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Original article

Genotypic and phenotypic spectrum of VWF exon 20 variants in type 1 Von Willebrand disease: Insights from Eastern Saudi Arabia

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Abstract. *Background and aim:* VWD is a genetic bleeding disorder, divided into three types VWD types 1,2, and 3. Aim was to determine whether a common SNP present at exon 20 of the VWD gene is associated with ABO groups, VWF levels, and platelet aggregation-related functional consequences in the Eastern Saudi Arabia. *Methods:* It included 160 cases and controls, 60 of whom were cases from 19 families, all participants were categorized into four groups: 22 index cases who had previously been diagnosed with type 1 VWD, 21 family members who had also been diagnosed with type 1 VWD, 17 unaffected family members, and 100 healthy Saudi controls. *Results:* The results indicate that the distribution of blood groups among different study groups was statistically significant. It also indicates that the VWF: Ag, VWF: RCO assay, VWF: RCO/VWF: Ag ratio and FVIII: C are significantly lower in index cases and affected family members than in non-affected family members and control groups. Among the 60 DNA samples, one variant (c.2555A>G) was found in all 60 samples, with 83.3% homozygous and 16.6% heterozygous. *Conclusions:* It appears that VWF levels are influenced by blood groups. One variant identified in exon 20 with a high prevalence in our population does not appear to be associated with disease status or may act in conjunction with other factors, so, further comprehensive genomic analyses of VWF are recommended to gain a better understanding of disease. (www.actabiomedica.it)

Key words: phenotype, genotype, von willebrand disease type1, vWF gene, exon 20, mutation analysis, genotype-phenotype correlation, coagulation disorders, genetic variation, Saudi Arabia

Introduction

Von Willebrand Disease (VWD) is a genetic bleeding disorder that affects the body's ability to form blood clots. The disease is named after Erik von Willebrand, a Finnish physician who first described it in 1926 (1-2). The prevalence of VWD in Eastern Mediterranean region (Saudi arabia, Iran and Oman) is estimated to be 1.5 to 2 per 100.00 patients and according to the 2019 annual global survey of the World

Federation of Hemophilia, the prevalence of Von Willbrand disease is estimated to be 1.5 per 100,000 patients (for all types of VWD)(Hemophilia, 2019) and from 0.1 to 8.8 per 100,000 in Western Pacific (3). This disease is characterized by a deficiency of von Willebrand factor (VWF), a glycoprotein that carries factor VIII (FVIII) and facilitates platelet adhesion, resulting from mutations in VWF genes that affect the gene expression (4). There are three main types of VWD, which differ in terms of disease severity and inheritance pattern. Types 1 and 3 VWD are characterized by quantitative defects in the VWF protein. Type 1 VWD involves partial quantitative deficiency of VWF and results in a mild to moderate bleeding phenotype. Type 3 VWD is the most severe form of the disease and results from near-complete absence of VWF. Type 2 VWD is a group of disease phenotypes that result from qualitative defects in VWF (5). Type 1 autosomal dominant von Willebrand disease is the most common form of the disease, accounting for approximately 60 to 80% of cases (6). People with type 1 VWD may experience mild to moderate bleeding symptoms, such as nosebleeds, bruising, and heavy menstrual period as well as bleeding following surgery or trauma. Diagnosis of this type is based on a combination of clinical symptoms, family history, and laboratory tests. Laboratory tests include measurement of VWF antigen, VWF activity, and factor VIII activity in addition to genetic analysis to identify the underlying genetic mutation (7). Genetic test can also be used for carrier testing and prenatal diagnosis in families with known VWD mutations, allowing for more accurate diagnosis and personalized treatment. The VWF gene is located on chromosome 12 and contains 52 exons. Its code for a large glycoprotein that consists of 2,813 amino acids. VWF has a complex structure with multiple functional domains that are involved in different stages of hemostasis. The protein is composed of a series of repeated domains, including the D1-D3 domains, which are responsible for binding to platelets and collagen in the extracellular matrix. The A1 domain of VWF binds to the platelet surface receptor glycoprotein (GPIb), which is crucial for platelet adhesion and aggregation. The A3 domain binds to collagen and other extracellular matrix proteins, anchoring the platelet to the site of vascular injury (6). Detecting mild cases of von Willebrand disease (VWD) can be challenging due to the inability to differentiate phenotypically (except for their bleeding manifestations) between persons with reduced, structurally normal VWF due to VWF gene mutations and healthy persons with VWF levels at the lower end of the normal range. Further complicating the diagnosis is incomplete disease penetrance and genetic and environmental influences (8). In order to gain a deeper understanding of VWD's phenotype, more phenotypic and genotypic studies have to be conducted to develop more effective treatment options. In Saudi Arabia, limited studies have been conducted on VWF at the phenotypic and genotypic levels, which may hinder the development of effective diagnostic tools and treatment. To bridge this gap, a study was conducted to determine whether common SNP present at exon 20 in particular is associated with ABO groups, VWF levels, and platelet aggregation-related functional consequences in the Eastern Province of Saudi Arabia.

Materials and methods

Ethical approval: All adult participants of this study provided written informed consent and completed the International Society on Thrombosis and Haemostasias (ISTH) bleeding score questionnaire (ISTH-SSC) to evaluate the level of bleeding symptoms, but for underage participants (children below 18 years ages) consent was obtained from their parents. The ethical approval for the present study was obtained by the institutional review board at Imam Abdulrahman Bin Faisal University (IRB-2017-03-009). *Study subjects:* The study included 160 individuals, 60 of whom were from 19 families, who were diagnosed with type 1 von Willebrand disease (VWD), with 11 males and 49 females. The participants were categorized into four groups: 22 index cases who had previously been diagnosed with type 1 VWD, 21 family members who had also been diagnosed with type 1 VWD, 17 unaffected family members, and 100 healthy Saudi controls. Their ages ranged from six to seventy. The diagnosis of type 1 VWD was confirmed using phenotypic analysis. Sampling and phenotype analysis: Each participant provided three blood samples, two of which were collected

in sodium citrate (light blue top) tubes and one sample was collected in an EDTA (Lavender top) tube (BD Vacutainer, USA). The phenotypic profiles of the participants were evaluated using a sodium citrate sample. This sample was utilized to perform various tests, including the activated partial thromboplastin time test (APTT), von Willebrand antigen level (VWF Ag), Factor VIII activity (FVIII:C), and ristocetin cofactor activity (VWF:RCo). The STAR Max® - Stago (Diagnostica Stago, France) hemostasis analyzer was used to measure VWF Ag, FVIII:C, and VWF: RCO. The Asserachrom® VWF Ag kit (Diagnostica Stago, France) was used to determine VWF Ag through the ELISA method. Factor VIII: C was quantified using the triniCHROM factor VIII: C kit (Diagnostica Stago, France) with a chromogenic method. The STA-VWF: RCO kit (Diagnostica Stago, France) and an automated method were used to assess VWF:RCO following the manufacturer's protocol. For genetic analysis and to determine the ABO blood group and platelet count, an EDTA sample was employed. Genotype analysis: Genotyping analysis was conducted on VWF exon 20, which was selected from the VWF variant database (http://www.vwf.group.shef.ac.uk/). This database contained the most frequent gene variants, indicating 73 variants were identified in this exon. The primers used for amplification of exon 20 (forward 5' CAGGTCCTCAACTTCCTTGG 3', reverse 5' GACCCCAGAGTTGTTTCTGC 3') were previously published, and their chemical synthesis was performed at a commercial facility (AlYami, 2014). For sanger sequencing, genomic DNA was extracted using the Relia prepTM blood gDNA mini prep system kit (Promega Corporation). Subsequently, the selected exon was PCR amplified using 100ng of genomic DNA in 30µl of final reaction mixture in each sample. In PCR tube 15µl of ready master mix (MOLEQULE-ON®, Auckland, New Zealand), which include Taq DNA polymerase, optimized Taq Green buffer, MgCl2, and nucleoside triphosphates containing deoxyribose (dNTPs) was added along with 9 µl of distilled water, 2µl (20-25pmol) of each forward and reverse primers and 2µl of (100 ng) genomic DNA. Next, the tubes were placed in the Thermal Cycler (MaxiTM thermal cycler by ESCO technologies). The main program for PCR reactions includes different cycles with different temperatures. The first step involves the denaturation cycle which increases the temperature to 94°C for one minute, followed by continuous 35 cycles of denaturation, annealing, and extension. The temperature remained at 94°C during the denaturation step lasting for one minute. The temperature decreased during annealing to 60°C, the suitable temperature for the primer used in the reaction. The temperature rose during the extension cycle to reach 72°C lasting for one minute. Another extension cycle was then applied and lasted for seven minutes at 72°C. A visual analysis was conducted for the PCR products using 2% (w/v) agarose gel to find out the size of the amplified PCR product, after which it was compared with DNA ladder (MOLEQULE-ON). Afterwards, the bands were visualized in an UV trans-illumination apparatus and the amplified PCR product was purified. Purification of the PCR products was done using MQ PCR/Gel product purification kit (MOLEQULE-ON). After that, the PCR amplicons were bidirectionally sequenced using the chain termination method developed by Frederick on the ABI-Prism 3730xl Genetic Analyzer (Applied Biosystems, USA). The Sanger sequencing files were aligned and compared to the reference sequence via by UCSC genome browser https://genome.ucsc.edu/ by the BLAT tool.

Results

In this study, 160 subjects were investigated. Those subjects were grouped in to four groups; 22 index cases, 21 affected cases, 17 unaffected cases, all compared to the fourth control group that has 100 volunteers. The 60 subjects that represent the non-control group are from 19 families and comprises 49 females and 11 males with variable age groups. Demographic data of all participants are presented in Table 1.

Phenotypic results

All participants were compared phenotypically in four different aspects: Blood groups, Bleeding symptoms, Bleeding score, and some other lab results. Distribution of blood groups among different study groups

Table 1. Participant's information

		Index cases	Affected family members	Non affected family members	Controls	P value
Number of subjects		22	21	17	100	
Gender	Males	0	7 (33%)	4 (24%)	72 (72%)	<.001
	Females	22 (100%)	14 (67%)	13 (76%)	28 (28%)	
Age	Mean	32.4	34.4	32.6	35.9	0.664
	Range	6-54	7-69	6-70	18-65	
	SD	12.55	20.28	17.86	11.96	

Table 2. Blood group distribution among study groups

Case group		Blood group		P value
		O group	Other	
IC	Count	19	3	<.001
	% within case code	86.4%	13.6%	
AFM	Count	20	1	
	% within case code	95.2%	4.8%	
NAFM*	Count	10	5	
	% within case code	66.7%	33.3%	
CONTROL	Count	54	46	
	% within case code	54.0%	46.0%	

*The blood groups of two participants were unknown.

was statistically significant with a P value of less than 0.001. The blood group O was dominant among all categories of the participants, with a highest representation in the AFM (95.2%) and a lowest in the controls (54%), while in the Index cases and NAFM it was 86.4% and 66.7% respectively (Table 2 and Figure 1).

Number of bleeding symptoms was significantly higher in the index case. The Kruskal Wallis H test median was 3:2:1 for the IC: AFM: NAFM, and it was significant with a p value of 0.003. (Figures 2 and 3).

When comparing the laboratory tests for all study groups (Tables 3 and 4), it was recognized that there was no significant difference between the study groups regarding platelet count and APTT (P value >.005). But it shows that VWF: Ag, VWF: RCO assay, VWF: RCO/VWF: Ag ratio and FVIII: C is significantly lower in the index cases and affected family members compared to non-affected family members and control groups. The P value <0.001 using Kruskal-Wallis Test.

Genotypic Results

After sequencing all DNA samples for exon 20 using Sanger sequencing, results were analyzed and compared to the reference sequence (Figure 4). One variant was identified in all 60 cases, with the majority of them (83.3%) to be homozygote (c.2555A>G) and only 16.6% were heterozygote (c.2555A>AG). This is a Synonymous variant where both the original and the variant alleles code for the same amino acid (Phenylalanine). All IC and AFM were heterozygote for this variant except for one NAFM in family number 6. Noticeably, this homozygote NAFM was the only with non-O blood group among all heterozygote cases.

Discussion

Von Willebrand disease is one of the most common inherited bleeding disorders in humans. There are

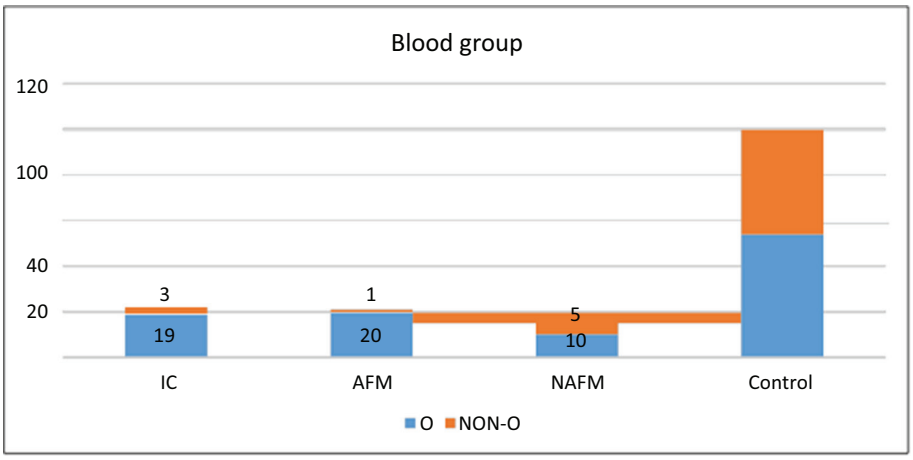

Figure 1. Stacked bar chart shows the distribution of blood groups among different study groups.

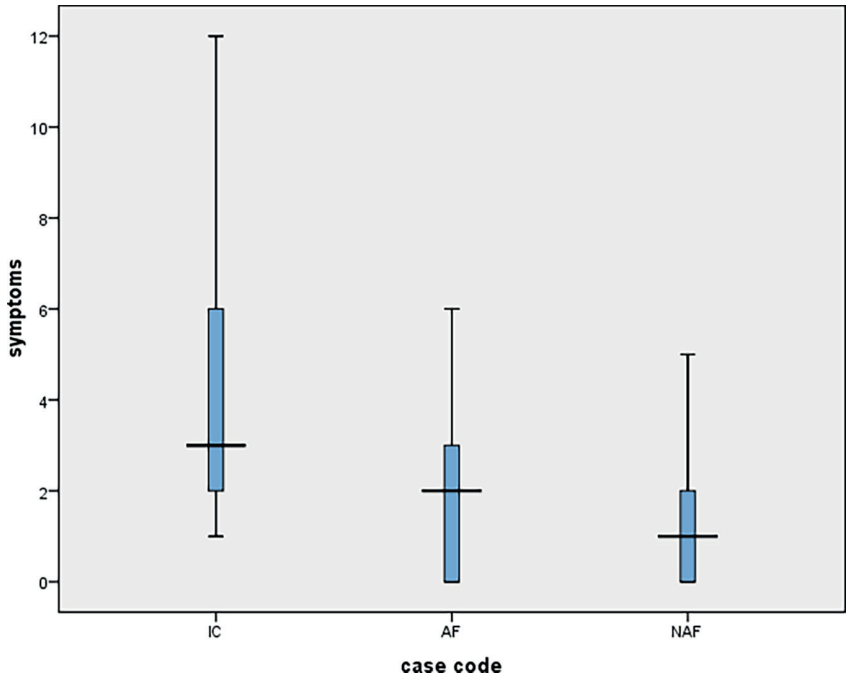

Figure 2. Box plot illustrating the difference in bleeding symptoms between the study groups.

different types of VWD but type 1 is the most common and challenging to diagnose and treat (9). Approximately 60% to 80% of VWD patients have type 1 VWD (6). It is characterized by a partial deficiency of structurally and functionally normal VWF due to mutations in the VWF gene (10-11). The purpose of this study is to determine whether a common SNP present at exon 20 of the VWD gene is associated with ABO groups, VWF levels, and platelet aggregation-related functional consequences in the Eastern Province of Saudi Arabia. The present study has utilized Sanger sequencing technology to analyze one of the most frequently mutated exons (exon20) in the vWF gene. This exon contains a single nucleotide variant rs216321

Table 3. The laboratory results of all study participants

			Index cases	Affected FM	Non-Affected FM	Control	P value
	Number		22	21	17	100	
1	Platelet number (140-450) * 10^3/ul	Median	276	273	307	263	0.593
		25^{th}-75^{th} percentile	225.5-338	218.7-319.5	216-330	213-308	
2	APTT(s) (26-40) s	Median	37.1	37.1	35.7	35.6	0.059
		25^{th}-75^{th} percentile	35.3-41	34-39.2	34-36.6	33.6-37.9	
3	VWF: Ag (50-150) U/dl	Median	53	58.5	90	100	<.001
		25^{th}-75^{th} percentile	42.2-56.5	46-66.7	76-96	85-130.2	
4	VWF: RCO Assay (58-172) U/dl	Median	37	41.5	73	92	<.001
		25^{th}-75^{th} percentile	27-41.5	33.5-50	64-83	68-133	
5	VWF: RCO/ VWF: Ag	Median	0.69	0.73	0.8	0.94	<.001
		25^{th}-75^{th} percentile	0.6-0.85	0.64-0.81	0.77-0.9	0.75-1.18	
6	FVIII:C (70-150) %	Median	82.5	84	120	131	<.001
		25^{th}-75^{th} percentile	68.4-94.7	68-110.7	110-139	102.7-169	

Table 4. Comparison of the laboratory tests results for all study groups against each other's

	Age	Platelets	APTT	VWF: Ag	VWF: RCO	VWF: RCO/ VWF: Ag	FVIII:C	Bleeding score	Number of symptoms
IC vs. AFM	.923	.923	.481	.096	.098	.632	.850	.001	.004
IC vs. NAFM	.788	.702	.034	<.001	<.001	.017	<.001	.001	.003
IC vs. control	.462	.516	.029	<.001	<.001	<.001	<.001		
AFM vs. NAFM	.908	.523	.123	<.001	<.001	.028	<.001	.908	.062
AFM vs. controls	.451	.531	.123	<.001	<.001	<.001	<.001		
NAFM vs. control	.419	.210	.597	.013	.008	.053	.559		

(c.2555A>G) p.Gln852Arg. The variant was reported to be located within the D'D3 domain of vWF protein and has pleiotropic effects on collagen-dependent platelet saggregation and FVIII concentration (12-14). rs216321 variant was observed in all index cases and in family members who had VWD as well as in non-affected family members. The variant is homozygous in 83.3% of participants (index cases 95.5%, family members with VWD 95.3%, and non-affected family members 76.5%), whereas the remaining participants are heterozygous (index cases 4.5%, family members with VWD 4.7%, and non-affected family members 23.5%) (Table 5). It is interesting to note that this variant is found in only 10.3% of the world's normal population (www.internationalgenome.org/, 2020), despite being highly prevalent in our population. It is possible and likely that the putative mutation described in this paper is not pathogenic. There is also a possibility that this variant may act in combination with other factors, both within the VWF gene itself, and at other sites, to cause the type 1 VWD phenotype.

The majority of the affected patients in this study were females. This indicates that although the disease affects both men and women equally, the symptoms of the disease are more prominent in women. This observation may be related to the fact that women are more likely to experience bleeding complications (menstruation, childbirth), and therefore seek treatment

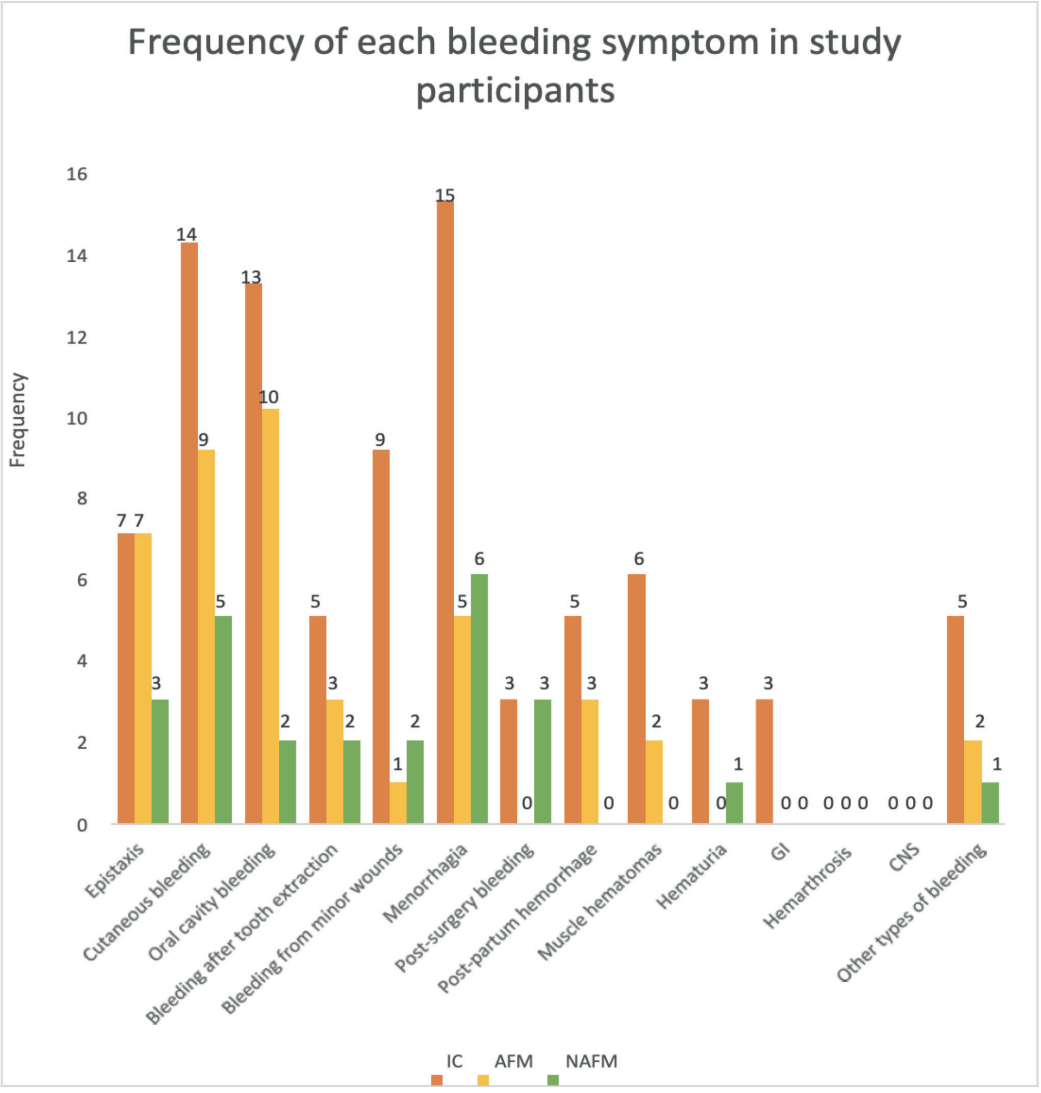

Figure 3. Clustered column chart illustrating the frequency of each bleeding symptom in study participants.

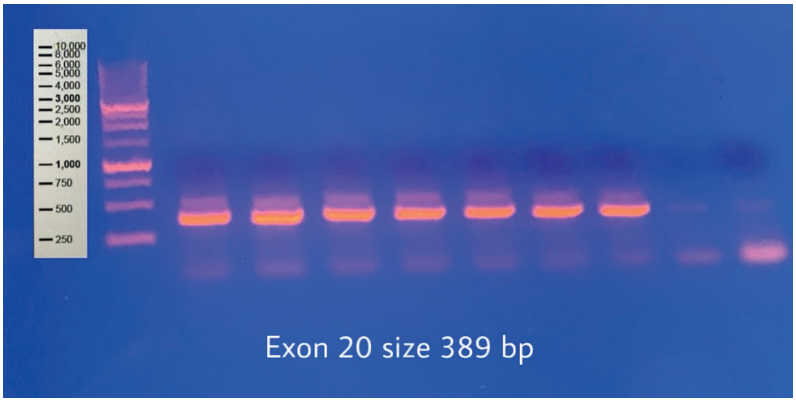

Figure 4. DNA electrophoresis for exon 20.

Table 5. Genotyping of rs216321 variant.

Nucleotide change	Location	Protein change	Rs number	C.S	Variant type	MAF (1000G)	No.	%
c.2555A>G	Exon 20	p. Arg852Gln	Rs216321	B	Missense	10.3%	50 Hom	83.3%
c.2555A>AG							10 Het	16.6%

and care more frequently (15-16). Several studies have investigated the effects of ABO blood type and ethnicity on the level of VWF protein in blood (17-18). As expected, the majority of participants were of type O (81.3% of the affected individuals, 76.5% of family members who were not affected and 54% of healthy individuals were of type O (control)). According to a previous study conducted in Sweden, type 1 VWD has been associated with the O blood group (19). This is consistent with the findings of the present study, which indicates that Saudi patients suffering from VWD are most likely to have the O blood group.

Conclusion

In conclusion, it appears that blood groups have an impact on the plasma level of VWF. One variant identified in exon 20 with a high prevalence in our population does not appear to be associated with disease status or may act in conjunction with other factors. However, we sincerely acknowledge a few limitations in the present investigation, 1- we analyzed only one exon of the VWF gene, 2- we examined only 19 families; therefore, the following recommendations are recommended: 1- run clinical whole exome sequencing so that all possible mutations will cover; 2- increase the number of families for a better understanding of the molecular basis of VWF disease in Saudi Arabian families.

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Ethics Committee Approval: This study was reviewed and approved by the institutional review board at Imam Abdulrahman Bin Faisal University (IRB-2017-03-009).

Author's Contribution: FMA, AAA, and SSS are the principal investigators. LAB, FMH, MAA and OAS contributed to the research concept and design. TNA, EAE, FAA, AMA, OAS and MAS contributed to the research concept, design, and statistical analysis of the data. All authors participated in draft preparation, manuscript revision, and content evaluation. All authors have read and agreed to the content of the manuscript and confirm the accuracy and integrity of every detail of this research.

Conflict of Interest: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

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