Supplementary Material to Batlle et al. "Molecular and clinical profile of von Willebrand disease in Spain (PCM–EVW–ES): Proposal for a new diagnostic paradigm" (Thromb Haemost 2015; 114.6)

Suppl. Material and Methods

Study design

A minor modification of the classification used was the inclusion of subtype 1H (historical) proposed by Montgomery et al[1] for cases in which an historical von Willebrand factor (VWF) level was confirmed but currently showing a very mild decrease or even a normal VWF plasma level. Despite not being recognized yet, this subclassification is appropriate as some patients with type 1 VWD show a progressive increase in VWF levels with age, but some still bleed despite having a normal VWF level, as shown in the Netherlands Win Study.[2] Type 1 diagnosis was restricted to patients with low levels of VWF at the recruitment moment of this study.

A minor additional modification is the inclusion of type 2A/2M which has been the subject of important controversy.[3]

Patients and controls

A 30 IU/dL VWF cut-off plasma level was used when a low VWF level was the only fulfilled criteria. There are several reasons for this: 1. The previous published view on type 1 VWD.[4] 2. The present work was not designed as an epidemiological study to establish either the prevalence of VWD or the distribution of its different types in Spain. Rather it was intended, as well as other objectives, to evaluate and validate the next generation sequencing (NGS) technology. 3. The rate of *VWF* mutations found in type 1 VWD decreases when VWF level >30 IU/dL.[5,6] 4. Due to financial and time constraints, to avoid submission (recruitment) of an overwhelming number of patients with borderline VWF levels.

Genetic analysis

DNA extraction. Genomic DNA was obtained from peripheral blood samples by using a QIAsymphony SP instrument and the QIAsymphony DNA Midi Kit (Qiagen). DNA concentration was adjusted to a range of 25 to 50 ng/µL.

Primer design. A total of 61 pairs of oligonucleotides have been designed (Accession NG_029217) capable of amplifying the relevant regions of the *VWF* gene (exons 1 to 52, intronic flanking regions and approximately 1300 bp of *VWF* promoter region). Primers used were designed taking into account the particular differences between the gene and pseudogene[7] to ensure highly specific amplification, as well as the absence of single nucleotide poylmorphisms (SNPs) in primer binding sequences that could result in preferential or single allele amplification, and lead to missing alleles (dbSNP [Build 138] and 1000 Genomes polymorphism database). Table S1 shows the primer sequences, locations in human genome sequence, and the polymerase chain reaction (PCR) product sizes.

PCR amplification. PCR sizes were adjusted to meet the required size criteria of the sequencing platform (between 376 and 450 bp bases/amplicon).

The Access Array™ System (Fluidigm) allows 2304 different PCR reactions by combining 48 samples with 48 pairs of primers (the 61 primer pairs were adjusted to 48 by creating 11 duplex and one triplex). The samples were loaded into the Access Array™ in combination with a distinctive short sequence identification label that acted as a barcode and which were incorporated into each set of amplicons from the same sample during the amplification.

Suppl. Results

Genetic results

Mutation versus variant

Indeed, genetic nomenclature has been and remains controversial in many ways and it is important to specify in each case the exact meaning of the terms used. We have attempted to use the term ‘mutation’ to indicate ‘a disease-causing change’. Conversely, we have used the word ‘variant’ for alterations with an unknown functional effect, or ‘variants of unknown significance’ (VUS). Similarly, the term ‘polymorphism’ was used both to indicate ‘a non disease-causing change’ and ‘a change found at a frequency of 1% or higher in any population included in the SNP databases’.

Types of mutations

Missense mutations. A total of 466 missense mutations (64% of all mutations) were detected (119 different) in 220 unrelated families. The top 10 mutations were found 194 times, and the five most frequent mutations (p.Arg1205His, p.Arg1374His, p.Pro2063Ser, p.Arg1374Cys, p.Arg854Gln), all of them described previously, represented 25% of missense mutations. The predicted impact of these mutations was

examined with two different *in silico* programs showing: 21 benign mutations, 20 possible damaging and 78 probably damaging for PolyPhen–2; 39 tolerated and 80 deleterious according to Sorting Intolerant From Tolerant (SIFT) analysis. Correlation in predictions between both algorithms was weak: only seven mutations (p.Pro2063Ser, p.Cys1899Arg, p.Cys1946Phe, p.Pro2646Thr, p.Cys2283Arg, p.Arg2118Trp, p.Cys2773Ser) were identified as non-deleterious (tolerated and benign) by both programs. None of these changes were included in the *VWF* mutation databases, except for the controversial p.Pro2063Ser, previously described both as a polymorphism and VWD causative mutation.

Null mutations. A total of 33 stop mutations (11 different) were found in 24 unrelated families, 20 patients were homozygous and 13 were heterozygous for stop mutations. The putative gene conversion mutation p.Gln1311Ter was found most frequently, in eight patients from six unrelated families.

Eighteen frameshift variants (10 different) were found in 15 unrelated families; one of these variants (c.376delT; p.Tyr126ThrfsTer49) was very prevalent in our population and was found in seven unrelated families. Remarkably, this mutation was always found with c.375G>C (synonymous change), c.4146G>T (synonymous change) and c.6197A>G (p.Asn2066Ser), indicating a founder effect. Furthermore, 12 in-frame mutations (five different, including three deletions and two insertions) were detected in six unrelated families.

Finally, among null mutations we detected two large deletions: one in the homozygous state comprising exons 16 to 43 detected by consecutive regions of zero coverage in NGS and then confirmed by multiplex ligation-dependent probe amplification (MLPA), and one heterozygous, including exons 11 to 32 and not detected by NGS, but which was then discovered by MLPA.

Variants in splice site regions. A total of 26 splice site variants (12 different) were found in 19 families (7%) as a putative causative mutation. Nine of these mutations comprised the consensus 5′ GT donor splice site or the 3′ AG acceptor splice site of several introns (5, 10, 13, 25, 31, 36, 37, 41 and 45) and would, therefore, have an undisputed effect on *VWF* mRNA processing and patient phenotype. The remaining three potential splice site mutations detected (c.7730-4C>G, c.8254-5T>G and c.8155+3G>C) encompassed neighboring consensus nucleotides. *In silico* analysis was able to predict the score variation of all the affected splice sites in introns 45, 50 and 51.[8]

Synonymous, intronic and upstream mutations. Besides mutations that have a very evident effect (stop, frameshift, acceptor or donor splice site, etc.) and missense mutations, whose effects are more variable, we have kept on the Registry all those

variants in introns, promoter and synonym changes whose frequency in the general population and in different populations studied in the project 1000 Genomes was below 1%. In fact for most of these variants the frequency was close to zero. Although it is hard to demonstrate, any nucleotide modification could be considered a potential candidate to alter the splicing, especially when an intronic or synonymous mutation appears next to an intron/exon junction.[9,10] We found a total of 166 (81 different) mutations in this group (for details see Figure S1). However, in only 20 patients from 13 families was one such mutation found alone as a putative exclusively responsible mutation. In such cases in particular it will be necessary to perform additional studies in order to demonstrate the underlying contribution of the mutation to clinical manifestations.

Suppl. Table 1. Primers used to amplify the *VWF*. Primers in black were designed by Fluidigm custom-designed assays and primers in green are those proposed in order to avoid the missed regions due to the proximity of the primers to the coding regions. SNPs positions have been taken into consideration when designing the primers and *in silico* PCR has been developed. The shadowed area contains specific primers designed to amplify the *VWF* in the pseudogene region (exons 23 to 34). The nucleotides outlined in red correspond to positions that are specific (different) for the gene in respect of the pseudogene. Forward primers are linked in 5′ to sequence ACACTGACGACATGGTTCTACA and reverse primers are linked in 5′ to sequence TACGGTAGCAGAGACTTGGTCT. Such universal tails are necessary to construct libraries with adapters specific for the MiSeq desktop sequencer and add 'the molecular barcodes' concomitantly with VWF amplification.

Name	Amplifiedregion	Forward primer (5′-3′)	Reverse primer (5′-3′)	PCR size (bp)	Genomeregion (hg19)
VWP1	Promoter	CCCCAAAACAAGTGGAGACAGG	GCCATCGGGATTTCCACTTCCA	434	chr12:6235188-6235621
VWP2		AAGAACATTTGTTGTATTTAGCACAG	GAGACAGTAGAAAAGGCCATTGG	446	chr12:6234855-6235300
VWP3		TGATCCCTTGACCTCGTGATCC	CTTCTTCCCAGCCTCCCATTCC	448	chr12:6234511-6234958
VWP4		TGCCCATTGCATCAGTTACTTATTTT	CCAGGGAAGTTGAGAAAAACACC	449	chr12:6234154-6234602
VWP5		CCAAATGGACTGTTTTTGTCAGGG	CAGGGGATTGGCCTCCTTTTAAT	446	chr12:6233844-6234289
VWF_E1	E1	AGGTCTCCCTCCAAACTCTACAA	GGATCAGTCAGTCCTGCATCTTC	424	chr12:6233525-6233948
VWF_E2	E2	GGGGTCACGAGGATCAATCTTTT	TCTGTGCCACCTTTATGCTTCTT	425	chr12:6232070-6232494
VWF_E3	E3	CACAGCCCAGTTTCTATCAGAGG	AAAGGCTGTTCCTCTCCTTGTTC	419	chr12:6230201-6230619
VWF_E4	E4	AAGACTTTTTGGGGCGTTTTCTG	TCTAACCCCAACCCCATGTTGTA	438	chr12:6219814-6220251
VWF_E5	E5	TTTGCACCATGTTCTGAACCCTA	CCAGGGAAGGCATGTTAGTGAAG	407	chr12:6219451-6219857
VWF_E6	E6	CAAAACCACCAGCAGACCTAGAA	ACTACACCAAGTCATTAGCCCAG	425	chr12:6204415-6204839
VWF_E7	E7	CAGGGCTAAGTCTCAGTGCC	CGTGACACAGCCCCGAAGCACC	383	chr12:6184410-6184792
VWF_E8	E8	GTAAGGGCCTCACAAGATGGAAG	TTCATCTCACTTCCCAAAGCCAA	406	chr12:6182713-6183118
VWF_E9	E9	GAACAAGTTCTTTGAGCTTCCTGG	CATTGCCTGCCACACCTGTCC	401	chr12:6181328-6181728
VWF_E10	E10	TTCGTTTGGGGACTGTGATAACT	GGCCTTACAGCTCATCAACCTTA	448	chr12:6180162-6180609
VWF_E11	E11	TTAGACTGGTTTGGGCAAAGGAC	CTGGGTTTCTGGATGAATGAGGA	434	chr12:6174141-6174574
VWF_E12	E12	TCTCTGATTAAGAGGGTCCTGGG	CCTTCTCTAGTCTTCCTCCTCCA	429	chr12:6173203-6173631
VWF_E13	E13	GCTCTTCAGTGCTACCATCCTTT	GAAGGGGCATCCAGAAAACAAAC	401	chr12:6171934-6172334
VWF_E14	E14	CTAAACAACTATGCCGCTGCTTT	CCCAGTTCAGATGATCAAGTGCT	424	chr12:6166849-6167272

Name	Amplifiedregion	Forward primer (5′-3′)	Reverse primer (5′-3′)	PCR size (bp)	Genomeregion (hg19)
VWF_E15	E15	GCACTGGGCTATTTCCAGGGG	CCTACGCCCTCTTTCCACAGG	416	chr12:6165915-6166330
VWF_E16	E16	CACAGCTACAAGGGGTGGCAAG	CATCCATGAAGTAAAGGACTTGGG	416	chr12:6161622-6162037
VWF_E17	E17	CGTTAGCAAGCTGTGCTTCAGG	AAACCCAGAAATGAAGGCGATCC	391	chr12:6155734-6156124
VWF_E18	E18	AGAAGAGCTGCCACCAATGTAAG	CACTCATCCCTGCCTACAAGAAA	447	chr12:6153395-6153841
VWF_E19	E19	CTTACCCGTAGGCTCAAGTCTCA	AGGATGGACACAGGTGATGAAAA	402	chr12:6145362-6145763
VWF_E20	E20	CTGTGTTCCTTCATTGCCTCCAT	CAGAGGGAAAATGGACAACCACT	427	chr12:6143653-6144079
VWF_E21	E21	CCAATCTTCTGGTCTGGTGAGAG	GCACTGTCGCTAAAATGGGATTG	424	chr12:6140388-6140811
VWF_E22	E22	CTGTTACCGTCTCTTGGTCATGG	AGGGAGCAGAAAACACTCCAAAG	446	chr12:6138446-6138891
VWF_E23	E23	TGTCCCGCTCCTCCTCCTCTT	TCCCACCCTGCAGCCACCTGG	431	chr12:6134984-6135414
VWF_E24	E24	ATGTCCCTCTGTCCCCATT**G**T**C**	GGTGCAGATCATGTCAAGACAC**G**	395	chr12:6134628-6135022
VWF_E25	E25	CTCCTTCTACCTG**GA**CCCCC**T**	GCCATCCAGTCCCTACTAA**CA**C**T**	409	chr12:6132665-6133073
VWF_E26	E26	CAACATAGCAAGACCCCATCT**G**	ACCTTTCCATCCATCCCTATCC	448	chr12:6131846-6132293
VWF_E27	E27	GAGGGATCAATCAAGGCACAAG**C**	CTTTTTACCCAAAACCTAGTCTCT**A**	410	chr12:6130980-6131389
VWF_E28a	E28	TCAGAAGTGTCCA**C**AGGTTCTT**C**	GCATACTTCACCTGGCTGGCAA**T**	433	chr12:6128535-6128967
VWF_E28b		**C**AGAAGTGGGTCCGCGTGGC**C**	CACAGAGGTAGCTAACGATCTC**G**	445	chr12:6128209-6128653
VWF_E28c		CCTCAAGCAGATCCGCCTCAT**C**	GCCTCGCTGAAGGGGTACTCC	402	chr12:6127920-6128321
VWF_E28d		GGC**G**TT**C**GTCC**T**GGAAGGAT**C**	CACCTGGATGTCTCCAGGCA**G**	379	chr12:6127709-6128087
VWF_E28e		CAGGGTGACCGGGAGCAGG**C**	CCAGGATTAGAACCCGAGTC**G**	376	chr12:6127429-6127804
VWF_E29	E29	TTGTAGGCCTGG**T**GGCCA**T**TG**T**	CTGCATCCAGCCTGTGGCACC	450	chr12:6125653-6126102
VWF_E30	E30	GGTGACGAAGAGGCTCTTTTTG**T**	CTTAAAAGCTGAATGATTCAGA**A**	413	chr12:6125479-6125891
VWF_E31	E31	CCACCGT**T**AAGACAGGGTGTC**G**	TTT**G**GTTTCCTAATCACATCGTG**G**	395	chr12:6125072-6125466
VWF_E32	E32	GAACATCTTCCTCATAGGGCTG**A**	CACCCTGGGGTCTCTTGAATA**C**	402	chr12:6122551-6122952
VWF_E33	E33	CACCTCAGCC**T**CATGTCCCTA**T**	ACTGACCCCTAGAATTGAACAC**A**	430	chr12:6121034-6121463
VWF_E34	E34	GCCTCCTTGCTGTGTAGGCC**T**	AGGGTACTTCTGGGCTGGTGG	398	chr12:6120679-6121076
VWF_E35	E35	TTTGGATAACGTCGCATCCATCC	GTGGTACTCCTCTCCTCCACTTA	401	chr12:6105030-6105430
VWF_E36	E36	AGTGTGATGAATGTGCAGGAACT	GCCATTCTTGATCCTCACCAGAA	427	chr12:6103443-6103869
VWF_E37	E37	CCAGGGCCACTCAGTTTATCTTT	GGCTGAGCAAGCCCAGTTAG	447	chr12:6102969-6103415
VWF_E38	E38	GTTGAATCAGCTGTGCCCATTTC	CCCTATCCCTAATGATCCCCGTA	406	chr12:6100851-6101256

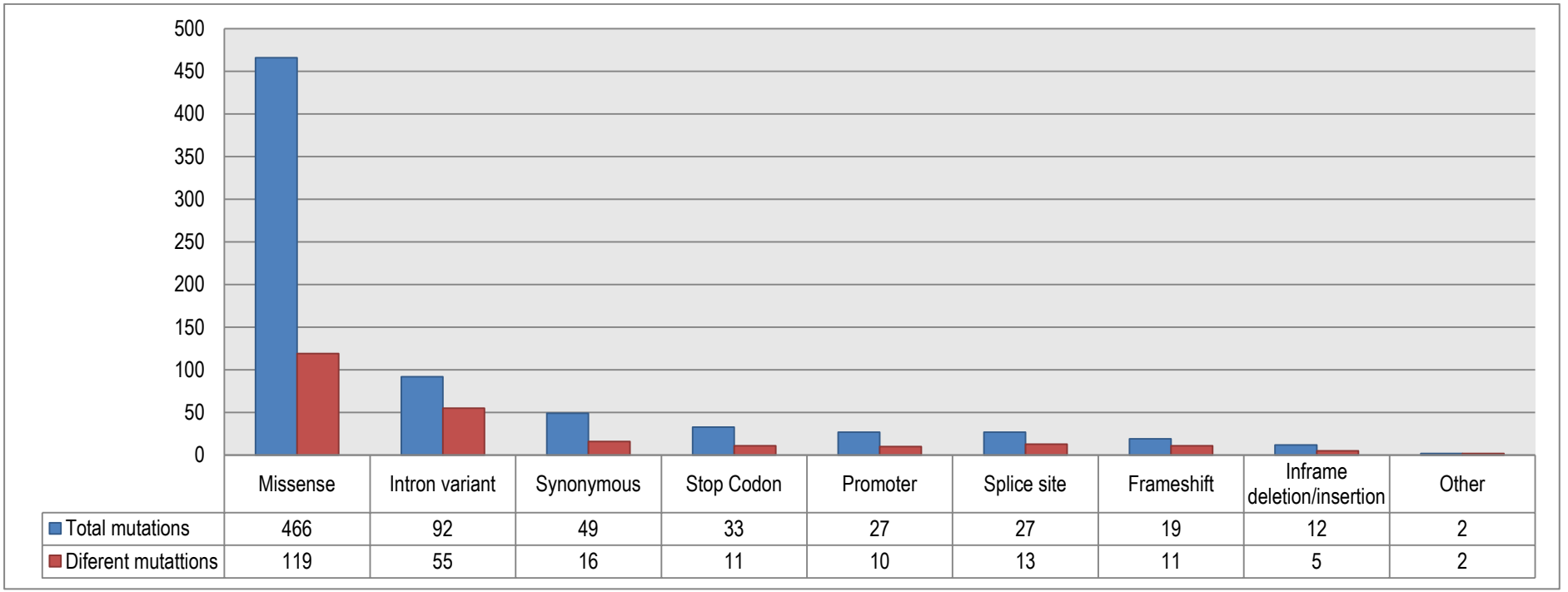
Name	Amplifiedregion	Forward primer (5′-3′)	Reverse primer (5′-3′)	PCR size (bp)	Genomeregion (hg19)
VWF_E39	E39	GGCTTCGTACCTAGAATGTCCTG	ACTCTTAAGTCTGACCGTTGCTG	404	chr12:6094481-6094884
VWF_E40	E40	GAGGGTAAATGTGAGGGTGTCAA	TTGGAGTATCCAGTCGGTCCTTA	405	chr12:6094070-6094474
VWF_E41	E41	GTAGCATCCCACTCACAGGTAAT	CAGCTGCTTCTTCATAAACCCAA	439	chr12:6092058-6092496
VWF_E42	E42	GGCACCCTATAGCATAGCTGAAT	ATGCTGGGTAGGATGCAAATCTT	447	chr12:6090788-6091234
VWF_E43	E43	CCCTGAGAGACTGGTCATAGCTT	CCTTCCTAAGATGCCCTCCTCTA	442	chr12:6085205-6085646
VWF_E44	E44	TGCAGCTGATGTAAGACTTCGTT	AAAGGGCAGGGAATGAGATGAAA	409	chr12:6080672-6081080
VWF_E45	E45	CCAGGCTTCACGTCTAGAAACC	GGTCCTATCCATTTCCCTAAGTTG	405	chr12:6078262-6078666
VWF_E46	E46	ATCCTCCCATTCATCCCATCTCT	GCTTTACAATGACTTGCCTGCTC	415	chr12:6077205-6077619
VWF_E47	E47	GATGGCTCATGGGAGCTATGG	TATTTCTGAGGCATGCAGTTTGG	408	chr12:6076471-6076878
VWF_E48	E48	GGCTGCTACAACTTGAGTGAGAT	CTGAACCAAACTTAGCCCCTCTT	417	chr12:6062564-6062980
VWF_E49	E49	GAGTCTACATAGCAGCCCTGTTC	GAGCTGGGATGCACACTATTCAG	427	chr12:6061477-6061903
VWF_E50	E50	TTACATCTGGGCACTTAAGCACA	GGAAAGAGATTGCTCCATGGGAT	414	chr12:6060757-6061170
VWF_E51	E51	GGCTGGCTTTATTTGGTTACTGTG	GGATTTGCTGAGTCTTTCCTCCA	440	chr12:6058733-6059172
VWF_E52	E52	AGATCAGACCTGCCTTGCTTG	AAGAGCTCAGCCTTTATTGTGGG	433	chr12:6058054-6058486

Suppl. Table 2. *F8* mutations validated by *Sanger* in patients of the Registry with a final diagnosis of hemophilia A and carriers.

Patient	Genotype	Consequence	Exon	Sift	PolyPhen	HGVSc	HGVSp	Described
*1 (hemophilic)	-	-	-	-	-	No mut	-	-
2 (hemophilic)	hem	missense_variant	15/26	deleterious(0.02)	probably_damaging(1)	c.5305G>A	p.Gly1769Arg	YES
3 (hemophilic)	hem	missense_variant	14/26	deleterious(0)	probably_damaging(0.997)	c.5123G>A	p.Arg1708His	YES
4 (hemophilic)	hem	missense_variant	9/26	deleterious(0.01)	probably_damaging(0.952)	c.1339T>C	p.Phe447Leu	NO
5 (hemophilic)	hem	missense_variant	9/26	deleterious(0.01)	probably_damaging(0.952)	c.1339T>C	p.Phe447Leu	NO
6 (hemophilic)	hem	missense_variant	9/26	deleterious(0.01)	probably_damaging(0.952)	c.1339T>C	p.Phe447Leu	NO
7 (hemophilic)	hem	missense_variant	18/26	deleterious(0.04)	probably_damaging(0.999)	c.5954G>A	p.Arg1985Gln	YES
8 (hemophilic)	hem	missense_variant	26/26	deleterious(0)	probably_damaging(0.999)	c.6968G>A	p.Arg2323His	YES
	hem	missense_variant	14/26	tolerated(0.19)	benign(0)	c.4264T>C	p.Tyr1422His	NO
9 (carrier)	het	missense_variant	15/26	deleterious(0.02)	probably_damaging(1)	c.5305G>A	p.Gly1769Arg	YES
10 (carrier)	het	missense_variant	14/26	deleterious(0)	probably_damaging(0.997)	c.5123G>A	p.Arg1708His	YES
11 (carrier)	het	synonymous_variant	2/26			c.222G>A	c.222G>A(p.=)	NO
12 (carrier)	het	missense_variant	9/26	deleterious(0.01)	probably_damaging(0.952)	c.1339T>C	p.Phe447Leu	NO
13 (carrier)	het	missense_variant	18/26	deleterious(0.04)	probably_damaging(0.999)	c.5954G>A	p.Arg1985Gln	YES

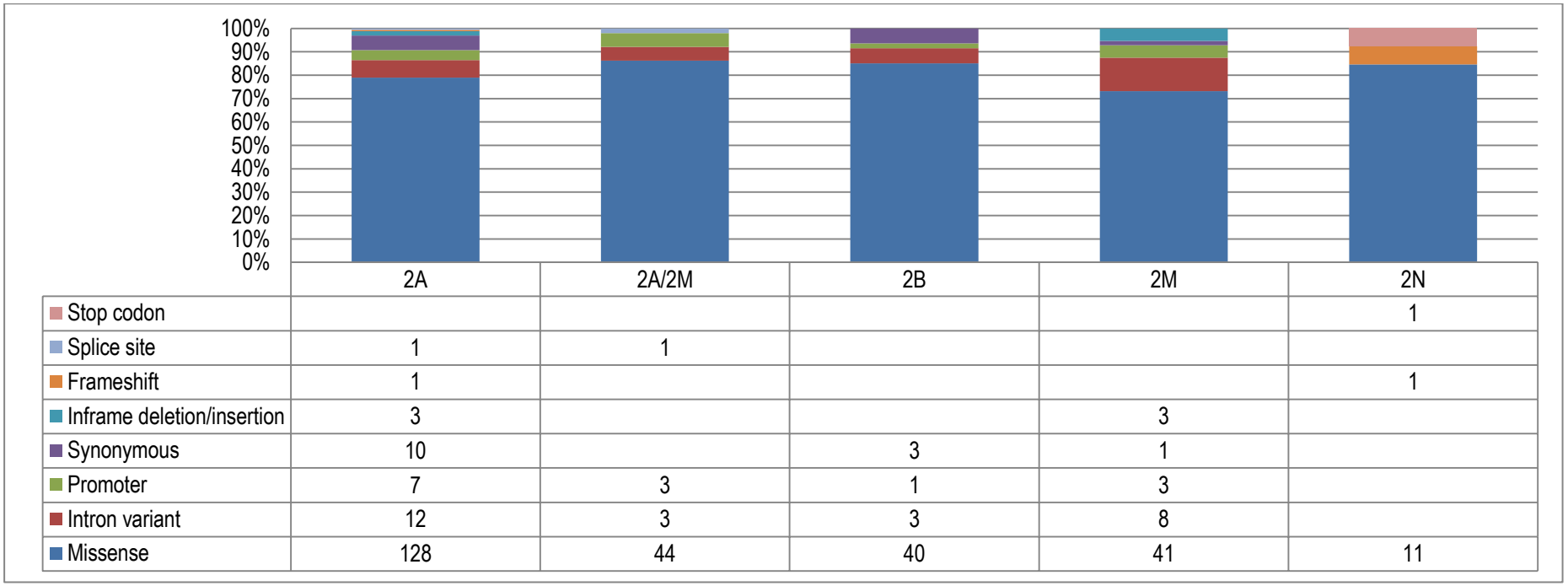
** Possible error in sampling. Pending of the study in a new sample.*

Suppl. Figure 1: Mutations (total and different) categorized by consequence found in patients included in the PCM–EVW–ES.



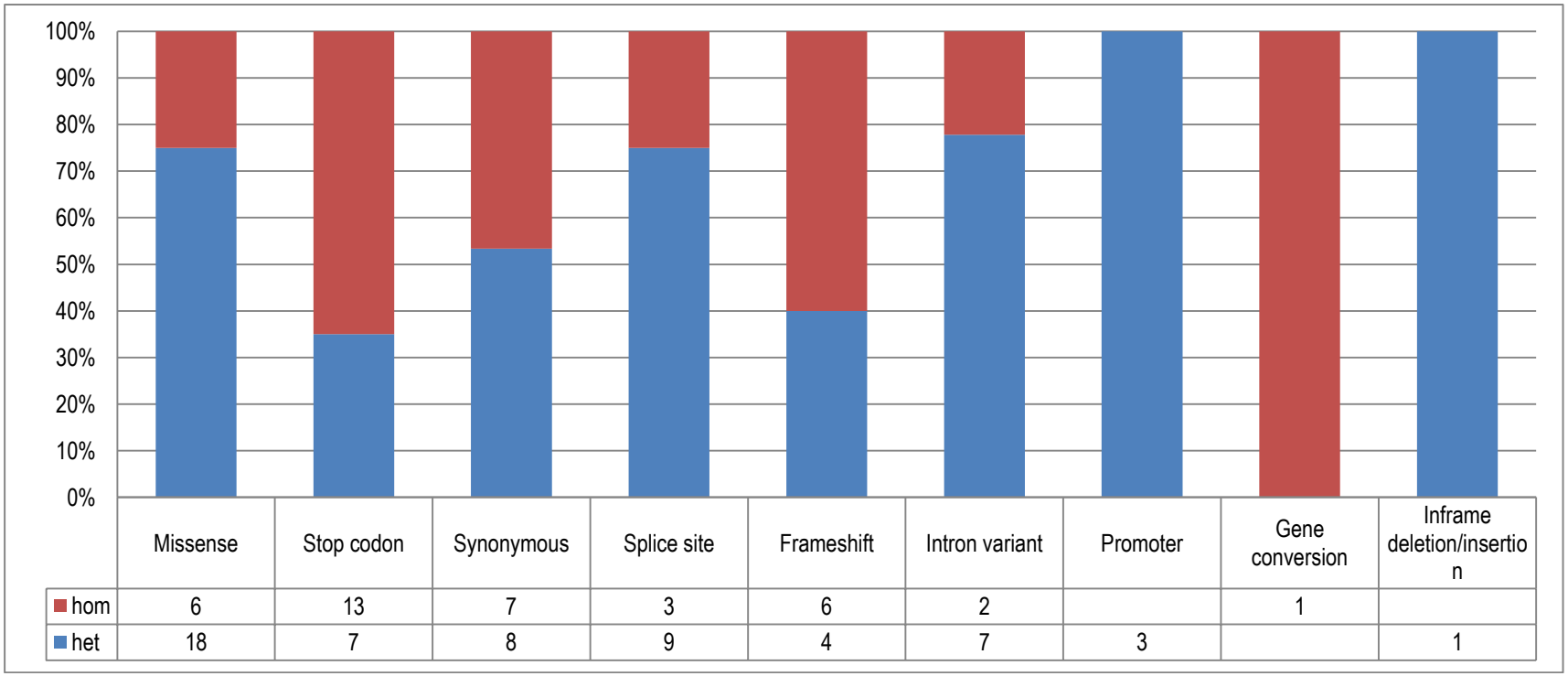
	Missense	Intron variant	Synonymous	Stop Codon	Promoter	Splice site	Frameshift	Inframe deletion/insertion	Other
Total mutations	466	92	49	33	27	27	19	12	2
Diferent mutattions	119	55	16	11	10	13	11	5	2

Suppl. Figure 2. Distribution of changes by type in qualitative defects.



	2A	2A/2M	2B	2M	2N
Stop codon					1
Splice site	1	1			
Frameshift	1				1
Inframe deletion/insertion	3			3	
Synonymous	10		3	1	
Promoter	7	3	1	3	
Intron variant	12	3	3	8	
Missense	128	44	40	41	11

Suppl. Figure 3. Distribution of changes by consequence and genotype state in type 3 VWD patients. Hom, homozygous; het, heterozygous.



	Missense	Stop codon	Synonymous	Splice site	Frameshift	Intron variant	Promoter	Gene conversion	Inframe deletion/insertion
hom	6	13	7	3	6	2		1	
het	18	7	8	9	4	7	3		1

Appendix: study group members

The members of the PCM-EVW-ES Study Group are:

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(†) Jesús M. César died on August 25th, 2014

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