombosis and Haemostasis*, *17*, 1018-1029.

Ward, S. E., O'Sullivan, J. M., & O'Donnell, J. S. (2020). The relationship between ABO blood group, von Willebrand factor, and primary hemostasis. *Blood*, *136*(25), 2864-2874. https://doi.org/10.1182/blood.2020005843

Watanabe, K., Ueno, M., Kamiya, D., Nishiyama, A., Matsumura, M., Wataya, T., Takahashi, J. B., Nishikawa, S., Nishikawa, S.-I., Muguruma, K., & Sasai, Y. (2007). A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nature biotechnology*, *25*(6), 681-686. https://doi.org/10.1038/nbt1310

Weibel, E. R., & Palade, G. E. (1964). New cytoplasmic components in arterial endothelia. *The Journal of cell biology*, *23*(1), 101-112.

Weidberg, H., Shvets, E., Shpilka, T., Shimron, F., Shinder, V., & Elazar, Z. (2010). LC3 and GATE−16/GABARAP subfamilies are both essential yet act differently in autophagosome biogenesis. *The EMBO journal*, *29*(11), 1792-1802. https://doi.org/10.1038/emboj.2010.74

Weiss, H. J., Baumgartner, H. R., Tschopp, T. B., Turitto, V. T., & Cohen, D. (1978). Correction by factor VIII of the impaired platelet adhesion to subendothelium in von Willebrand disease. *Blood*, *51*(2), 267-279. https://doi.org/10.1182/blood.V51.2.267.267

Weiss, H. J., Hoffmann, T., Yoshioka, A., & Ruggeri, Z. M. (1993). Evidence that the arg1744 gly1745 asp1746 sequence in the GPIIb-IIIa-binding domain of von Willebrand factor is involved in platelet adhesion and thrombus formation on subendothelium. *The Journal of laboratory and clinical medicine*, *122*(3), 324-332. https://doi.org/10.5555/uri:pii:002221439390079E

Weiss, H. J., Hoyer, L. W., Rickles, F. R., Varma, A., & Rogers, J. (1973). Quantitative assay of a plasma factor deficient in von Willebrand's disease that is necessary for platelet aggregation Quantitative Assay of a Plasma Factor Deficient in von Willebrand's Disease that is Necessary for Platelet Aggregation. *Journal of clinical investigation*, *52*(11), 2708-2716. https://doi.org/10.1172/JCI107465

Weiss, H. J., Meyer, D., Rabinowitz, R., Pietu, G., Girma, J. P., Vicic, W. J., & Rogers, J. (1982). Pseudo-von Willebrand's disease: an intrinsic platelet defect with aggregation by unmodified human factor

VIII/von Willebrand factor and enhanced adsorption of its high-molecular-weight multimers. *New England Journal of Medicine*, *306*(6), 326-333. https://doi.org/10.1056/NEJM198202113060603

Weiss, H. J., Sussman, I. I., & Hoyer, L. W. (1977). Stabilization of factor VIII in plasma by the von Willebrand factor: studies on posttransfusion and dissociated factor VIII and in patients with von Willebrand's disease. *The Journal of clinical investigation*, *60*(2), 390-404. https://doi.org/10.1172/JCI108788

Werner, E. J., Broxson, E. H., Tucker, E. L., Giroux, D. S., Shults, J., & Abshire, T. C. (1993). Prevalence of von Willebrand disease in children: a multiethnic study. *The Journal of pediatrics*, *123*(6), 893-898. https://doi.org/10.1016/S0022-3476(05)80384-1

Westbury, S. K., Turro, E., Greene, D., Lentaigne, C., Kelly, A. M., Bariana, T. K., Simeoni, I., Pillois, X., Attwood, A., Austin, S., Jansen, S. B., Bakchoul, T., Crisp-Hihn, A., Erber, W. N., Favier, R., Foad, N., Gattens, M., Jolley, J. D., Liesner, R., … Freson, K. (2015). Human phenotype ontology annotation and cluster analysis to unravel genetic defects in 707 cases with unexplained bleeding and platelet disorders. *Genome medicine*, *7*(1), 1-15. https://doi.org/10.1186/s13073-015-0151-5

Whiffin, N., Minikel, E., Walsh, R., O'Donnell-Luria, A. H., Karczewski, K., Ing, A. Y., Barton, P. R., Funke, B., Cook, S. A., MacArthur, D., & Ware, J. S. (2017). Using high-resolution variant frequencies to empower clinical genome interpretation. *Genetics in Medicine*, *19*(10), 1151-1158. https://doi.org/10.1038/gim.2017.26

White, G. C. (2003). The partial thromboplastin time: defining an era in coagulation. *Journal of Thrombosis and Haemostasis*, *1*(11), 2267-2270. https://doi.org/10.1046/j.1538-7836.2003.00459.x

Wilcock, J. (2015). New oral antiplatelets and anticoagulants in general practice: Part 1. *British Journal of Family Medicine*, *3*(5).

Wilkinson, B., & Gilbert, H. F. (2004). Protein disulfide isomerase. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, *1699*(1-2), 35-44. https://doi.org/10.1016/j.bbapap.2004.02.017

Williams, I. M., & Wu, J. C. (2019). Generation of endothelial cells from human pluripotent stem cells: methods, considerations, and applications. *Arteriosclerosis, thrombosis, and vascular biology*, *39*(7), 1317-1329. https://doi.org/10.1161/ATVBAHA.119.312265

Williams, S. B., McKeown, L. P., Krutzsch, H., Hansmann, K., & Gralnick, H. R. (1994). Purification and characterization of human platelet von Willebrand factor. *British journal of haematology*, *88*(3), 582-591. https://doi.org/10.1111/j.1365-2141.1994.tb05077.x

Williams, S. R., Hsu, F. C., Keene, K. L., Chen, W. M., Dzhivhuho, G., Rowles III, J. L., Southerland, A. M., Furie, K. L., Rich, S. S., Worrall, B. B., Sale, M. M., Sale, M. M., & GARNET (The Genomics and Randomized Trials Network) Collaborative Research Group. (2017). Genetic drivers of von Willebrand factor levels in an ischemic stroke population and association with risk for recurrent stroke. *Stroke*, *48*(6), 1444-1450. https://doi.org/10.1161/STROKEAHA.116.015677

Williams, S. R., Yang, Q., Chen, F., Liu, X., Keene, K. L., Jacques, P., Chen, W.-M., Weinstein, G., Hsu, F.-C., Beiser, A., Wang, L., Bookman, E., Doheny, K. F., Wolf, P. A., Zika, M., Selhub, J., Nelson, S., Gogarten, S. M., Worrall, B. B., … Framingham Heart Study. (2014). Genome-wide meta-analysis of homocysteine and methionine metabolism identifies five one carbon metabolism loci and a novel association of ALDH1L1 with ischemic stroke. *PLoS genetics*, *10*(3), e1004214. https://doi.org/10.1371/journal.pgen.1004214

Wilson, K. D., & Wu, J. C. (2015). Induced pluripotent stem cells. *JAMA*, *313*(16), 1613-1614. https://doi.org/10.1001/jama.2015.1846

Windyga, J., Dolan, G., Altisent, C., Katsarou, O., López Fernández, M. F., Zülfikar, B., & EHTSB. (2016a). Practical aspects of DDAVP use in patients with von Willebrand Disease undergoing invasive procedures: a European survey. *Haemophilia*, *22*(1), 110-120. https://doi.org/10.1111/hae.12763

Windyga, J., Dolan, G., Altisent, C., Katsarou, O., López Fernández, M. F., Zülfikar, B., & EHTSB. (2016b). Practical aspects of factor concentrate use in patients with von Willebrand disease undergoing invasive procedures: a European survey. *Haemophilia*, *22*(5), 739-751. https://doi.org/10.1111/hae.12955

Windyga, J., von Depka-Prondzinski, M., & European Wilate® Study Group. (2011). Efficacy and safety of a new generation von Willebrand factor/factor VIII concentrate (Wilate®) in the management of perioperative haemostasis in von Willebrand disease patients undergoing surgery. *Thrombosis and haemostasis*, *105*(06), 1072-1079. https://doi.org/10.1160/TH10-10-0631

Wise, R. J., Pittman, D. D., Handin, R. I., Kaufman, R. J., & Orkin, S. H. (1988). The propeptide of von Willebrand factor independently mediates the assembly of von Willebrand multimers. *Cell*, *52*(2), 229-236. https://doi.org/10.1016/0092-8674(88)90511-9

Wong, H., & Curry, N. (2018). Do we need cryoprecipitate in the era of fibrinogen concentrate and other specific factor replacement options? *ISBT Science Series*, *13*(1), 23-28. https://doi.org/10.1111/voxs.12376

Woods, C. G., Cox, J., Springell, K., Hampshire, D. J., Mohamed, M. D., McKibbin, M., Stern, R., Raymond, F. L., Sandford, R., Sharif, S. M., Karbani, G., Ahmed, M., Bond, J., Clayton, D., & Inglehearn, C. F. (2006). Quantification of homozygosity in consanguineous individuals with autosomal recessive disease. *The American Journal of Human Genetics*, *78*(5), 889-896. https://doi.org/10.1086/503875

World Bank. (2020). *The World Bank | Data Catalog*. World Bank national accounts data, and OECD National Accounts data files. Retrieved 01 26, 2022, from https://data.worldbank.org/indicator/NY.GDP.PCAP.CD

World Federation of Hemophilia. (2021). *Report on the Annual Global Survey 2020*. World Federation of Hemophilia. Retrieved 01 26, 2022, from https://www1.wfh.org/publications/files/pdf-2045.pdf

World Health Organisation (WHO). (2019). *International Statistical Classification of Diseases and Related Health Problems 10th Revision*. ICD-10 Version:2010. Retrieved 11 02, 2021, from https://icd.who.int/browse10/2010/en

Wright, I. S. (1962). The nomenclature of blood clotting factors. *Thrombosis and Haemostasis*, *7*(02), 381-388. https://doi.org/10.1055/s-0038-1655480

Xie, F., Wang, X., Cooper, D. N., Lan, F., Fang, Y., Cai, X., Wang, Z., & Wang, H. (2007). Compound heterozygosity for two novel mutations (1203insG/Y1456X) in the von Willebrand factor gene causing type 3 von Willebrand disease. *Haemophilia*, *13*(5), 645-648. https://doi.org/10.1111/j.1365-2516.2007.01514.x

Yadegari, H., Biswas, A., Ahmed, S., Naz, A., & Oldenburg, J. (2021). von Willebrand factor propeptide missense variants affect anterograde transport to Golgi resulting in ER retention. *Human mutation*, *42*(6), 731-744. https://doi.org/10.1002/humu.24204

Yadegari, H., Biswas, A., Akhter, M. S., Driesen, J., Ivaskevicius, V., Marquardt, N., & Oldenburg, J. (2016). Intron retention resulting from a silent mutation in the VWF gene that structurally influences the 5′ splice site. *Blood*, *128*(17), 2144-2152. https://doi.org/10.1182/blood-2016-02-699686Yadegari, H., Biswas, A., Akhter, M. S., Driesen, J., Ivaskevicius, V., Marquardt, N., & Oldenburg, J. (2016). Intron retention resulting from a silent mutation in the VWF gene that structurally influences the 5′

Yadegari, H., Driesen, J., Pavlova, A., Biswas, A., Hertfelder, H. J., & Oldenburg, J. (2012). Mutation distribution in the von Willebrand factor gene related to the different von Willebrand disease (VWD) types in a cohort of VWD patients. *Thrombosis and haemostasis*, *108*(10), 662-671. https://doi.org/10.1160/TH12-02-0089

Yadegari, H., Jamil, M. A., Marquardt, N., & Oldenburg, J. (2022). A Homozygous Deep Intronic Variant Causes Von Willebrand Factor Deficiency and Lack of Endothelial-Specific Secretory Organelles, Weibel–Palade Bodies. *International journal of molecular sciences*, *23*(6), 3095. https://doi.org/10.3390/ijms23063095

Yago, T., Lou, J., Wu, T., Yang, J., Miner, J. J., Coburn, L., López, J., Cruz, M. A., Dong, J.-F., McIntire, L. V., McEver, R. P., & Zhu, C. (2008). Platelet glycoprotein Ibα forms catch bonds with human WT vWF but not with type 2B von Willebrand disease vWF. *The Journal of clinical investigation*, *118*(9), 3195-3207. https://doi.org/10.1172/JCI35754

Yamamizu, K., Kawasaki, K., Katayama, S., Watabe, T., & Yamashita, J. K. (2009). Enhancement of vascular progenitor potential by protein kinase A through dual induction of Flk-1 and Neuropilin-1. *Blood*, *114*(17), 3707-3716. https://doi.org/10.1182/blood-2008-12-195750.

Yamamoto, F. I., Clausen, H., White, T., Marken, J., & Hakomori, S. I. (1990). Molecular genetic basis of the histo-blood group ABO system. *Nature*, *345*(6272), 229-233. https://doi.org/10.1038/345229a0

Yan, J., & Marr, T. G. (2005). Computational analysis of 3′-ends of ESTs shows four classes of alternative polyadenylation in human, mouse, and rat. *Genome research*, *15*(3), 369-375. https://doi.org/10.1101/gr.3109605

Yang, J., Yang, F., Campos, L. S., Mansfield, W., Skelton, H., Hooks, Y., & Liu, P. (2017a). Quenching autofluorescence in tissue immunofluorescence. *Wellcome Open Research*, *2*(79), 79. https://doi.org/10.12688/wellcomeopenres.12251.1

Yang, L., Stanworth, S., & Baglin, T. (2012). Cryoprecipitate: an outmoded treatment? *Transfusion medicine*, *22*(5), 315-320. https://doi.org/10.1111/j.1365-3148.2012.01181.x

Yang, S., Lincoln, S. E., Kobayashi, Y., Nykamp, K., Nussbaum, R. L., & Topper, S. (2017). Sources of discordance among germ-line variant classifications in ClinVar. *Genetics in Medicine*, *19*(10), 1118-1126. https://doi.org/10.1038/gim.2017.60

Yarovoi, H. V., Kufrin, D., Eslin, D. E., Thornton, M. A., Haberichter, S. L., Shi, Q., Zhu, H., Camire, R., Fakharzadeh,, S. S., Kowalska, M. A., Wilcox, D. A., Sachais, B. S., Montgomery, R. R., & Poncz, M. (2003). Factor VIII ectopically expressed in platelets: efficacy in hemophilia A treatment. *Blood*, *102*, 4006-4013.

Ye, J., Couloris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. L. (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC bioinformatics*, *13*(1), 1-11. https://doi.org/10.1186/1471-2105-13-134

Yehia, L., & Eng, C. (2019). Large scale population genomics versus deep phenotyping: Brute force or elegant pragmatism towards precision medicine. *npj Genom. Med*, *4*(6). https://doi.org/10.1038/s41525-019-0080-0

Yilmaz, D., Karapinar, B., Yeniay, S. B., Balkan, C., Bilenoglu, B., & Kavakli, K. (2005). Epidemiological study in İzmir about prevalence of von Willebrand disease. *Ege Pediatri Bülteni*, *12*, 151-159.

Yorde, L. D., Hussey, C. V., Yorde, D. E., & Sasse, E. A. (1979). Competitive enzyme-linked immunoassay for Factor VIII antigen. *Clinical chemistry*, *25*(11), 1924-1927. https://doi.org/10.1093/clinchem/25.11.1924

Yu, J., Vodyanik, M. A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J. L., Tian, S., Nie, J., Jonsdottir, G. A., Ruotti, V., Stewart, R., Slukvin, I. I., & Thomson, J. A. (2007). Induced Pluripotent Stem Cell Lines Derived from Human Somatic Cells. *Science*, *318*(5858), 1917-1920. https://doi.org/10.1126/science.1151526

Yuan, L., Chan, G. C., Beeler, D., Janes, L., Spokes, K. C., Dharaneeswaran, H., Mojiri, A., Adams, W. J., Sciuto, T., Garcia-Cardena, G., Molema, G., Kang, P. M., Jahroudi, N., Marsden, P. A., Dvorak, A., Regan, E. R., & Aird, W. C. (2016). A role of stochastic phenotype switching in generating mosaic endothelial cell heterogeneity. *Nature communications*, *7*(1), 1-16. https://doi.org/10.1038/ncomms10160

Yusa, K., Rashid, S. T., Strick-Marchand, H., Varela, I., Liu, P. Q., Paschon, D. E., Miranda, E., Ordonez, A., Hannan, N. R., Rouhani, F. J., Darche, S., Alexander, G., Marciniak, S. J., Fusaki, N., Hasegawa, M., Holmes, M. C., Di Santo, J. P., Lomas, D. A., Bradley, A., & Vallier, L. (2011). Targeted gene correction of α1-antitrypsin deficiency in induced pluripotent stem cells. *Nature*, *478*(7369), 391-394. https://doi.org/10.1038/nature10424

Zanardelli, S., Crawley, J. T., Chan Kwo Chion, C. K., Lam, J. K., Preston, R. J., & Lane, D. A. (2006). ADAMTS13 substrate recognition of von Willebrand factor A2 domain. *Journal of biological chemistry*, *281*(3), 1555-1563. https://doi.org/10.1074/jbc.M508316200

Zavelia Padilla−Romo, M. G., Ornelas−Ricardo, D., Luna−Záizar, H., & Jaloma−Cruz, A. R. (2021). Diagnosis of von Willebrand disease in Western Mexico. *Haemophilia*, *27*(1), e78-e87. https://doi.org/10.1111/hae.14203

Zenner, H. L., Collinson, L. M., Michaux, G. M., & Cutler, D. F. (2007). High-pressure freezing provides insights into Weibel-Palade body biogenesis. *Journal of cell science*, *120*(12), 2117-2125. https://doi.org/10.1242/jcs.007781

Zhang, C., Kelkar, A., & Neelamegham, S. (2019). von Willebrand factor self-association is regulated by the shear-dependent unfolding of the A2 domain. *Blood advances*, *3*(7), 957-968. https://doi.org/10.1182/bloodadvances.2018030122

Zhang, H. B., & Wu, C. (2001). BAC as tools for genome sequencing. *Plant Physiology and Biochemistry*, *39*(3-4), 195-209. https://doi.org/10.1016/S0981-9428(00)01236-5

Zhang, L., Li, H., Zhao, H., Zhang, X., Ji, L., & Yang, R. (2003). Retrospective analysis of 1312 patients with haemophilia and related disorders in a single Chinese institute. *Haemophilia*, *9*(6), 696-702. https://doi.org/10.1046/j.1351-8216.2003.00826.x

Zhang, Y., Chen, F., Yang, A., Wang, X., Han, Y., Wu, D., Wu, Y., & Zhang, J. (2021). The disulfide bond Cys2724-Cys2774 in the C-terminal cystine knot domain of von Willebrand factor is critical for its dimerization and secretion. *Thrombosis Journal*, *19*(1). https://doi.org/10.1186/s12959-021-00348-w

Zhang, Y. Z., Kemper, C., Bakke, A., & Haugland, R. P. (1998). Novel flow cytometry compensation standards: Internally stained fluorescent microspheres with matched emission spectra and long−term

stability. *Cytometry*, *33*(2), 244-248. https://doi.org/10.1002/(SICI)1097-0320(19981001)33:2<244::AID-CYTO20>3.0.CO;2-T

Zhang, Z. P., Blombäck, M., Egberg, N., Falk, G., & Anvret, M. (1994). Characterization of the von Willebrand factor gene (VWF) in von Willebrand disease type III patients from 24 families of Swedish and Finnish origin. *Genomics*, *21*(1), 188-193. https://doi.org/10.1006/geno.1994.1241

Zhang, Z. P., Lindstedt, M., Falk, G., Blombäck, M., Egberg, N., & Anvret, M. (1992). Nonsense mutations of the von Willebrand factor gene in patients with von Willebrand disease type III and type I. *American journal of human genetics*, *51*(4), 850-858.

Zhao, H., Sun, Z., Wang, J., Huang, H., Kocher, J. P., & Wang, L. (2014). CrossMap: a versatile tool for coordinate conversion between genome assemblies. *Bioinformatics*, *30*(7), 1006-1007. https://doi.org/10.1093/bioinformatics/btt730

Zhao, J. V., & Schooling, C. M. (2018). Coagulation factors and the risk of ischemic heart disease: a Mendelian randomization study. *Circulation*, *11*(1), e001956. https://doi.org/10.1161/CIRCGEN.117.001956

Zheng, X., Chung, D., Takayama, T. K., Majerus, E. M., Sadler, J. E., & Fujikawa, K. (2001). Structure of von Willebrand factor-cleaving protease (ADAMTS13), a metalloprotease involved in thrombotic thrombocytopenic purpura. *Journal of Biological Chemistry*, *276*(44), 41059-41063. https://doi.org/10.1074/jbc.C100515200

Zhou, T., Benda, C., Duzinger, S., Huang, Y., Li, X., Li, Y., Guo, X., Cao, G., Chen, S., Hao, L., Chan, Y.-C., Ng, K.-M., Ho, J. C., Wieser, M., Wu, J., Redl, H., Tse, H.-F., Grillari, J., Grillari-Voglauer, R., … Esteban, M. A. (2011). Generation of induced pluripotent stem cells from urine. *Journal of the American Society of Nephrology*, *22*(7), 1221-1228. https://doi.org/10.1681/ASN.2011010106

Zhou, Y. F., & Springer, T. A. (2014). Highly reinforced structure of a C-terminal dimerization domain in von Willebrand factor. *Blood, The Journal of the American Society of Hematology*, *123*(12), 1785-1793. https://doi.org/10.1182/blood-2013-11-523639

Zhu, Q., Yamakuchi, M., Ture, S., de la Luz Garcia-Hernandez, M., Ae Ko, K., Modjeski, K. L., LoMonaco, M. B., Johnson, A. D., O'Donnell, C. J., Takai, Y., Morrell, C. N., & Lowenstein, C. J. (2014). Syntaxin-binding protein STXBP5 inhibits endothelial exocytosis and promotes platelet secretion. *The Journal of clinical investigation*, *124*(10), 4503-4516. https://doi.org/10.1172/JCI71245

Zhukov, O., Popov, J., Ramos, R., Vause, C., Ruden, S., Sferruzza, A., Dlott, J., & Sahud, M. (2009). Measurement of von Willebrand factor−FVIII binding activity in patients with suspected von Willebrand disease type 2N: application of an ELISA−based assay in a reference laboratory. *Haemophilia*, *15*(3), 788-796. https://doi.org/10.1111/j.1365-2516.2009.01995.x

Zimmerman, T. S., Ratnoff, O. D., & Powell, A. E. (1971). Immunologic differentiation of classic hemophilia (factor VIII deficiency) and von Willebrand's disease: with observations on combined deficiencies of antihemophilic factor and proaccelerin (factor V) and on an acquired circulating anticoagulant(add rest)). *The Journal of clinical investigation*, *50*(1), 244-254. https://doi.org/10.1172/JCI106480

Ziv, O., & Ragni, M. V. (2004). Bleeding manifestations in males with von Willebrand disease. *Haemophilia*, *10*(2), 162-168. https://doi.org/10.1111/j.1365-2516.2003.00871.x

Zucker, M. B., Broekman, M. J., & Kaplan, K. L. (1979). Factor VIII-related antigen in human blood platelets: localization and release by thrombin and collagen. *The Journal of Laboratory and Clinical Medicine*, *94*(5), 675-682.

Zwaginga, J. J., Sakariassen, K. S., King, M. R., Diacovo, T. G., Grabowski, E. F., Nash, G., Hoylaerts, M., & Heemskerk, J. W. (2007). Subcommittee of the SSC of the ISTH. Can blood flow assays help to identify clinically relevant differences in von Willebrand factor functionality in von Willebrand disease types 1–3? *J Thromb Haemost*, *5*, 2547–9. https://doi.org/10.1111/j.1538-7836.2007.02807.x