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Effect of the *VWF* promoter $(GT)_n$ repeat and SNP c.-2527G>A on circulating VWF levels under normal conditions

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Circulating von Willebrand factor (VWF) protein levels in plasma (VWF:Ag) of healthy individuals can be affected by a number of factors, either physiological and environmental such as stress, or genetic such as ABO blood group [1]. It has previously been suggested that VWF:Ag levels could be partly determined by four single nucleotide polymorphisms (SNP) in the promoter region of *VWF*; c.-3268C>G, c.-2709T>C, c.-2661G>A and c.-2527G>A, segregating predominantly as two haplotypes, GCAG (haplotype 1) or CTGA (haplotype 2), with the latter being associated with lower VWF:Ag levels [2,3]. Shear stress has been shown to enhance *VWF* promoter activity, with a dinucleotide $(GT)_n$ short tandem repeat (STR) located at c.-2144_-2105 in the *VWF* promoter exhibiting size-dependent mediation of this stress-induced transactivation [4]. Studies on Cushing's syndrome patients suggest that both the SNPs and STR influence a glucocorticoid-induced increase in plasma VWF:Ag level [5,6]. However, other studies have failed to reproduce this SNP and STR association [7,8]. The current study reports the largest investigation of the association between SNP c.-2527G>A, the $(GT)_n$ STR, and VWF:Ag levels in healthy controls (HC) from the MCMDM-1VWD study [9] under normal conditions, while controlling for the effect of ABO blood group.

ABO blood group, age and VWF:Ag levels were available for 1115 European male and female healthy controls (HC) from nine countries [9]. None had been referred to a specialist centre due to bleeding problems. VWF:Ag levels were determined at a single centre [9]. Genotypes for the c.-2527G>A SNP were determined using TaqMan allelic discrimination (ABI 7900; Applied Biosystems, Europe BV, Warrington, UK). The *VWF* region containing the $(GT)_n$ STR was amplified by PCR (primer sequences available on request), sequenced and analysed on an ABI PRISM™ 3730 DNA analyser (Applied Biosystems). Analysis of two homozygous samples showed that $(GT)_n$ alleles 181bp and 185bp long corresponded to $(GT)_{19}$ and $(GT)_{21}$ respectively. Hardy-Weinberg equilibrium, allele frequencies and

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linkage disequilibrium analyses were performed using SNPStats: http://bioinfo.iconcologia.net/index.php?module=Snpstats (accessed August 2010). Associations between genotypes and VWF:Ag levels were assessed using Kruskal Wallis tests (GraphPad Prism v5.00, GraphPad Software Inc, San Diego, CA, USA). p values <0.05 were considered to be statistically significant in all tests.

Genotype data for the c.-2527G>A SNP were used for investigation of a genotype-VWF:Ag relationship. There was no significant association between VWF:Ag level and SNP genotype before or after controlling for the effect of ABO blood group (Table 1A), thus contradicting previous results of a smaller study on blood group O Canadian HC (n=250) [3] which identified a significant association (p=0.006). Presence of an extended founder haplotype in the Canadian population with an effect on VWF level could be a possible explanation. 100 European HC from two countries were also genotyped for the four promoter SNP examined in the Canadian study and the SNP were shown to be in complete linkage disequilibrium (D'=0.999, p<0.0001), segregating as the two previously reported haplotypes GCAG (haplotype 1) and CTGA (haplotype 2). No association of genotype with VWF:Ag levels was found in this subgroup (data not shown).

Genotyping of the STR identified alleles ranging in size from $(GT)_{16}$ to $(GT)_{27}$ with a bimodal distribution, i.e. a peak at $(GT)_{19}$ (37.2%) and another at $(GT)_{21}$ (32.2%). The genotype $(GT)_{19}/(GT)_{21}$ was the most frequent (23.8%), followed by $(GT)_{19}/(GT)_{19}$ (13.3%) and $(GT)_{21}/(GT)_{21}$ (10.2%). GT repeat alleles were divided into two subclasses for ease of analysis [8]. Small (S) alleles included all those $<(GT)_{20}$ and large (L) included those $\geq(GT)_{20}$. Based on this approach, 42.8% of alleles were $(GT)_S$ and 57.2% were $(GT)_L$; 18% HC had genotype S/S, 50% had S/L and 32% had L/L, while genotype distribution was consistent with Hardy-Weinberg equilibrium (p=0.42).

In contrast to the results of a smaller study (n=394 HC) reporting a lack of association between $(GT)_n$ genotypes and VWF:Ag levels in HC [8], in this study VWF:Ag values were lower in HC homozygous for S alleles compared to HC heterozygous or homozygous for L alleles (S/S vs S/L vs L/L = 92.0 vs 97.0 vs 100.0 IU/dL, p=0.027) (Table 1B). This association was not maintained when HC of only blood group O were examined (S/S vs S/L vs L/L = 80.0 vs 80.0 vs 82.0 IU/dL, p=0.524), but was observed when examining HC of non-O blood groups (S/S vs S/L vs L/L = 101.0 vs 107.5 vs 114.0 IU/dL, p=0.020) (Table 1B). These data suggest an effect of GT repeat length on VWF levels of ~10% and are in agreement with recent findings suggesting that longer GT repeats induce a larger increase in *VWF* promoter activity than shorter ones under shear stress conditions [4]. A statistically significant association amongst O blood group HC may only be observed with analysis of a larger number of individuals in this subcategory.

A small degree of linkage was observed between the c.-2527G>A SNP and the $(GT)_n$ repeat (D'=0.661, R^2=0.597, p<0.0001). The two most frequent haplotypes (S/G and L/A) accounted for ~80% of HC (49.9% and 30.5% respectively). The most frequent genotypes resulting from homozygosity or heterozygosity for the above haplotypes were S/L-G/A (32.8%), L/L-A/A (23.6%) and S/S-G/G (10.1%). Other allele combinations comprised the remaining 33.5% of genotypes. The association between the three genotypes (S/L-G/A, L/L-A/A, S/S-G/G) and VWF:Ag levels was examined in order to identify a potential cooperative effect of the STR and SNP loci on VWF:Ag levels. The results shown in Table 1C suggest that these two polymorphic regions have no significant cooperative effect on VWF:Ag levels in the EU population, before or after controlling for the effect of ABO blood group on VWF:Ag levels (Table 1C). This suggests that an effect on VWF:Ag levels is mediated only by the $(GT)_n$ repeat.

In conclusion, this study has provided evidence that the c.-2527G>A *VWF* promoter SNP does not have a significant effect on levels of VWF in the EU population. In contrast, the *VWF* $(GT)_n$ STR exhibited a small effect on VWF levels, mediated by its length. A SNP/STR cooperative effect on the regulation of VWF:Ag levels in HC was excluded. This is the largest study examining the potential influence of these loci on VWF levels in HC while controlling for the effect of ABO blood group and is the first study to show a significant effect of the c.-2144_-2105 locus on VWF levels under normal conditions.

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Table 1

Association of genotype at *VWF* locations (A) c.-2527, B) $(GT)_n$ and C) (GT)n plus c.-2527 with median levels of VWF in healthy controls from the MCMDM-1VWD study controlling for blood group.

Analysis	Genotype and ABO blood group	Mean age (SD)	n*	Median VWF:Ag level (IU/dL) (Range†)	p value
A) c.-2527G>A	**All‡**		**1115**		
	G/G	40.7 (12.5)	162	96.5 (74.8–119.5)	
	G/A	40.2 (11.9)	521	98.0 (77.0–120.0)	0.961
	A/A	40.5 (11.4)	432	96.0 (75.0–122.0)	
	O		**410**		
	G/G	41.6 (11.3)	51	84.0 (66.0–106.0)	
	G/A	42.1 (12.5)	191	80.0 (64.0–98.0)	0.747
	A/A	41.2 (10.6)	168	78.5 (66.0–99.0)	
	Non-O		**678**		
	G/G	40.8 (13.1)	104	106.0 (81.0–133.0)	
	G/A	39.3 (11.4)	319	107.0 (89.0–126.0)	0.852
	A/A	40.2 (12.0)	255	112.0 (86.0–129.0)	
B) $(GT)_n$	**All**		**1105**		
	S/S	41.0 (11.5)	196	92.0 (70.3–114.5)	
	S/L	40.3 (11.8)	555	97.0 (76.0–120.0)	**0.027**
	L/L	40.6 (12.3)	354	100.0 (78.0–125.0)	
	O		**408**		
	S/S	41.8 (11.0)	71	80.0 (62.0–99.0)	
	S/L	42.0 (11.7)	199	80.0 (65.0–102.0)	0.524
	L/L	40.9 (11.6)	138	82.0 (67.8–101.3)	
	Non-O		**569**		
	S/S	41.1 (11.7)	118	101.0 (77.8–124.3)	
	S/L	39.3 (11.8)	344	107.5 (86.0–127.0)	**0.020**
	L/L	40.7 (12.8)	207	114.0 (91.0–132.0)	
C) c.-2527G>A & $(GT)_n$	**All**		**727**		
	S/S-G/G	41.3 (12.0)	110	92.0 (71.8–116.3)	0.314

Analysis	Genotype and ABO blood group	Mean age (SD)	n*	Median VWF:Ag level (IU/dL) (Range†)	p value
	S/L-G/A	39.7 (11.7)	358	96.5 (77.8–120.0)	
	L/L-A/A	40.7 (11.9)	259	98.0 (76.0–125.0)	
	O		**278**		
	S/S-G/G	41.5 (10.6)	39	84.0 (66.0–101.0)	
	S/L-G/A	41.8 (12.0)	134	81.5 (65.8–104.0)	0.714
	L/L-A/A	40.8 (10.7)	105	85.0 (67.0–103.0)	
	Non-O		**432**		
	S/S-G/G	42.1 (12.5)	65	102.0 (76.5–134.5)	
	S/L-G/A	38.6 (11.4)	219	107.0 (87.0–126.0)	0.387
	L/L-A/A	40.8 (12.9)	148	112.5 (88.0–130.0)	

*ABO blood group data were available for 1088 out of 1115 HC.

†Range = 25th–75th percentile.

‡All: all blood groups; O: blood group O only; Non-O: blood groups other than O.

SD: Standard deviation

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