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# Genotype-Phenotype Interaction Analyses in Hemophilia

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## 1. Introduction

As with monogenic diseases, hemophilia A and hemophilia B have a direct relationship between factor VIII and factor IX gene mutations, respectively, and their causative effect on protein deficiency either in function or reduced antigen level in plasma. These aspects are related to, but do not totally explain, a more complex clinical phenotype such as the age of initial symptom onset, bleeding tendency, inhibitor development, arthropathy tendency, carrier bleeding symptoms, etc., which currently are critical complications under study in various clinical protocols in order to improve medical care in patients with hemophilia.

The complex relationship between clinical behavior and genetics of hemophilia is changing the approach to diagnosis and research methods, expanding the scope of analysis to other related genes (bleeding tendency, immune system, regulatory genes of X-chromosome expression, etc.). In addition, novel functional approaches can provide prognostic parameters of clinical behavior such as gene expression assays and biochemical analyses including kinetics of inhibitors to factor VIII or thrombin generation assay by the standardized method of calibrated automated thrombography. This method describes the overall clotting capacity of patients' plasma *in vitro* and *ex vivo*. This chapter will focus on certain studies regarding genotype-phenotype interactions in hemophilia that have been applied in Mexican hemophilia families for molecular diagnosis and genetic counseling. These studies have also been used to determine prognostic factors for clinical behavior and treatment response in hemophilia patients in order to improve hematological management as well as to optimize the use of therapeutic resources, an important consideration in developing countries such as Mexico.

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## 2. Mutation–phenotype correlation in hemophilia

### 2.1 Origin of mutations in hemophilia

Because of the high mutation rate of *factor VIII* gene ( $2.5\text{--}4.2 \times 10^5$ ), ~50% of the severely affected families have only one affected case (isolated), pointing to a recent mutation occurring in the grandparental or parental generation. Family studies reveal that most mutations in hemophilia A originate in male germ cells with a predominance of point mutations. Some deletions occur in female gametes and *de novo* mutations may occur in early embryogenesis from a somatic or germinal mosaicism. This mechanism, if minor, remains underestimated by routine analysis and originates predominantly from females in the case of somatic mosaicism. This may represent a frequent event in hemophilia that must be considered in genetic counseling of isolated cases that mainly involve point mutations (Leuer et al., 2001).

In the case of *factor IX* gene, a considerably lower mutation rate than for *factor VIII* has been reported ( $3.2 \times 10^9$ ) (Koeberl et al., 1990). However, in hemophilia B there is also a high proportion of recent germline mutations originating in the last three generations of isolated cases that have been studied in different populations. A similar mutation pattern has been found, suggesting an endogenous mechanism for the genetic changes in *factor IX* gene causing hemophilia B. The endogenous mechanism indicates that genetic characteristics of the gene (rather than environmental conditions) account for most human germline mutations (Sommer 1995, as cited in Jaloma-Cruz et al., 2000). Point mutations are the most common mutations in hemophilia, being present in >90% of the patients. This is followed by deletions in 5-10% of the cases. Less frequent are the insertion/inversion rearrangements with the exception of intron 22 inversion of *factor VIII* gene in hemophilia A. This is the most common genetic rearrangement demonstrated in severe disease, comprising 40-50% of cases (Bowen, 2002).

### 2.2 Mutation pattern in hemophilia

There is a high degree of heterogeneity in the location and type of mutations in *factor VIII* and *factor IX* genes causing hemophilia; >90% are located in coding and promoter regions as well in junction sites of intron-exon. Types of mutation and their relative frequencies are determined by genetic mechanisms according to the genetic sequence (mutation hot-spots such as CpG dinucleotides, gene size, nucleotide repeats, etc.). A general pattern of mutations is found as follows: single nucleotide changes: transitions > transversions; deletions > insertions > complex rearrangements (Sommer, 1995, as cited in Jaloma-Cruz et al., 2000).

### 2.3 Unusual mutagenesis mechanism in the Mexican hemophilia B population

From a study of nine independent Mexican hemophilia B families (Jaloma-Cruz et al., 2000), we found a particular mechanism of recurrent mutagenesis by four single independent substitutions in two similar non-CpG sites at nucleotide positions 17,678 (C88Y, C88F) and 17,747 (C111S, C111Y) of *factor IX* gene (Table 1). Using a statistical test considering a mutation target of 439 nucleotide position non-CpG sites in the coding region of *factor IX* (where >96% of *factor IX* gene mutations occur), it was demonstrated that the observed mutations were nonrandom events ( $p = 0.0004$ ) (Jaloma-Cruz et al., 2000). These mutations were considered as first-line evidence of a mechanism of recurrent mutation in hemophilia involving unusual hot-spot sites. It will be interesting to continue these types of studies in

epidemiological analyses to explore the subjacent mechanism of causative mutagenesis in particular populations.

Proband (Clinical) <sup>a</sup>	Mutation	Effect	Origin <sup>b</sup>	Geographical Region	Haplotype <sup>c</sup>
HB758 (M)	17747G>C	C111S	Familial	Michoacan, Central Pacific Coast	C/-/I/-/-/IV/-
HB759 (S)	Exons F,G&H	LD <sup>d</sup>	Familial	Michoacan, Central Pacific Coast	C/-/I/-/-/-
HB760 (M)	17678G>A	C88Y	<i>de novo</i> MF	Jalisco, Central Pacific Coast	T/-/I/-/-/IV/-
HB762 (S)	20519G>A@CpG	R180Q	<i>de novo</i> MM	Nuevo Leon, Northeast	C/-/I/-/-/II/-
HB763 (M)	31284-5del AG	fs <sup>e</sup>	Familial	Michoacan, Central Pacific Coast	C/-/I/-/-/II/+
HB764 (S)	20519G>A@CpG	R180Q	Sporadic	Sinaloa, North Pacific Coast	C/-/I/-/-/II/-
HB765 (M)	17747G>A	C111Y	<i>de novo</i> M	Nuevo Leon, Northeast	C/-/I/-/-/II/-
HB766 (S)	Exons G&H	LD <sup>d</sup>	Familial	Michoacan, Central Pacific Coast	C/-/I/+/-/+/-
HB767 (N)	17678G>T	C88F	Sporadic	Puebla, Central	C/-/I/-/-/II/+

Table 1. Summary of Mutations in Mexican Patients with Hemophilia B

<sup>a</sup>S = Severe; M = Moderate; N = No data (White, 2001).  
<sup>b</sup>HB760: the sequence change at 17678 was present in the index patient and mother, but absent from both maternal grandmother and grandfather. The 3'RY(i) polymorphism, established the maternal grandfather (MF) as the origin. HB762: the mother was a carrier, but the sequence change at 20,519 was not present in 13 maternal aunts and uncles or in the maternal grandfather. Extragenic polymorphisms, *DXS* 1211 and *DXS* 1232, established the maternal grandmother (MM) as the origin. HB765: the sequence change at 17,747 was present only in the index patient, establishing the mother (M) as the origin.  
<sup>c</sup>*MseI*/*BamHI*/*HinfI*/*XmnI*/*TaqI*/3'RY(i)/*HhaI*.  
<sup>d</sup>LD, large deletions. The *MCF2* gene, located about 80 kb downstream of the *F9* transcription start site is present in HB759 but absent in HB766, indicating deletions of about 60 kb and more than 100 kb, respectively.  
<sup>e</sup>Microdeletion resulting in frameshift (fs) at G388 and stop codon at I408.  
(Modified from Jaloma et al., 2000)

2.4 Genotype-phenotype correlation in hemophilia

As with monogenic diseases, hemophilia A and B have a direct relationship between *factor VIII* and *factor IX* gene mutations, respectively, and their causative phenotype effect at the clinical level secondary to the caused protein deficiency, either in function or reduced antigen level in plasma (Koeberl et al., 1990). In general, there is good correlation between the type of mutation (location in the amino-acid position and domain in the protein) and their functional outcome, yielding a predictable clinical severity. However, some authors affirm that this correlation is rare and, in the majority of cases, a clear correlation has not been shown (Bowen, 2002) with the exception in a very few cases of self-evident molecular defects such as large deletions,

nonsense mutations causing premature translation stop and truncated protein, corrupted mRNA splicing, etc.

Because of the difficulty in presenting a clear correlation, in 2001 the International Committee of Standardization in Thrombosis and Hemostasis established the clinical severity classification according to the clotting level in plasma and not to clinical manifestations (more complex phenotype). A direct relationship between the genetic defect and protein activity is expected (Table 2) (White, 2001).

Factor Level	Classification
<0.01 IU/ml (<1% of normal)	Severe
0.01 – 0.05 IU/ml (1%-5% of normal)	Moderate
> 0.05 - <0.40 IU/ml (>5% - <40% of normal)	Mild

Table 2. Classification of hemophilia A and B. Normal is 1 IU/ml of factor VIII:C (valid also for factor IX:C) as defined by the current World Health Organization International Standard for Plasma Factor VIII:C (as distributed by The National Institute for Biological Standards and Control, Potters Bar, Hertsfordshire, UK). (Modified from White et al., 2001).

The limitation of the criterion of clotting activity of deficient factors VIII and IX to establish clinical severity in hemophilia may be due to the fault of reproducibility of laboratory tests of coagulation. These may be due to assay problems such as sample management (preanalytical factors), quality of reagents, zero point activity, etc., that must be carefully controlled in order to have a confident result and the relationship with clinical severity (Barrowcliffe, 2004). These problems also lead to investigation of newer methods with closer physiological correlation, higher sensitivity and less coefficient variation values such as thrombin generation test or thromboelastography (Barrowcliffe, 2004).

3. Structure-function relationship of factor IX gene mutations at the subcellular level

During the search for mechanisms that cause mutations in hemophilia and its impact on the relationship between the structure-function of mutant proteins, several analytical investigations have been developed at the cellular level of the interaction of factor VIII (FVIII) and factor IX (FIX) mutant proteins with other intracellular components involved in their posttranslational processing and secretion.

3.1 Structure-function relationship in mutant FIX proteins

In the study of factor IX mutations causing a severe phenotype, two mutations were analysed. These were located at the first and second epidermal growth factor (EGF) domain,



C71Y and C109Y, respectively, both affecting a cysteine site and, therefore, the folding native structure of FIX protein (Enjolras et al., 2004). Different analyses of posttranslational processing and intracellular trafficking revealed that neither mutant was secreted nor accumulated in the intracellular space due to their interaction with chaperones from endoplasmic reticulum (ER) that cause their arrest and lead to their degradation into proteasomes (Enjolras et al., 2004). A related study was carried out for the relevant *factor IX* mutations identified in Mexican hemophilia B patients that were caused by a recurrent mutagenesis at non-CpG sites (Jaloma-Cruz et al., 2000). They also affected the structure-function of FIX by changes of a cysteine position in the second epidermal growth factor (EGF2) domain of *factor IX* gene (C111S and C111Y mutations).

### 3.2 Directed mutagenesis and functional analysis of FIX mutations

The two mutations at cysteine 111 cause severe hemophilia B in Mexican hemophilia B patients. To analyze their impact on the structure-function relationship of FIX, the effect of inhibitors of intracellular trafficking was studied comparing C111 wild-type (wt) and the C111S and C111Y mutations that were inserted by directed-site mutagenesis into an expression vector (pcDNA 3.1®) containing *factor IX* wild type (wt) gene. Transfection by Fugene6® was evaluated in Cos-7 cells after 48 h. Intracellular production and secretion of FIX were quantified by ELISA assay. Transfected cells were incubated for 6 h with different inhibitors of intracellular trafficking classified by their solubility properties; hydrosolubles: NH<sub>4</sub>Cl and leupeptin, which are lysosomal inhibitors, and liposolubles: Brefeldin A, that blocks protein transport from ER to the Golgi complex; cycloheximide, inhibitor of synthesis protein, N-acetyl-leu-leu-norleucinal (ALLN) and clasto-lactacystin beta-lactone (calpain), both proteasomal inhibitors (Mantilla-Capacho et al., 2008).

The mutants showed a decreased FIX secretion (20%) and intracellular accumulation of 140% (C111Y) and 160% (C111S) with respect to wt *factor IX*. The inhibitors caused higher intracellular accumulation, which evidenced a degradation primarily in lysosomes (NH<sub>4</sub>Cl) of both mutants. C111S mutation showed a strong effect of Brefeldin A, suggesting an adequate transport from ER to Golgi complex in contrast to C111Y, which showed higher proteasomal degradation, evidenced by the effect of ALLN (Figure 1) (Mantilla-Capacho et al., 2008).

The study concluded that the disruption of the disulfide bond in the mutants has an important effect on the native folding of FIX due to their accumulation in the intracellular space in regard to wt FIX. C111Y showed a higher impact than C111S on its transport through ER with a predominant degradation at proteasomes (Mantilla-Capacho et al., 2008). Other *factor IX* mutations previously identified in the Mexican population have also been analyzed using related approaches for the analysis of genotype-phenotype interaction at the subcellular level. These demonstrate evidence of posttranscriptional regulation mechanisms and behavior of mutant proteins that reveal the importance of key sites of protein function. Further studies are essential for a better understanding of the properties of FVIII and FIX and the biochemical phenotype of hemophilia.

## 4. Molecular diagnosis for carrier testing

The wide mutational heterogeneity in both types of hemophilia compels the use of intragenic polymorphisms for carrier testing. Different polymorphisms have been described in *factor VIII* and *factor IX* genes such as single nucleotide polymorphisms (SNPs) identified

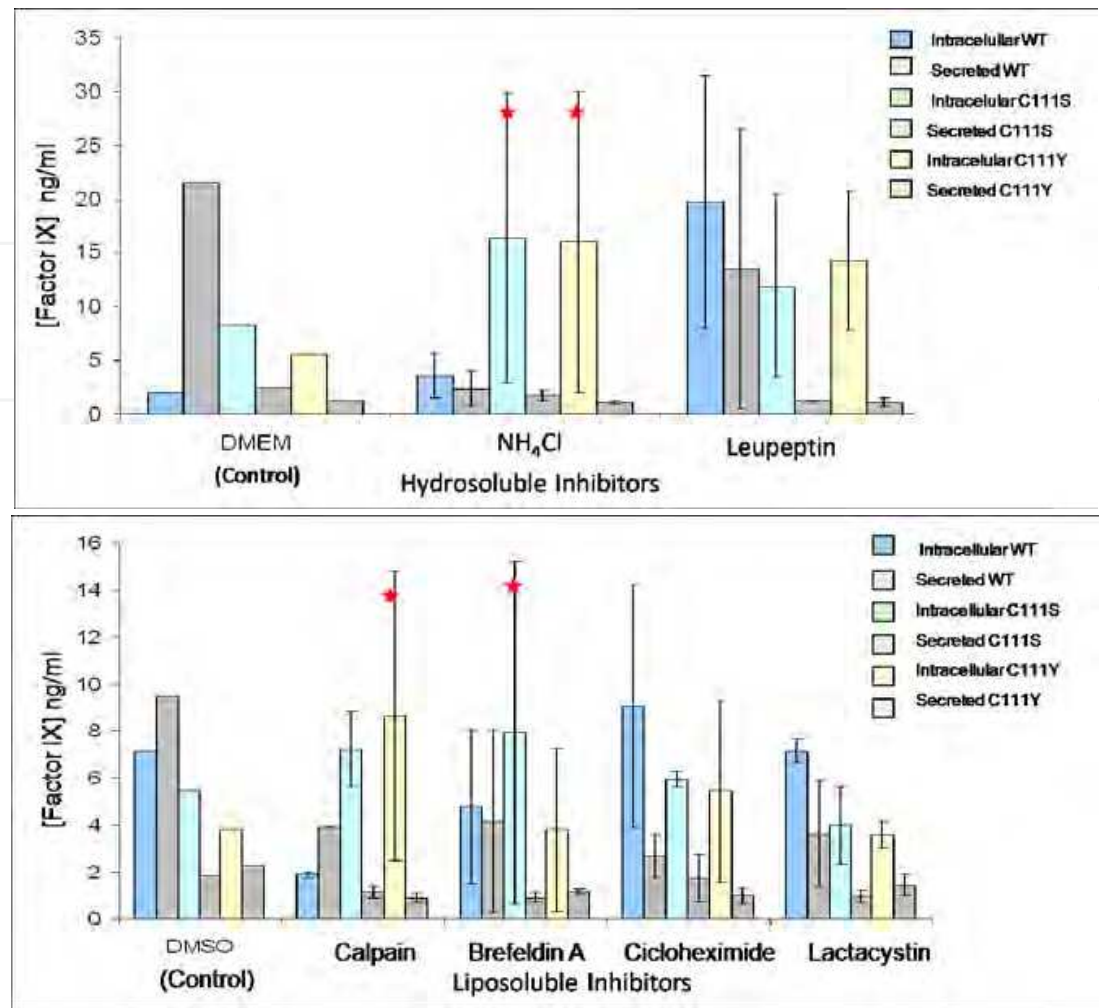


Fig. 1. Effect of inhibitors of cellular trafficking on factor IX production (intracellular and secretion) of C111S and C111Y mutations of *factor IX* gene. Significant effect is highlighted by red stars.

by restriction fragment length enzyme polymorphisms (RFLPs), variable number of tandem repeats (VNTRs) or microsatellites defined by the length of the repeated units [1-4 nucleotides, short tandem repeats (STRs), >5 nucleotides, VNTRs] (Bowen, 2002). According to recent knowledge of the human genome and the high variability among individuals, a difference is expected of one base for every 1250 base pairs on average. According to the gene sizes of *factor VIII* (186 kb) and *factor IX* (34 kb), both genes would be expected to be ~144 and 27 SNPs, respectively. However, fewer polymorphisms have been identified, which means a paucity of polymorphisms or a detection problem, this last reason being more plausible because new polymorphisms continue to be described (Figure 2) (Bowen, 2002; Kim, 2005).

4.1 Linkage analysis in hemophilia A and B

Automatic sequencing methods and high-yield analysis techniques of the human genome have extended the detection of mutations in both types of hemophilias. In the case of *factor VIII* and *factor IX* genes, diverse polymorphisms have been used for carrier diagnosis as a low cost, alternative and rapid method. Use of intragenic polymorphisms by linkage analysis shows high confidence (>95%) according to the linkage disequilibrium between

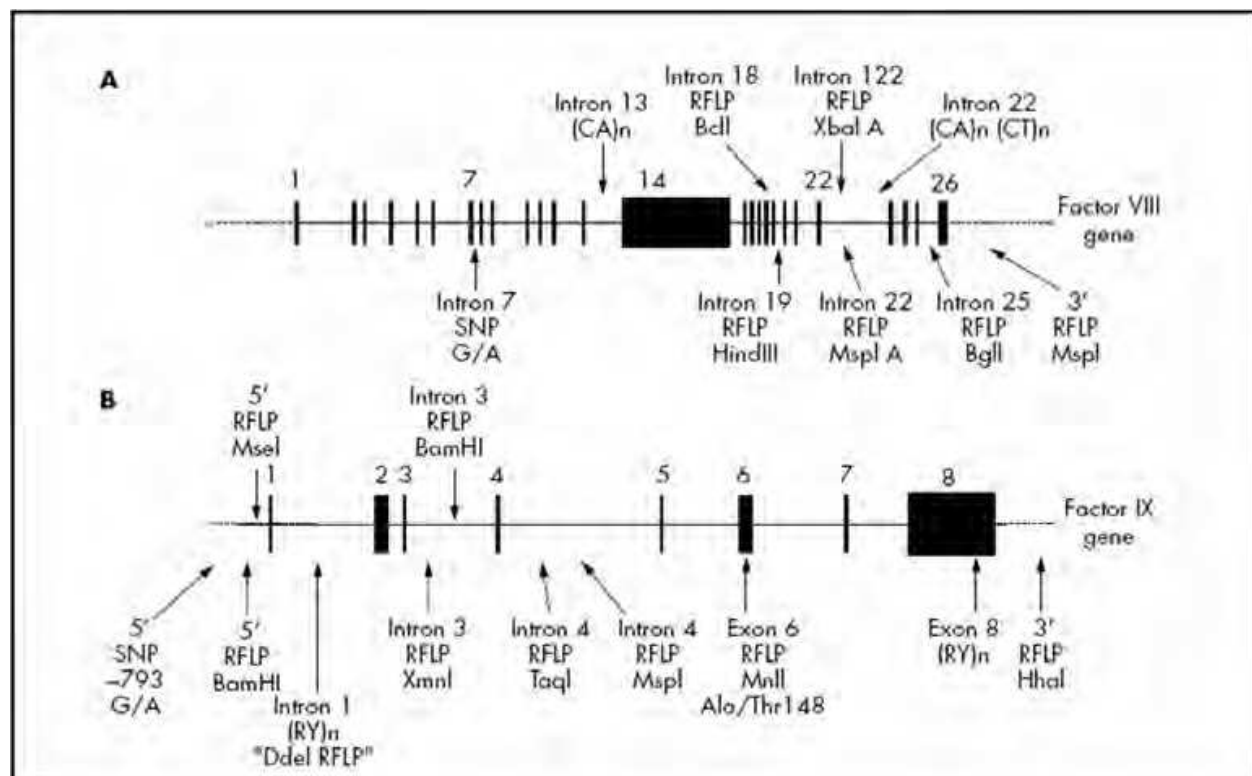


Fig. 2. Polymorphisms of *factor VIII* and *factor IX* genes. Some of the known polymorphisms in the human genes for (A) *factor VIII* and (B) *factor IX*.

a) *Factor VIII* gene: intron 7 G/A, intron 13 (CA)<sub>n</sub>, intron 18 *BclI*, intron 19 *HindIII*, intron 22 *XbaI* A, intron 22 *MspI* A, intron 22 (CA)<sub>n</sub>, intron 25 *BglI*, 3' *MspI*.

b) *Factor IX* gene: 5' -793 G/A, 5' *BamHI*, 5' *MseI*, intron 1 *DdeI*, intron 3 *XmnI*, intron 3 *BamHI*, intron 4 *TaqI*, intron 4 *MspI*, exon 6 *MnlI*, exon 8 (RY)<sub>n</sub>, and 3' *HhaI*.

(Figure and source references of polymorphisms as cited in Bowen, 2002).

polymorphisms and the causative mutation of the hemophilia (Mantilla-Capacho et al., 2005). This strategy requires sampling of all family members to trace the segregation of the polymorphism linked to the mutated X-chromosome for *factor VIII* or *factor IX* genes. The main limitation of the strategy is the informativeness of the polymorphism that is defined by its heterozygosity in a population, with a maximum value of 50%, corresponding to the highest probability of finding two alleles in the obligated carrier of one family. The described polymorphisms must be analyzed in each population in order to identify the useful markers.

#### 4.2 Carrier diagnosis strategy in the Mexican population

For molecular analysis of carrier diagnosis, we developed a strategy based on intragenic polymorphism linkage analysis. According to their diagnostic informativeness percentage in the Mexican population, for *factor VIII* gene we initially used the following: the microsatellite of (CA)<sub>n</sub> of intron 13 (75%) and the RFLPs *BclI*- intron 18 (50%) and *AlwNI*-intron 7 (20%) (Mantilla-Capacho et al., 2005).

In order to improve the technical feasibility and informative level of carrier diagnosis in Mexican families with hemophilia A, we used the method of Kim et al. (2005) based on fluorescent PCR of four intragenic dinucleotide-repeat polymorphisms analyzed by automated Genescan®. Preliminary data show that the use of dinucleotide repeats at introns



1, 13 and 22 achieved a significant increase in informativeness (>85%), which is useful for carrier testing in more than 200 hemophilia A families (González-Ramos et al., 2010).

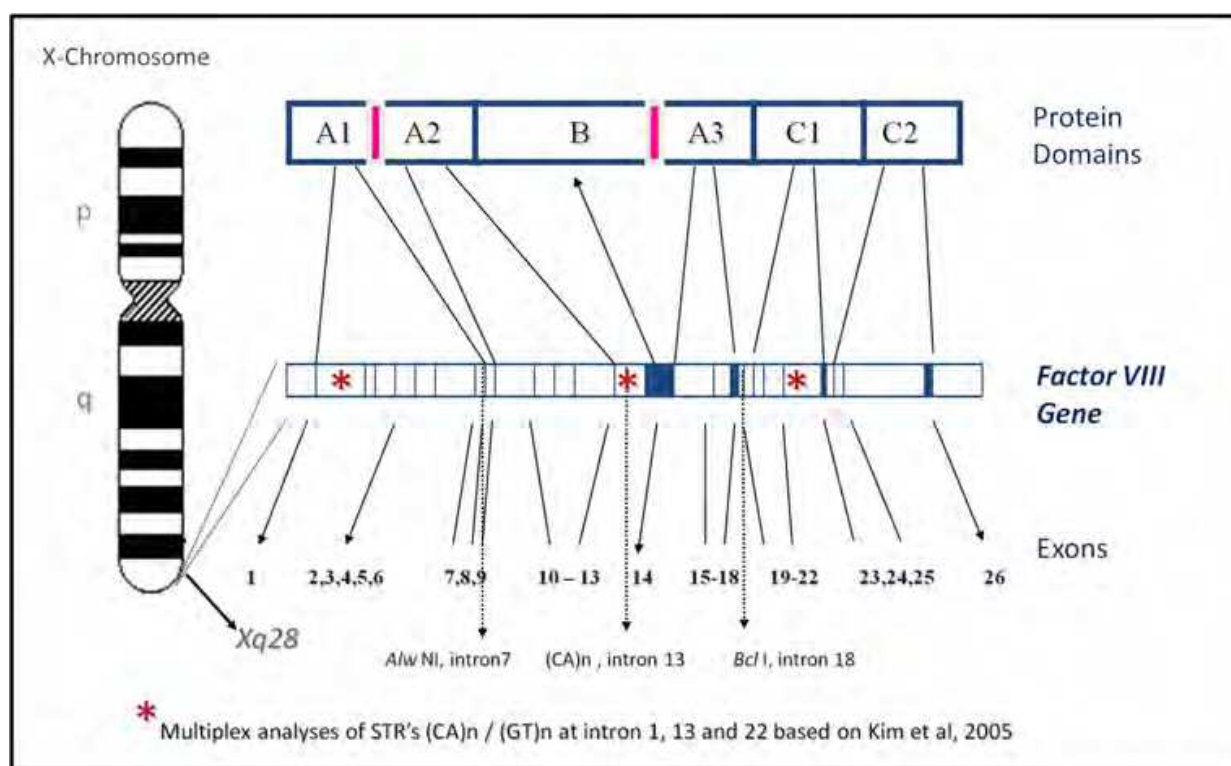


Fig. 3. Intragenic polymorphisms of *factor VIII* gene used for carrier diagnosis in hemophilia A in the Mexican population

In the case of hemophilia B we used four RFLPs of *factor IX* gene in order of heterozygosity: *HhaI*-3' terminal region (50%); *NruI* and *SalI* in the promoter region (40-20%); *TaqI*-intron D (30%) and *HinfI*-intron A (25%). Together the polymorphisms are highly informative and most families (>90%) are diagnosed for carrier status using these markers (Mantilla-Capacho et al., 2005).

#### 4.3 Detection of common mutations in hemophilia A

The inversion of intron 22 in *factor VIII* gene is the most common mutation in hemophilia A. It is the cause of the severe form of the disease due to the inversion of *factor VIII* gene at intron 22 and its disruption from the rest of the gene caused by an intrachromosomal recombination of a region at intron 22 and two copies located at 400 kb toward the telomeric region (Lakich et al., 1993; Naylor et al., 1993). This rearrangement is responsible for 40-50% of severe hemophilia A cases. A similar mechanism at intron 1 accounts for 1-5% of severe cases (Bagnall et al., 2002).

Using the method of long-distance PCR (Liu et al., 1998) we found a frequency of 45% of the intron 22 inversion in patients with severe hemophilia A. We did not find the intron 1 inversion in the Mexican population with severe hemophilia A (n=65) (Mantilla-Capacho et al., 2007). Intron 22 inversion as the complex rearrangement causing the absence of FVIII protein has been identified as a moderate genetic risk factor for inhibitor development in patients with the severe form of the disease (Mantilla-Capacho et al., 2007).

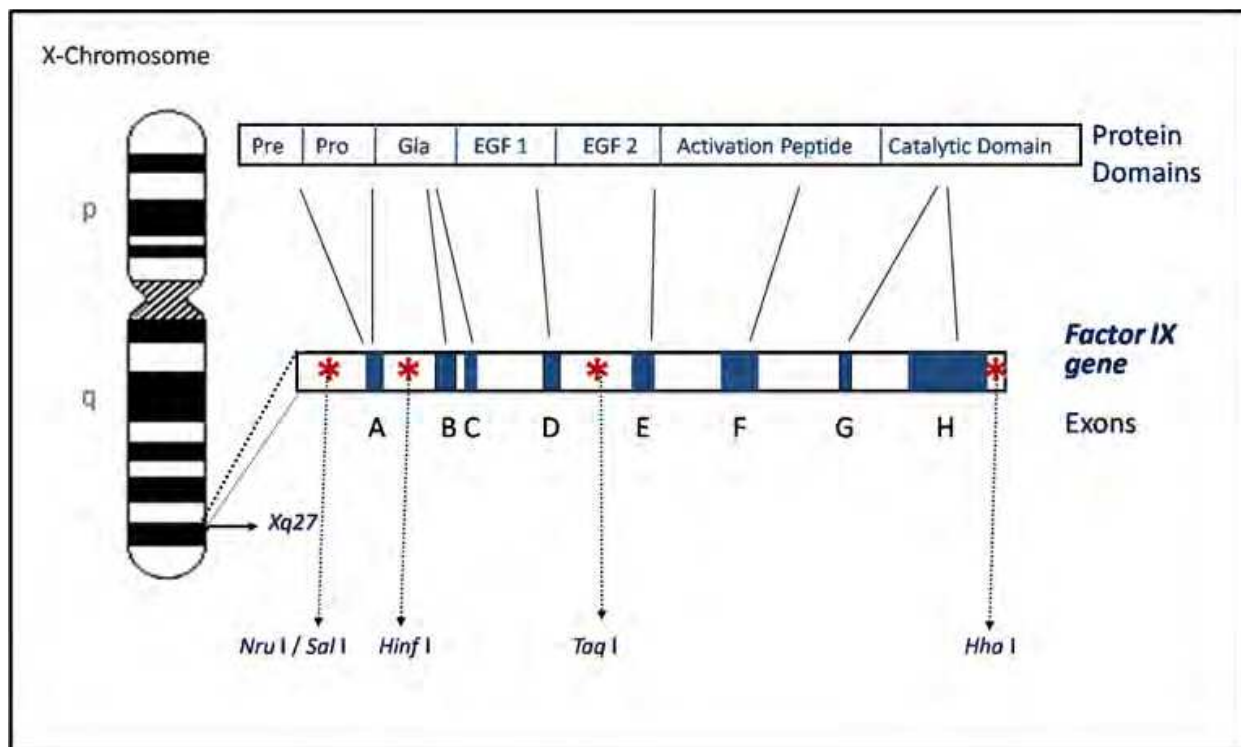


Fig. 4. Intragenic polymorphisms of *factor IX* gene used for carrier diagnosis in hemophilia B in the Mexican population

A new procedure was recently developed for the simultaneous detection of both inversions and the discrimination between distal and proximal rearrangements of intron 22 inversions (inv22-type 1 and inv22-type 2, respectively) by a novel inverse shifting PCR (IS-PCR) approach (Rossetti et al., 2008). This genotyping method includes the following: a) genomic digestion by *Bcl*II enzyme followed by b) self-ligation of the digested fragments containing the sequences of intron 22 of factor VIII gene and their telomeric copies and c) a final PCR standard with different primers to detect normal and recombined fragments. The method also includes complementary diagnostic testing for detection of nondeleterious variants (normal products and duplications) produced by intron 22 rearrangements (Rossetti et al., 2008).

This procedure has recently been established in our laboratory and tested in a group of 24 patients with severe hemophilia A from independent families showing similar results in frequencies previously reported for the inversions in the Mexican population of severe hemophilia A patients (46% intron 22 inversion; 0% intron 1 inversion). From this study it was also possible to discriminate between both types of inv22. We found a frequency of 73% of type 1 inv22 and 27% of type 2 inv22 (Valdés-Galván, 2011). A high-quality DNA sample and the self-ligation step conditions are important in order to obtain consistent results. IS-PCR is the first-choice method for genetic analyses and carrier diagnosis in familial and sporadic cases of severe hemophilia A.

## 5. Hemorrhage phenotype attenuation in hemophilia by prothrombotic genes

In monogenic diseases such as hemophilia A and B, good correlation is expected between genotype and phenotype, i.e., type of mutation in *factor VIII* and *factor IX* genes causing

functional deficiency of the respective proteins to determine clinical severity. This is valid at the biochemical level (clotting activity) but not directly related to the bleeding symptoms in patients because there is clinical variability due to components other than deficient FVIII and FIX (White, 2001).

Different reports and meta-analyses have shown that, despite similar mutations in *factor VIII* or *factor IX* genes, there is an expected clinical variability in hemophilia. This is due to natural anticoagulant and fibrinolytic genes (Shetty et al., 2007, as cited in López-Jiménez et al., 2009) or the concomitant presence of mutations causative of prothrombotic risk factors (Factor V G1691A and Factor II 20210A). These cause the attenuation of hemorrhagic symptoms such as onset of bleeding episodes and frequency of hemarthroses as well as treatment requirements (Nichols et al., 1996; Kurnick et al., 2007; Tizzano et al., 2002; as cited in López-Jiménez et al., 2009) and from an extensive review of literature, FVLeiden has demonstrated to decrease hemophilia severity most consistently (Van Dijk et al., 2004). These studies have also demonstrated thrombosis risk in hemophilia patient carriers of prothrombotic genes such as reported for a patient with hemophilia B who suffered a venous thromboembolism as a result of exposure to high doses of replacement treatment during a surgical procedure (Pruthi et al., 2000).

Descriptive studies of hemophilia A and B families with some affected members with a striking attenuation of bleeding symptoms have demonstrated evidence of attenuation of bleeding phenotype attributable to the presence of prothrombotic markers such as Factor V G1691A and Factor II 20210A. To some extent, these are also related to allelic polymorphisms of the methylenetetrahydrofolate-reductase (MTHFR) gene related to the activity of the enzyme (C677T) and the regulatory domain (A1298C). From a study in Mexican families with hemophilia A and B, the effect of Factor V G1691A and Factor II 20210A was demonstrated in the attenuation of hemorrhagic symptoms in hemophilia patients (López-Jiménez et al., 2009). The attenuation of hemophilia phenotype was mainly observed in the delay of bleeding symptom onset and secondly in a lower frequency of bleeding episodes (López-Jiménez et al., 2009). There was no evidence of an additional effect of attenuation on hemorrhagic symptoms by MTHFR polymorphisms, confirming the main contribution of Factor V G1691A and Factor II 20210A mutations, which are modulating genes of the hemophilia phenotype. On the basis of the feasible molecular analysis by routine PCR of prothrombotic genes and their relative frequency in different populations (1-5%), screening is recommended in those hemophilia patients with noncongruent clinical behavior in regard to severity by clotting activity of factor VIII or factor IX proteins.

## **6. Thrombin generation assay to evaluate clinical severity and treatment response in hemophilia**

In search of objective criteria for the classification of clinical severity in hemophilia and prognostic factors with regard to treatment response in patients, functional approaches reflecting overall hemostatic behavior have shown important usefulness in providing parameters for clinical evaluation and investigation. The fundamental premise of the method is based on thrombin as a central molecule of coagulation whose increase or decrease reflects any alteration from the hemostasis equilibrium caused by hemorrhagic or thrombotic factors. The thrombin generation assay (TGA) was originally analyzed as a research source beginning in the 1950s with significant limitations due to labor-intensive requirements by subsampling and its application being restricted to only very specialized laboratories (Hemker, 2000).

Subsequently, Hemker and coworkers (2003) continued the research and improvement of the method until automation in calibrated automated thrombography.

### 6.1 TGA and correlation with clinical severity in hemophilia

Using calibrated automated thrombography, we studied 23 hemophilia A patients from nine families. Correlation analysis was done for clinical severity (according to an annual number of hemarthroses) and by the clotting activity of FVIII. The study showed that TGA was not able to discriminate differences among familial members but showed correlation with the general bleeding tendency of the clinical severity of patients according to FVIII:C levels (Beltrán-Miranda et al., 2005). Different parameters of the TGA may be useful to correlate with clinical severity in addition to endogenous thrombin potential (ETP), such as the peak and rate of thrombin generation.

### 6.2 TGA and inhibitor behavior in hemophilia A patients

In search of prognostic factors of treatment response in patients positive to inhibitors, we describe the kinetic study of FVIII:C inhibitors and TGA *in vitro* in poor platelet plasma (PPP) of hemophilia A patients positive to inhibitors to correlate with clinical parameters of response to available treatments in Mexico (Luna-Záizar, 2008).

The activity of FVIII:C in plasma was measured by one-stage clotting method and inhibitors to factor VIII was investigated using the Nijmegen-Bethesda method. Inhibitor kinetics was determined by plasma dilutions. TGA was measured in the inhibitor-positive PPP previously spiked and incubated with two treatments: FVIII and Activated Prothrombin Complex Concentrate (APCC, FEIBA™) by the Calibrated Automated Thrombography. Response to treatment by clinical criterion was assessed by 30 hematologists from 25 health institutions according to a questionnaire that assessed specific parameters of reduction of bleeding and improvement from the damage by decreasing pain and inflammation. We detected inhibitor antibodies in 71 patients (37.8%): 46 high responders (5-1,700 NB-U/mL) and 25 low-responders (0.6-4.7 NB-U/mL). When the plasmas of patients with high-responding inhibitors were incubated with the therapeutic product we found some changes in the thrombogram parameters. We found a significant association between inhibitor type and clinical treatment response to FVIII ( $p=0.0003$ ,  $n=42$ ) and between type kinetics vs. FVIII response evaluated with ETP ( $p=0.0021$ ,  $n=47$ ).

Concordance of FVIII response under clinical criteria and ETP was 71%, 86% and 67% among patients with type I, II and III inhibitors, respectively. The inhibitor kinetics was a prognostic parameter of response to FVIII replacement therapy in 74% of the patients. The change in the ETP parameter showed a relationship between inhibitor type and clinical treatment response. TGA permitted an individual evaluation of treatment response and showed usefulness such as objective criterion of responsiveness for a better selection of therapeutic resources, such as observed in one studied patient (Figure 5) (Luna-Záizar, 2008).

Other studies have also demonstrated the usefulness of TGA for monitoring treatment response to bypassing agents in patients positive to inhibitors in approaches carried out *in vivo* (Varadi et al, 2003 as cited in Dargaud et al., 2005) and *ex vivo* (Dargaud et al., 2005, 2010), which use the TGA as an important tool for direct clinical application in regard to medical decisions such as treatment doses and management of hemophilia patients with inhibitors.



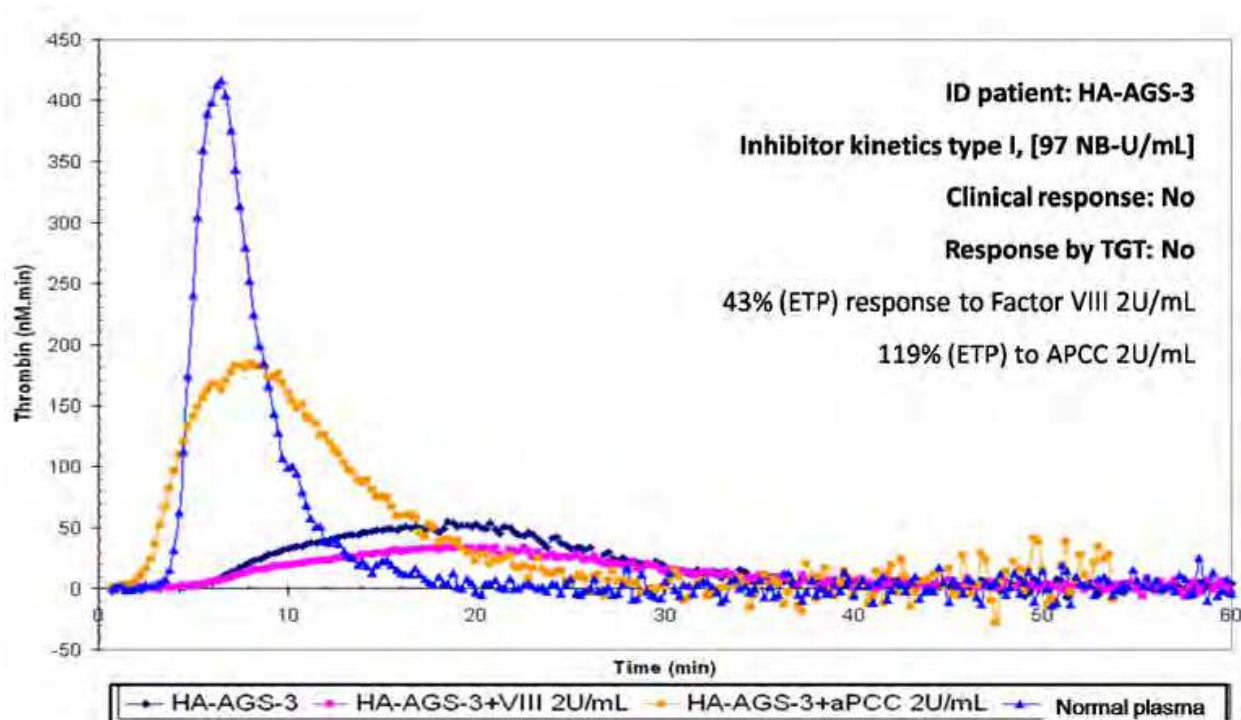


Fig. 5. TGA parameters in a hemophilia A patient positive to inhibitors and treatment response. Response to factor VIII and APCC by ETP increment from basal levels evaluated in platelet poor plasma of hemophilia A patients with inhibitors (Luna-Záizar, 2008).

## 7. X-chromosome inactivation pattern in hemophilia carriers with bleeding symptoms

Because hemophilia A and B are X-linked recessive disorders, males are affected, whereas females are carriers and usually asymptomatic due to the lyonization phenomenon. The lyonization process allows expression of only one allele of the genes located in the X active chromosome. For this reason, females are mosaic for the expression of maternal and paternal alleles and each chromosome contributes ~50% of gene expression (Puck & Willard, 1998), which is sufficient to prevent females from the manifestations of the disease. X-chromosome inactivation is a stochastic event that occurs early in female embryonic development to achieve dosage compensation with males. Certain genetic mechanisms affect the normal process causing a skewed X-inactivation pattern that has clinical relevance in female carriers of X-linked recessive disorders such as hemophilia (Mundo-Ayala & Jaloma-Cruz, 2008).

In probabilistic terms, the X-inactivation process follows a normal distribution pattern; however, it is possible to observe skewed and extremely skewed values (Amos-Landgraf et al., 2006). In some instances, skewness is due to the variation of the process itself when the inactivation ratio among X chromosomes is close to the mean value (75:25) or (80:20). Skewness higher than these proportions may indicate a genetic cause (Amos-Landgraf et al., 2006).

Genetic mechanisms that can explain extreme skewness of the X-inactivation process include mutations in genes that participate in the lyonization phenomenon. A mutation on



the promoter region of the XIST gene has been described that affects the randomness of the process resulting in a skewed X inactivation (Plenge et al., 1997; Tompkins et al., 2002).

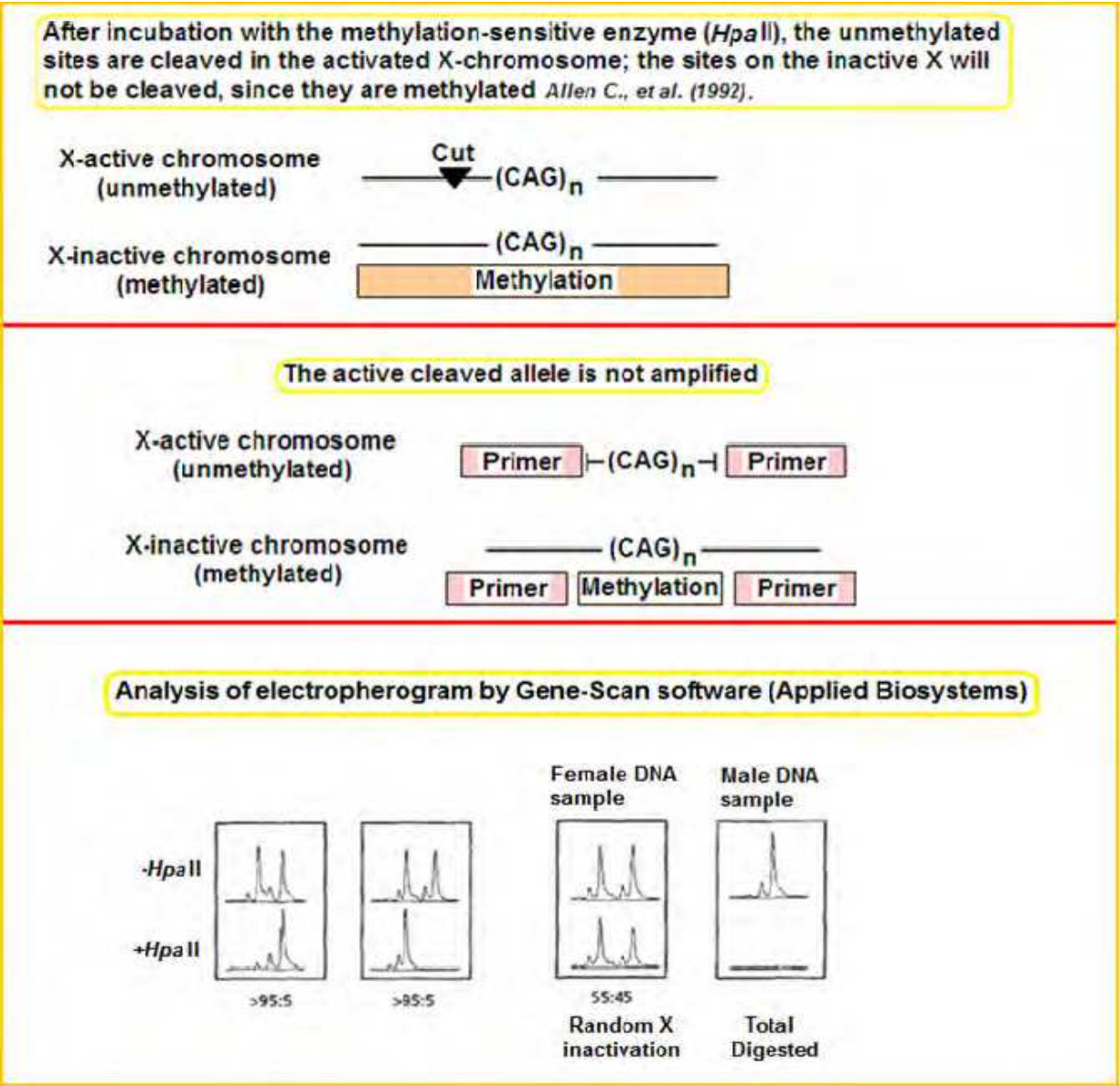


Fig. 6. Scheme of the HUMARA assay. Analysis of the X-inactivation pattern using DNA samples and the Gene-Scan software (Applied Biosystems®).

7.1 Molecular diagnosis of skew in the X-chromosome inactivation pattern in symptomatic hemophilia carriers

A symptomatic hemophilia carrier may request genetic counseling due to the presence of bleeding such as menorrhagia, epistaxis, bruising, gingivitis, etc. (Mundo-Ayala & Jaloma-Cruz, 2008). In case of symptoms in a hemophilia B carrier and after ruling out von Willebrand disease (in a symptomatic carrier of hemophilia A) or chromosomal anomalies such as Turner syndrome to explain the bleeding symptoms, geneticists and molecular biologists should consider analysis of the X-inactivation pattern in the DNA samples of the patient and her parents (Mundo-Ayala & Jaloma-Cruz, 2008).

The gold standard for the analysis of the X-inactivation pattern is the human androgen receptor assay (HUMARA) developed by Allen et al. (1992). We recently used a modified protocol for automatic genotyping of HUMARA by fluorescent Genescan® described by Karasawa et al. (2001) with some modifications to achieve a precise reading in the GC-rich region of the polymorphic region of HUMARA (Ishiyama et al., 2003) and to improve the yield of PCR product and digestion to discriminate the active/inactive alleles by methylation (Mundo-Ayala & Jaloma-Cruz, 2008; Mundo-Ayala, 2010). The methodology is illustrated in Figure 6 and our group has described it in detail for the automatic fluorescent Genescan® (Mundo-Ayala & Jaloma-Cruz, 2008; Mundo-Ayala, 2010). Use of this technique in bleeding carriers and females with hemophilia allows identifying whether their hemorrhagic symptoms are due to an unfavorable lyonization.

7.2 Analysis of a Mexican hemophilia A family with a symptomatic carrier

We describe the study of X-chromosome inactivation pattern in a family with hemophilia A and a symptomatic carrier (Figure 7). Members of this family are affected males, and two of four sisters (II:6 and II:7) were confirmed as carriers after molecular diagnosis by *factor VIII* polymorphisms. Sister (II:7) is a symptomatic carrier who presented clinical manifestations of hemophilia A and a significantly reduced level of FVIII:C (2.5%). Using molecular analysis to determine the X-inactivation pattern, a nonrandom X inactivation was found. The results showed that the healthy X chromosome inherited from the father was preferentially inactive, whereas the affected chromosome from maternal origin was expressed in ~96% of the patient’s total organism (Mundo-Ayala, 2010).

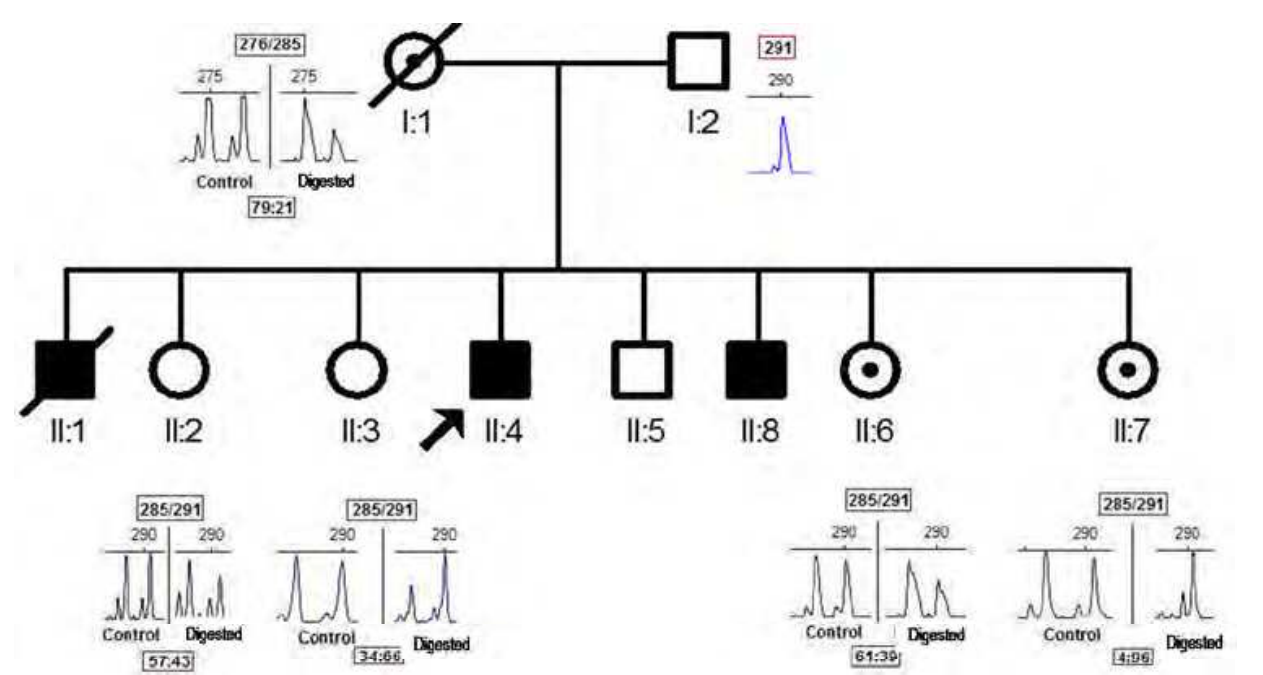


Fig. 7. Symptomatic carrier from a family of moderate hemophilia A studied by HUMARA assay. The obligate carrier (I:1) had HUMARA alleles of 276/285 bp. There were three affected males; the proband (II:4) is indicated with an arrow. There were four females; two (II:6, II:7) were carriers of hemophilia A as confirmed by molecular diagnosis. Symptomatic carrier (II:7) showing an extreme X-inactivation pattern that explains her bleeding manifestations due to the preferential inactivation of the paternal X chromosome.

Clinical bleeding manifestations in the symptomatic carrier (II:7) of this family with hemophilia A occur as a result of the nonrandom X inactivation pattern, which favorably silences the healthy X chromosome inherited from her father. After a negative result of mutations at the promoter region of XIST gene, the molecular origin remains unknown of the skewness in the symptomatic carrier (Mundo-Ayala, 2010).

From the study of different symptomatic carriers of hemophilia from Mexican families, we conclude that it is important to provide genetic counseling due to the possibility of inheriting a nonrandom pattern of X-chromosome inactivation.

Clinical implications from the skewed pattern of X-chromosome must be considered for genetic counseling and hematological control in symptomatic carriers. Furthermore, analysis of X-inactivation pattern is necessary for understanding the human X-chromosome inactivation process (Mundo-Ayala & Jaloma-Cruz, 2008).

## 8. Conclusions

The various studies presented in this chapter emphasize the importance of a comprehensive overview in hemophilia, considering multiple interactions among genes, metabolic pathways and different approaches including molecular data, biochemical analysis and clinical aspects. All these factors are important in order to consider an integrative evaluation of the clinical aspects of hemophilia so as to improve medical management and to obtain prognostic factors for clinical behavior and treatment response.

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The authors of this chapter dedicate all the work described here carried out during 17 years to the **Federación de Hemofilia de la República Mexicana, A.C.** and to all the affiliated associations in the country, especially to the Jalisco Association **“Unidad y Desarrollo, Hermanos con Hemofilia, A.C.”**, with hope that all the developed research may contribute to the welfare and improvement in the quality of life of Mexican hemophilia patients and their families.

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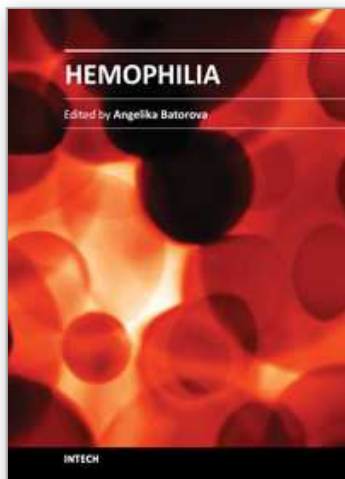


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This book demonstrates the great efforts aimed at further improving the care of the hemophilia, which may bring further improvement in the quality of life of hemophilia persons and their families.

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