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# Detection of *F8* mutations in carriers and patients with severe hemophilia A. Identification of a novel mutation.

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**Keywords:** carrier; factor VIII; molecular diagnosis; mutation; hemophilia A; Venezuela.

**Abstract.** The molecular diagnosis of haemophilia A (HA) patients has many benefits including diagnosis confirmation and inhibitor risk development prediction. In female carries of a mutation, the molecular diagnosis allows for genetic counseling and prenatal diagnosis, which have become part of the comprehensive care for HA in many countries. Therefore, the aim of this study was to determine the *F8* mutations in severe HA (sHA) patients and female carriers. In 12 patients with sHA, the presence of the intron 22 and intron 1 inversions was investigated using an inverse and a conventional PCR method, respectively. In patients negative for the inversions, the *F8* gene was screened through conformation sensitive gel electrophoresis (CSGE) and further sequencing. The causative mutation was successfully identified in 6/12 patients, including the novel mutation p.G190C. The mothers of these six patients and those of seven other sHA patients molecularly diagnosed in a previous work were investigated for the presence of the genetic alterations found in their sons. All mothers were found to be carriers. This is the first study conducted in Venezuela which directly analyzes the *F8* gene in potential carrier mothers to specifically identify the presence of the mutation that was detected in their sons, and complements a previous study on sHA patients. Our findings will facilitate the implementation of regular screening of HA carriers in our country and will allow a better care of bleeding symptoms and genetic counseling.

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# Detección de mutaciones en el gen *F8* en mujeres portadoras y en pacientes con hemofilia A. Identificación de una mutación nueva.

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**Palabras clave:** portadoras; factor VIII; diagnóstico molecular; mutación; hemofilia A; Venezuela.

**Resumen.** El diagnóstico molecular de pacientes con hemofilia A (HA) tiene múltiples beneficios, incluyendo la confirmación del diagnóstico y la predicción del riesgo de desarrollar inhibidores. En mujeres portadoras de una mutación, el diagnóstico molecular permite el consejo genético y el diagnóstico prenatal, los cuales son parte de la atención integral de HA en muchos países. Así, el objetivo de este estudio fue determinar mutaciones en el gen *F8* en pacientes con HA severa (HAs) y en mujeres portadoras. En 12 pacientes con HAs, la presencia de la inversión del intrón 22 y el intrón 1 fue investigada utilizando una PCR inversa y una convencional, respectivamente. En pacientes negativos para cualquiera de las inversiones, el gen del *F8* fue analizado a través de la técnica de electroforesis en gel sensible a conformación (CSGE) y posterior secuenciación. La mutación causante de la enfermedad fue identificada en 6/12 pacientes, incluyendo la mutación nueva p.G190C. Las madres de estos seis pacientes y las de otros siete pacientes de HAs diagnosticados en un estudio previo y fueron investigadas para la presencia de alteraciones genéticas encontradas en sus hijos. Todas las madres resultaron ser portadoras. Éste es el primer estudio realizado en Venezuela donde se analiza directamente el gen *F8* en portadoras potenciales para identificar específicamente la presencia de una mutación que fue detectada en sus hijos, y complementa un estudio previo con pacientes HAs. Nuestros hallazgos facilitarán la implementación del análisis regular de portadoras de HA en nuestro país y permitirán un mejor cuidado de los síntomas de sangrado y consejo genético.

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## INTRODUCTION

Hemophilia A (HA) is an X-linked recessive bleeding disorder caused by the total or partial absence or dysfunction of blood coagulation factor VIII (FVIII) (1). Severe HA (sHA) is characterized by plasma levels of FVIII < 0.01 IU $mL^{-1}$ (< 1% of normal levels) (2) and is commonly caused by the intron 22 inversion (frequency of approximately 45%) (3) or the intron 1 inversion (frequency of approximately 2%) (4) in the *F8* gene; the rest of the causative mutations is greatly diverse. In the Factor VIII Variant Database, 2,015 unique mutations have been reported to date (5).

Because of the X-linked inheritance pattern of HA, mostly men are affected, while their female relatives may be heterozygous for the mutation, also referred to as carriers. HA carriers are expected to have FVIII plasma concentrations of nearly half of that of healthy individuals, which is generally sufficient for normal

hemostasis. However, a wide range of FVIII levels has been reported in carriers: from very low, resembling male patients, to normal (6). For this reason, molecular diagnosis is the method that offers the most certainty about the carrier status of HA (7).

Similarly to male HA patients, the severity of the bleeding tendency of carriers is correlated to the type of mutation in the *F8* gene (8). It is important to assess the bleeding risk in HA carriers to improve their care, an aspect often not recognized by physicians and health care professionals. Prophylactic factor substitution or alternative treatments may prevent abnormal bleeding such as menorrhagia, in surgical and dental procedures, childbirth, or the treatment of injuries. Also, the molecular diagnosis of HA carriers allows for genetic counseling and prenatal diagnosis of the disease, which have become part of the comprehensive care for HA in many countries (9). On the contrary, this is not an established practice in the Venezuelan healthcare system. The only other study reported was the indirect diagnosis of carriers realized by the analysis of intragenic polymorphisms in the *F8* gene (10).

In this study, we report the results of the first direct molecular diagnosis of HA carriers in Venezuela. In addition, we report the causative mutations in the *F8* gene of 6 Venezuelan sHA non-related male patients, including a mutation that has not been previously reported.

## PATIENTS AND METHODS

### Patients and potential carriers

Twelve non-related male sHA patients, diagnosed biochemically at the National Hemophilia Center located at the Banco Metropolitano de Sangre (Caracas, Venezuela), were studied. The mothers of sHA patients with identified causative mutations [both from the present study and from a previous work (11)] were investigated for the presence of the genetic alterations found in their sons. Informed consent was obtained from all participants**.**

### Detection of *F8* mutations

Genomic DNA extraction was performed from peripheral blood samples by standard protocols using chloroform purification and ethanol precipitation (12). Detection of *F8* mutations was initially performed in all sHA patient samples, and once a mutation was identified, it was investigated in their mothers by the same method. The presence of the intron 22 inversion was investigated using a touchdown inverse PCR method, as previously described (11). Patients negative for the intron 22 inversion were then analyzed for the presence of the intron 1 inversion, as previously reported (4). Finally, the *F8* was screened in all patients negative for both inversions. The entire coding region, including the flanking splice sites, the 5'- and 3'-UTR, was amplified through multiplex PCR (M-PCR), as previously described (11). The M-PCR products were screened for mutations through a mildly denaturing conformation-sensitive gel electrophoresis (CSGE) (12.5% polyacrylamide) (13). Samples displaying abnormal CSGE patterns were sequenced in the forward and reverse directions using an automated ABI 377 genetic analyzer (Applied Biosystems) at the UEGF-IVIC (Caracas, Venezuela). Mothers of patients with point mutations were analyzed by sequencing the potentially affected region of *F8*. Mutation nomenclature was according to the Human Genome Variation Society (HGVS) (14).

### *In silico* analysis of missense mutations

The pathological authenticity of novel (not previously reported in the Factor VIII Variant Database) or not previously analyzed *F8* gene mutations was determined through the fo-

llowing steps: 1) In UniProtKB (15), the domain and chain location of the mutation was determined. Information was obtained on whether the mutation occurred at an amino acid involved in cleavage, post-translational modification or disulfide bond. 2) The potential effect of amino acid substitutions was analyzed with the software UCSF Chimera, version 1.7 (16), using the tridimensional model of the crystal structure of FVIII available at the Protein Data Bank (17, 18) (PDB ID: 2R7E). 3) MutPred (19) and PolyPhen-2 (20) (version 2.2.2) were used to corroborate the probability of an amino acid substitution being deleterious. 4) Evolutionary sequence conservation of the substituted amino acids was evaluated through sequence alignment of *F8* orthologues from eight vertebrate species: *Homo sapiens* (NP_000123), *Bos taurus* (NP_001138980), *Canis lupus familiaris* (AAB87412), *Mus musculus* (NP_032003), *Oryctolaguscuniculus* (ACA42556), *Rattus norvegicus* (NP_899160), *Susscrofa* (NP_999332), and *Takifugu rubripes* (NP_001027922). Sequence alignment was performed with the DNAMAN® software (21) and the sequences were obtained from Gene Bank.

## RESULTS

### Molecular diagnosis of sHA patients

*F8* variants were identified in eight out of 12 non-related male sHA patients (Table I). No mutations were identified in 33.33% of the patients studied. The intron 22 inversion was detected in three patients, while point mutations were identified in five patients: two nonsense (p.S872* and p.R1985*) and three missense (p.G190C, p.D1260E and p.M2257V). The mutation p.D1260E was detected in two unrelated patients, while p.G190C has not been previously described and therefore we report it as a novel mutation for sHA. *In silico* analysis suggests a possible pathogenic effect of this mutation, as discussed later. Only the intron 22 inversion, p.S872* and p.R1985* mutations have been previously associated with a pathogenic effect. Altogether, were detected 6 distinct sHA causative mutations, as also discussed later. The intron 1 inversion was not found in the patients analyzed.

### Molecular diagnosis of mothers of sHA patients

The six distinct causative mutations detected in this study and those found in seven patients from a previous work (11) [3 intron 22 inversions, 2 missense (p.R1966* and p.S524*), one splicing (c.6654-1G>A) and one small deletion/insertion (del_TTGT209-212) mutations] were used to identify the carrier status among the mothers of these patients. All 13 possible carriers were found to have the mutation, for a carriership frequency of 100%.

## DISCUSSION

### Intron 22 and intron 1 inversions

The intron 22 inversion was the most common mutation found in this study, with an incidence of 25%. In a previous study (11) we found a 41% frequency for this mutation, which is consistent with the 45% reported in other studies (3, 4). Additionally, no intron 1 inversions were found, while it is reported to cause approximately 2% of HA cases (4). We assume that the lower than expected frequencies are most likely due to the small number of patients studied. However, it should be noted that the intron 1 inversion, the second most common causative mutation of sHA, is yet to be found in Venezuelan patients, as it was not detected in this study and neither in the 54 sHA patients studied in the first and only other work of this kind in Venezuela (11).

### Nonsense and missense mutations

We found two nonsense mutations that have

**TABLE I**
MUTATIONS IDENTIFIED IN THE *F8* GENE IN 8 OF 12 sHA MALE PATIENTS

| Patient ID | Mutation* | Amino acid change* | Type of mutation | Exon/intron | FVIII domain | previous reports† |
|---|---|---|---|---|---|---|
| H178 | Intron 22 inversion | – | Complex rearrangement | – | – | Several |
| H180 | Intron 22 inversion | – | Complex rearrangement | – | – | Several |
| H182 | Intron 22 inversion | – | Complex rearrangement | – | – | Several |
| H211 | c.568G>T | p.G190C | Missense | 4 | A1 | 0 |
| H196 | c.2615C>G | p.S872X | Nonsense | 14 | B | 3‡ |
| H162 | c.3951C>G | p.D1260E§ | Missense | 14 | B | 2 |
| H158 | c.3951C>G; | p.D1260E§ | Missense | 14 | B | 2 |
| | c.6940A>G | p.M2257V§ | Missense | 25 | C2 | 5 |
| H183 | c.5953C>T | p.R1985X | Nonsense | 18 | A3 | 30 |

*Mutation nomenclature was according to the Human Genome Variation Society (HGVS) (13).
†Number of previous reports in non-related individuals according to the Factor VIII Variant Database (5).
‡2 reports in the Factor VIII Variant Database and 1 in a previous work in Venezuela (10).
§Non-deleterious mutations.

been previously reported as sHA causative mutations: p.S872* and p.R1985* (22, 23). In addition, we detected two missense mutations that have been previously reported: p.D1260E and p.M2257V. However, the effects of these amino acid changes on the function of FVIII have not been analyzed in earlier publications.

Mutation p.D1260E was found in two unrelated patients in this study: H158 and H162 (Table I). It has been previously reported as a polymorphism (rs1800291) and the mutant allele has been associated with reduced FVIII:C levels (24, 25). However, no other missense mutation in the Asp1260 residue has been reported, it is not conserved, and PolyPhen and MutPred analysis both suggest it has no deleterious effect on the FVIII protein (Table II).

Mutation p.M2257V, also reported as a polymorphism (rs1800297), was first detected in 4/42 non-related Brazilian patients with moderate HA in whom no other mutation was found (26). The researchers considered the possibility that this relatively common alteration was polymorphic and thus analyzed 100 Dutch and 63 Brazilian unaffected individuals, none of which had the mutation. It was found in a later study in 1/7 Jamaican sHA patients (27). This patient, however, had another mutation in the *F8* gene (intron 5 671-2A>G), to which the authors attribute the cause of the disease. Furthermore, the p.M2257V mutation was present in healthy family members of this patient, and the authors found a 42% heterozygosity rate of the mutation in 31 unrelated Jamaican individuals. No other missense mutations have been reported in the Met2257 residue and it is conserved

**TABLE II**
*IN SILICO* ANALYSIS OF NOVEL OR PREVIOUSLY NOT ANALYZED MISSENSE MUTATIONS

| Mutation* | Amino acid change* | Degree of conservation† | PolyPhen score | MutPred probability of deleterious mutation |
|---|---|---|---|---|
| c.6940A>G | p.M2257V | M/M/M/M/M/V/V/− | Benign (0.15) | 0.60 |
| c.3951C>G | p.D1260E | D/E/E/V/T/T/G/I | Benign (0.00) | 0.10 |
| c.568G>T | p.G190C | G/G/G/G/G/G/G/G | Probably damaging (1.00) | 0.85 |

*Mutation nomenclature was according to the Human Genome Variation Society (HGVS) (13).
†Amino acid residue present in the Factor VIII sequence for each of the following species: *Homo sapiens/ Bos Taurus/ Canis lupus familiaris/ Mus musculus/ Oryctolagus cuniculus/ Rattus norvegicus/ Sus scrofa/ Takifugu rubripes.*

in 5/8 of the species analyzed (Table II). While Met2257 generates one clash with Cys2345 (Fig. 1a), Val2257 is predicted to generate two clashes of higher overlap values with Phe2253 (Fig. 1b, orange lines). Since both valine and phenylalanine are hydrophobic amino acids, these unfavorable interactions may cause a steric hindrance effect in the C2 domain, which may affect its stability or conformation and potentially its interaction with von Willebrand Factor (VWF) and/or phospholipids. PolyPhen predicts the amino acid substitution to be benign (0.15); however MutPred calculates the probability of it being a deleterious mutation at 0.60. Even though the previous evidence is not conclusive, it would seem that this mutation is not causative of sHA because it has been found in healthy male subjects (27).To date we have not found reports of patients or healthy individuals carrying both polymorphisms (p.D1260E and p.M2257V). The allelic frequencies reported in world population are 0.19 and 0.03 for p. D1260E and p.M2257V, respectively (28). The low frequency of allele Val2257, could explain the absence of individuals with both mutations. Even though patient H158 has two apparently innocuous mutations in *F8* (p.D1260E and p.M2257V) and another pathogenic mutation was not found, an additive effect of both alterations in FVIII could not be discarded. However, this could not be further explored in the crystal structure of FVIII because it does not contain the B domain, which includes amino acid D1260.

We also detected the novel mutation p. G190C. A missense mutation in the same amino acid position, p.G190D, has been previously reported as a sHA causative mutation, but no mechanism was proposed (29). Gly190 is a highly conserved residue (Table II) located in the A1 domain, specifically in the plastocyanin-like 1 domain (18). As shown in the tridimensional structural model of FVIII, the cysteine residue may cause a steric hindrance effect (Fig. 1d); in fact, five clashes are generated in the mutant protein, four of them with carbon atoms of residue Leu69. These unfavorable interactions may affect the stability or conformation of this domain, which could affect its copper-binding property. PolyPhen predicts the amino acid substitution to be probably damaging (1.00) and MutPred calculates that the probability of it being a deleterious mutation to be 0.85 (Table II).

No mutations were identified in 33.33% of the patients studied. It is possible that the

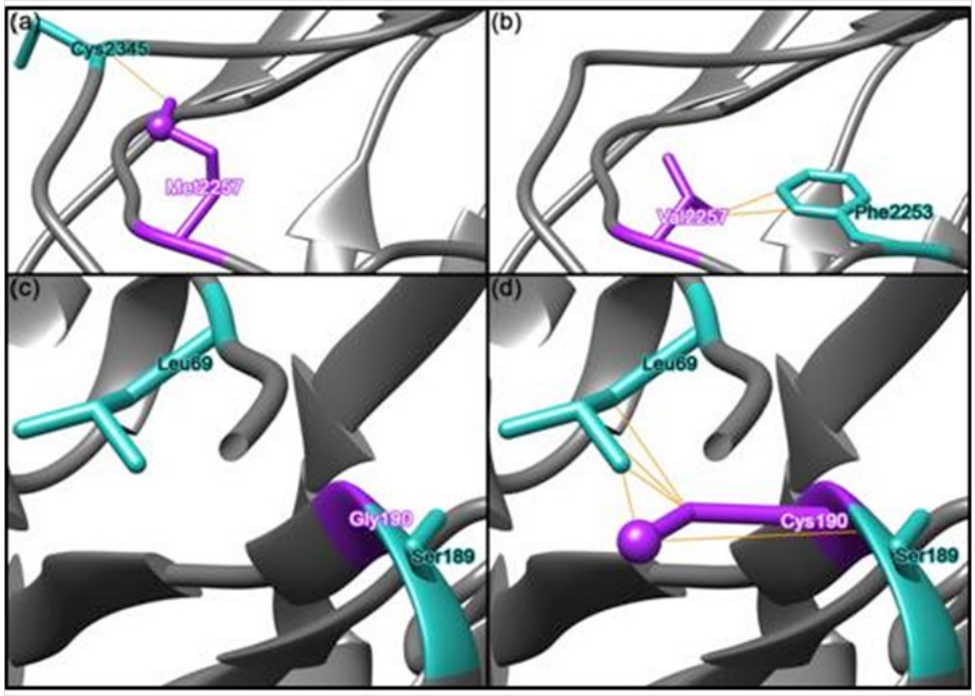


**Fig. 1.** Three dimensional representations of amino acidic residues interactions in two missense mutations: (a and b) Effect of mutation p.M2257V. (c and d) Effect of mutation p.G190C. The affected amino acids are shown in purple; the interacting amino acids are shown in cyan; clashes are represented as orange lines; sulfur atom on Cys190 is represented as a ball.

M-PCR–CSGE approach may have missed some mutations or that the mutation is outside of the region analyzed, like intronic sequence changes that might affect transcription or translation. In addition, the size of the fragment is important for the CSGE sensitivity (30) and some of the fragments analyzed in this study exceeded the recommended size (200- 450 bp) because of the adoption of previously described primers. However, this technique showed a 91% of sensibility in our previous study (11).

## Carriership of severe hemophilia A

We found that all mothers of patients with sHA were carriers. In spite of the small number of mothers studied, this frequency is within the expected range. It has been calculated that the mother of a patient with sporadic HA has a probability of 0.85 of being a carrier, while in the case of familiar HA, the probability is 1.00 (31). Since we had no information about the occurrence of the disease in the families analyzed in this study, we expected a carrier state frequency between 85 and 100%.

All six mothers of patients with the intron 22 inversion were carriers of the mutation. This is consistent with other studies in which 20/20, 43/43 and 49/50 mothers were carriers of the inversion (32-34). This high probability of intron 22 inversion carriership is due to the fact that the recombination that produces the inversion is more probable during male meiosis because the homologous pairing of X chromosomes that occurs during female meiosis inhibits the bending

of the tip of X chromosomes (35). In fact, it has been reported that the sex ratio frequency of this mutation is >10-fold-higher in male germ cells (22). Therefore, intron 22 inversion has a higher probability of originating in the male germ cells of the maternal grandfather than in the female germ cells of the mother.

The point mutation carriership frequency found in this study was of 100% (7/7). This result matches another study that found that point mutations have a sex ratio frequency 5 to10-fold higher in male germ cells (22). It has been suggested that this tendency is due in part to the fact that methylation at CpG sites in the female germ line is considerably reduced (36). Since methylated cytosine is prone to deamination and further mutation to thymidine, transitions C to T and G to A occur more frequently in male germ lines. This mechanism is particularly relevant in HA because 40% of causative point mutations occur in one of 70 CpG sites of the *F8* gene, even though these regions represent only 2% of the coding region of the gene (37).

The results obtained in this study highlight the importance of implementing the molecular diagnosis of HA carriers in Venezuela, given the high probability for a patient's mother to be a carrier. This information is useful for family members of yet unknown HA status who could benefit from genetic counseling for future pregnancies, which is highly relevant in third-world countries. Knowledge of the causative mutation can improve clinical care of both HA carriers and patients, as the mutation type is correlated to phenotype severity (8) and, in the case of male patients, to the risk of developing inhibitors (38).

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