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# Microsatellite $(GT)_n$ is part of the von Willebrand factor (VWF) promoter region that influences the glucocorticoid-induced increase in VWF in Cushing's syndrome

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ABSTRACT

*Introduction:* The cortisol-induced increase in von Willebrand factor (VWF) in Cushing's syndrome (CS) seems to depend on single nucleotide polymorphisms (SNPs) of the VWF promoter, haplotype 1 (-3268G/-2709C/-2661A/-2527G) being the susceptible pattern.

*Materials and Methods:* This study focused on a new variable region of the VWF promoter, the -2144$(GT)_n$ locus, to establish whether different GT-repeat lengths are also involved in modulating the cortisol-induced increase in VWF. Sixty-nine CS patients were investigated, divided into groups A (high VWF) and B (normal VWF).

*Results:* Analysing the $(GT)_n$ locus revealed a similar allele distribution in CS patients and normal subjects, $(GT)_n$ variants ranging from 15 to 24 repeats and $(GT)_{19}$ and $(GT)_{21}$ being the two most represented. However, when groups A and B were analysed separately, a different allele distribution was observed: short GT-repeats (15-19, $GT_S$) were more frequent in group A, long GT-repeats (20-24, $GT_L$) in group B ($p=0.01$). About genotype distributions, $(GT)_S/(GT)_S$ was higher in group A and rare in group B (22.5% and 3.4%, respectively), whereas $(GT)_L/(GT)_L$ was higher in group B than in group A (55.2%, 27.5%) ($p=0.021$). Odds-ratio analysis revealed a risk of a cortisol-dependent increase in VWF three times higher for alleles $(GT)_S$ than for $(GT)_L$, and 13-fold for genotype $(GT)_S/(GT)_S$ respect to $(GT)_L/(GT)_L$.

*Conclusions:* In conclusion, not only the SNPs haplotypes in the VWF gene promoter, but also the variable-length $(GT)_n$ locus predict the risk of developing high VWF levels under conditions of glucocorticoid excess; the combination of $(GT)_S$ and haplotype 1 represents the susceptible pattern.



## Introduction

Cushing's syndrome (CS) is a rare endocrine disorder sustained by excessive concentrations of circulating free cortisol and caused by corticotropin-dependent and -independent pathogenic mechanisms [1–4]. A hypercoagulable state is described in CS patients, relating to increased levels of clotting factors, especially von Willebrand factor (VWF) and factor VIII (FVIII) [5–9]. VWF is a multimeric glycoprotein synthesised by endothelial cells and megakaryocytes. It mediates platelet adhesion to damaged subendothelium and acts as a carrier, stabilising circulating FVIII [10,11]. An excess of circulating VWF is responsible for a hypercoagulable condition predisposing patients to coronary heart disease and generally raising the risk of thrombotic events [12–15], particularly in conjunction with other risk factors such as diabetes or atherosclerosis [16,17]. In normal conditions, VWF concentrations are extremely variable, ABO blood group and age being the two main modulators: individuals with non-O blood groups and those over 40 years old have higher VWF levels than O blood group people and those under 40 [18–21]. A contribution to VWF levels has also been reported for SNPs -3268 C>G, -2709 T>C, -2661 G>A and -2527 G>A of the VWF gene promoter, haplotype 1 (GCAG) being associated with higher levels of VWF than haplotype 2 (CTGA) [22,23]. Many environmental factors raise VWF levels, including trauma, inflammatory conditions, pregnancy and chronic conditions such as diabetes, kidney failure and atherosclerosis [24,25]. Glucocorticoid (GC) excess is also known to upregulate VWF levels and stimulate the production of unusually large multimers which are haemostatically the most efficient [26–28]. The resulting hypercoagulable state predispose CS patients to thromboembolic events, particularly after surgery. It has been calculated that the cardiovascular mortality

*Abbreviations:* (CS), Cushing's syndrome; (VWF), von Willebrand factor; (FVIII), factor VIII; (SNP), single nucleotide polymorphism; (GC), glucocorticoid; (LD), linkage disequilibrium; $(GT)_S$, short GT-repeat; $(GT)_L$, long GT-repeat; (VWF:Ag), von Willebrand factor Antigen; (UFC), urinary free cortisol; (OR), Odds-ratio; (GRE), glucocorticoid response element.

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observed in CS is four to five times higher than in the normal population [29]. Not all CS patients have increased FVIII and VWF levels, however, because VWF responds differently to cortisol excess, depending on SNPs of the VWF gene promoter: haplotype 1 confers a higher risk of developing increased VWF levels than haplotype 2 [30]. It has also been demonstrated recently that such SNPs haplotypes are part of a larger linkage disequilibrium (LD) region that includes the c.-2144GT(15_24) locus of the VWF promoter, with short $(GT)_n$ variants, $(GT)_{15\text{-}19}$, mostly segregating with haplotype 1 and long ones, $(GT)_{\geq 20}$, with haplotype 2 [31]. Although no variations in VWF levels have been associated with the $(GT)_n$ locus in normal individuals [31], polymorphic GT-repeat nonetheless seems to modulate the shear stress-induced activation of the VWF promoter [32].

In this study, we assessed the variable-length $(GT)_n$ locus of the VWF gene promoter in a cohort of CS patients to ascertain whether a different number of dinucleotide repeats correlated with a different VWF response to cortisol excess.

## Materials and methods

Patients and controls were studied after obtaining their written informed consent in accordance with the Helsinki Declaration and the study was approved by our institutional review board.

### *Subjects*

Sixty-nine CS patients being monitored at the Endocrinology unit of the University of Padua Medical School were studied: 15 males and 54 females, with a mean age of 42.5 ± 13.2 years. Fifty-one patients had cortisol hypersecretion due to a pituitary adenoma, 14 had CS of adrenal origin, and 4 had ectopic corticotropin secretion. All patients studied were in an active phase of their disease and were not receiving medication. CS was diagnosed on the basis of clinical features and hormonal evaluations, as previously reported [30]. A group of 160 normal individuals matched for gender and age was also studied, drawn from the Padua blood bank.

### *Haemostatic analysis*

Blood samples were drawn from the antecubital vein and anticoagulated using 3.8% sodium citrate (1/10 vol/vol). Plasma VWF antigen (VWF:Ag) levels were measured using a home-made enzyme-linked immunosorbent assay (ELISA) [33].

### *Genetic analysis*

Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp®DNA Blood Mini kit (Qiagen, Hilden, Germany). SNPs -3268 C>G (rs7966230), -2709 T>C (rs7964777), -2661 G>A (rs7954855) and -2527 G>A (rs7965413) were analysed by direct sequencing in an ABI 3100 Genetic Analyzer (Applied Biosystems, AB, Foster City, CA, USA), as described elsewhere [30]. The GT-repeat region of the VWF gene promoter was amplified by PCR using the primers PROgt (*F*) = 5'TGCCCATTGCATCAGTTACTT3' and PROgt (*R*) = 5'TGGGGAGTGATGGTTTGAGT 3' ($T_A$ = 63 °C), the forward primer being labelled with the fluorescent dye 6-FAM. Fluorescent PCR products were analysed on an ABI 3100 Genetic Analyzer (AB) and the correct number of GT was determined as reported elsewhere [31].

### *Statistical analysis*

Fisher's exact test, the two-sample t-test and the chi-square test were used to compare patients in groups A and B as regards age, sex, ABO distribution and the nature of their disease. Multiple comparisons between CS patient groups A and B, and healthy individuals were performed by one-way ANOVA and Tukey's test. The association between patient groups and classes of $(GT)_n$ alleles was established by Correspondence Analysis [34]. The chi-square test was used to verify the LD between $(GT)_n$ and SNPs-haplotype, and to compare allele and genotype distributions between groups A and B, and between each patient group and controls, for both SNPs-haplotypes and $(GT)_n$ locus.

## Results

### *Haemostatic parameters and urinary free cortisol (UFC) values*

CS patients were divided into groups A and B, depending on whether their VWF levels were increased or normal, respectively. Since ABO blood group is the major inherited determinant of VWF levels, two different normal VWF ranges were used, i.e. 62-116 U/dL for O blood group cases, and 67-171 U/dL for non-O cases, based on calculations in 160 normal individuals. Group A contained 40 patients (VWF:Ag 232.1 ± 15.6 U/dL, UFC 1788.5 ± 453.7 nmol/24 h), and group B 29 (VWF:Ag 119.9 ± 4.7 U/dL, UFC 1024.9 ± 154.4 nmol/24 h). The two patient groups were comparable in terms of mean age, sex, nature of their disease, ABO blood group distribution and UFC levels, while a significant difference emerged for VWF levels ($p<0.0001$) (Table 1). UFC and VWF:Ag values correlated in patients with high VWF:Ag levels (group A, $r = 0.66$, $p<0.0001$), but not in patients with normal VWF:Ag levels (group B, $r = 0.052$, $p = 0.79$).

### *$(GT)_n$-repeat in the VWF gene promoter*

In healthy controls, the $(GT)_n$ variants showed a bimodal distribution, $(GT)_{21}$ being the major allele (39.4%), followed by $(GT)_{19}$ (34.1%), $(GT)_{20}$ (10.6%) and $(GT)_{22}$ (9.1%), while $(GT)_{15\text{-}17\text{-}18\text{-}23\text{-}24}$ were below 5% (Fig. 1a). Much the same allele distribution was seen in the CS population (Fig. 1b), but when patient groups A and B were analysed separately $(GT)_{19}$ was more frequent than $(GT)_{21}$ in group A (42.5% vs 30.0%), while the frequency of $(GT)_{19}$ was much reduced (22.4%) and $(GT)_{21}$ rose to 48.3% in group B (Fig. 1c,d). One-way ANOVA confirmed that the alleles were distributed differently among the groups analysed ($p = 0.043$), and particularly between patient groups A and B (Tukey's test, $p<0.05$), while the differences detected between either group A or group B and the controls were not significant.

Correspondence Analysis revealed the preferential association between group A (high VWF) and alleles with 17, 18 or 19 GT-repeats, and between group B (normal VWF) and alleles with 20, 21 or 22 GT-repeats (Fig. 2). Given the bimodal distribution of allele frequencies, and taking the above-mentioned preferential association into account, $(GT)_n$ variants were grouped into two classes: short GT-repeats, $(GT)_S$, if the number of dinucleotide repeats varied from 15 to 19, or long GT-repeats, $(GT)_L$, if the number of GT-repeats was ≥20. The analysis of genotype frequencies for the $(GT)_n$ locus showed that the $(GT)_S/(GT)_S$ genotype was common in group A (22.5%), but present in only one patient in

**Table 1**
Main characteristics of patient groups A and B.

| | Group A | Group B | *p values* |
|---|---|---|---|
| **Patients— No.** | 40 | 29 | |
| **Males/Females— No.** | 11/29 | 4/25 | 0.24° |
| **P/A/E — No.** | 28/8/4 | 23/6/0 | 0.21§ |
| **O/nonO — No.** | 20/20 | 11/18 | 0.34° |
| **Age — yr** | 44.3 ± 14.9 | 40.3 ± 10.7 | 0.24^ |
| **UFC (nmol/24 h)** | 1788.5 ± 453.7 | 1024.9 ± 154.4 | 0.17^ |
| **VWF:Ag (U/dL)** | 232.1 ± 15.6 | 119.9 ± 4.7 | <0.0001^ |

P: Pituitary Cushing's Syndrome.
A: Adrenal Cushing's Syndrome.
E: Ectopic Cushing's Syndrome.
UFC: Urinary Free Cortisol (Mean ± SEM).
VWF: von Willebrand factor (Mean ± SEM).
°Fisher's exact test; §Chi-square test; ^ Student's t-test.