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ORIGINAL ARTICLE

Phenotype–genotype correlations in hemophilia A carriers are consistent with the binary role of the phase between *F8* and X-chromosome inactivation

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Summary. *Background:* The recessive X-linked disorder hemophilia A (HA) is rarely expressed in female carriers, most of whom express about half of normal factor VIII activity (FVIII:C). *Objective:* To propose an integrative assessment model for the binary role of the phase between the mutated *F8* and the active X-chromosome (Xa) in FVIII:C in HA carriers. *Methods:* We studied 67 females at risk of severe HA, comprising five symptomatic females (FVIII:C < 1.5 IU dL^{-1}) and 14 controls. A correlation study between FVIII:C (observed vs. expected) and X-chromosome inactivation (XCI) patterns (XIPs; androgen receptor gene [*AR*] system) in blood leukocyte DNA was performed in carriers, by comparison of a model correlating FVIII:C and XIP with arbitrary models devoid of biological significance, and with FVIII:C levels in non-carriers (mean model) as a proxy from background data dispersion not influenced by XIP. *Results:* We provide proof-of-concept example from a family presenting with extremely skewed XIPs in which the severe HA phenotype appeared in a heterozygous carrier of a crossover between *AR* and *F8* loci that phased the mutated *F8* with the maternally inherited Xa. Furthermore, four cases of severe HA affected women who had a combination of a heterozygous *F8* mutation and extremely skewed XIPs in leukocytes or oral mucosa are presented. Correlation analyses between FVIII:C levels and XIPs in carriers ($n = 38$) but not in non-carriers ($n = 20$) showed highly significant differences between the proposed correlation model and models without biological significance. The data support a binary influence of XCI, either increasing or decreasing the FVIII:C, subject to the underlying phase set between the *F8* mutation and XCI. *Conclusions:* Our evidence suggests that the phase between XCI and mutated *F8* acts as a molecular switch conditioning FVIII:C levels and HA expression in carriers.

Keywords: F8 protein, human; hemophilia A; X chromosome; X chromosome inactivation; X-linked genetic diseases.

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Introduction

Hemophilia A (HA) (OMIM#306700) is an X-linked disorder characterized by defects in the factor VIII gene (*F8*). As a recessive X-linked disease, the HA phenotype is manifested in hemizygous males, whereas heterozygous females (carriers) are usually asymptomatic, showing normal or intermediate FVIII activity (FVIII:C) levels.

X-chromosome inactivation (XCI) is a normal epigenetic phenomenon that occurs early in the embryogenesis of female mammals, when, randomly, one of the two X-chromosomes is transcriptionally silenced to compensate for X-chromosome linked gene dose between 46(XX) females and 46(XY) males. This random inactivation creates female mosaics with two populations of cells that differ in the

X-chromosome that is inactive [1]. XCI involves *cis*-specific expression of a long, non-coding RNA, *XIST* (X-inactive specific transcript) from and coating the inactive X-chromosome (Xi) [2].

Essentially, HA carriers express half of normal FVIII:C, reflecting a balanced somatic cell mosaicism because of random XCI, whereby approximately the same number of cells have either the normal or the mutated active X-chromosome (Xa). Symptomatic carriers express FVIII:C similar to that of HA-affected male patients.

Skewed XCI refers to a significant deviation of the random inactivation. Skewing may be linked to: (i) a low number of tissue-pioneer cells involved at the late embryonic stage; (ii) a specific mutation on the X-chromosome that affects cell survival, resulting in a selective disadvantage that leads to a skewed pattern in mature somatic tissue; and (iii) a *cis*-mutation affecting the X-inactivation machinery (e.g. mutations in *XIST* regulatory elements; CTCF point mutations [3]), resulting in non-random selection of the X-chromosome to be inactivated [4,5].

Most reported cases of HA symptomatic carriers showing skewed XCI have been explained by the X-chromosome gametic-phase hypothesis, whereby the mutated *F8* allele (*F8**) acts *in cis* with the preferentially active X-chromosome in somatic cells. Despite the gametic-phase being widely applied, there are contradictory experimental results about its significance for the phenotype–genotype correlations. No correlation between XCI patterns and FVIII:C levels was found in peripheral blood leukocytes (PBLs) from carriers of hemophilia [6]. However, Renault *et al*. [7] found a correlation between FVIII:C and the percentage of lymphocytes with the normal *F8* allele ($F8^N$) on the Xa in six carriers in a large family pedigree affected with HA. Here we integrate the theoretical elements supporting the molecular basis of hemophilia expression in human females, highlighting the role of the phase between the *F8* mutation and the Xa in carriers. First, we report typical cases of female hemophilia: four women affected with severe HA as a result of heterozygous *F8* mutation associated with extremely skewed XCI. Second, we report a proof-of-concept case, in which the severe HA phenotype appeared for the first time in a carrier who showed evidence of a crossover phasing the *F8* mutation with the preferentially activated X-chromosome, owing to extremely skewed XCI. Third, we demonstrate a correlation between FVIII:C and XCI patterns (XIPs) in PBLs from HA carriers by contrasting different novel models.

Materials and methods

Study population

The study group included 67 females at risk of being carriers (age range 1–64 years; average 27 years) from 54 families affected with severe HA. The study group included five symptomatic females with severe HA with FVIII:C ranging from < 1 UI dL^{-1} to 1.5 UI dL^{-1}. In addition, 14 female individuals from the general Argentinian healthy population (age range 20–74 years; average 48.6 years) were included as normal controls. Our Local Institutional Ethical Committee approved this study, and written informed consent was obtained.

FVIII:C

The plasma concentration of FVIII:C was determined with a one-stage method (IU dL^{-1}) [8].

Symptomatic carriers with HA

All cases displayed a normal human female karyotype (46, XX), no FVIII inhibitors, and no phenotypic or biochemical evidence of von Willebrand disease (VWD) or other coagulation defects apart from HA.

Sample purification methods

Genomic DNA (gDNA) was extracted from PBLs with a standard salting-out method [9]. gDNA from oral mucosa cells (OMCs) was obtained with a modified micro-salting-out method [9].

Total RNA was extracted with Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA) from 5 mL of PBLs. Total RNA to copy DNA (cDNA) reverse transcription was carried out with 200 U μL^{-1} Moloney murine leukemia virus reverse transcriptase and 250 ng μL^{-1} random hexamer primers under conditions recommended by the manufacturer (Promega, Madison, WI, USA).

F8 *genotyping*

F8 intron 22 and intron 1 inversions were investigated with inverse shifting PCR [10]. All relevant *F8* sequences were amplified to detect large *F8* deletions, and positive amplimers were screened for small mutations by conformation-sensitive gel electrophoresis (CSGE) and Sanger DNA sequencing on the amplimers exhibiting anomalous CSGE mobility patterns [11]. Multiplex ligation-dependent probe amplification (MLPA) was carried out with SALSA probemix P178-B2, according to the manufacturer's recommendations (MRC Holland, Amsterdam, The Netherlands) to screen for copy number variations (CNVs) in *F8*. Amplification products were analyzed on an AB1 3130XL. The dosage quotient was calculated with COFFALYSER software (MRC Holland). *F8* variation nomenclature followed the rules of the Human Genome Variation Society (HGVS) on RefSeq NM_000132.3 and NP_000123.1.

The HA-causative *F8* mutation was characterized in the family proband. Carrier diagnosis was performed in females at risk by investigation of the family-specific mutation. A second *F8* mutation in symptomatic carriers was ruled out by MLPA analysis and complete *F8* CSGE screening.

X-chromosome inactivation

The XIP in PBLs and OMC DNA was determined by PCR analysis at the first exon of the androgen receptor gene (*AR*, Xq12) with the methylation-based assay and primers shown in Table S1 [12]. Allele profiles and areas under the curve (AUCs) were obtained with capillary electrophoresis and SoftGenetics GENEMARKER V2.2.0 software (SoftGenetics-LLC, Pennsylvania, USA). The XIP percentage (range 50–100%) was calculated with the formula XIP = 100% – 50% $(A'/A)(A + B)/(A' + B')$, where A and B correspond to mock-digested alleles' AUCs, and A' and B' to *Hpa*II alleles' AUCs. XIP values of 90% or 85% were used as cut-offs, over which XIPs were classified as extremely skewed or moderately skewed, respectively.

XIST genomic analysis

The 5′ region and all six exons of *XIST* were screened for reported polymorphisms by CSGE and Sanger sequencing with a new PCR amplification scheme (Table S1). *XIST* DNA sequence analysis was performed against RefSeq NG_016172.1, and sequence variation notation followed the rules of the HGVS.

XIST and F8 RNA expression analysis

XIST-linked single-nucleotide polymorphism (SNP) expression was analyzed by RT-PCR amplification with specific primers (Table S1), with the same conditions and thermocycling as used for *XIST* gDNA sequencing. Relative expression levels of the *F8* mutated and normal alleles in cases 10, 31 and 32 and controls were analyzed by nested PCR with primers E1-8/1 and E1-8/4 in a 40-cycle first round and primers E1-8/2 and E1-8/R in a 35-cycle second round, as previously described [13,14] (Table S1). The *XIST* non-coding RNA Sequence RefSeq is NR_001564.2.

Statistical analysis

Contingency tables were analyzed by the use of odds ratios, 95% confidence intervals, and Fisher's exact test. A non-parametric Mann–Withney *t*-test of the differences between observed (experimental) and expected (theoretically associated with a specific model) data was used to evaluate each FVIII:C vs. XIP correlation model.

Results

Characterization of female study groups

F8 genotyping of the 67 females at risk from severe HA-affected families allowed the diagnosis of 44 carriers of distinct severe *F8* mutations and 23 non-carriers (Table S2). Each observed *F8* mutation associated with severe HA in the family proband passed the genotype–phenotype assignment criteria [15].

The $(CAG)_n$ short tandem repeat (STR) in *AR* exon 1 was informative in 38 (86%) of 44 HA carriers and in 20 (87%) of 23 non-carriers. XIP estimation revealed mainly random patterns of XCI, but few cases exhibited extremely skewed XCI (> 90%) (Table S2). Figure S1 shows the distribution of FVIII:C and XIP percentages from the groups of carriers and non-carriers. HA carriers (n = 44) exhibited an average FVIII:C of 48.6 IU dL^{-1}, and non-carriers (n = 23) an average of 96.2 IU dL^{-1}.

The study group included five unrelated females with clinical symptoms of severe HA. All symptomatic cases carried a heterozygous *F8* mutation. Complete CSGE analysis did not show any additional variant, and further *F8* MLPA analysis ruled out eventual CNV (i.e. large deletions or duplications) (Fig. S2A).

A severe HA phenotype is associated with extremely skewed XCI in carriers of severe F8 mutations

Our patient series comprised four female cases with the most prevalent presentation of symptomatic carriers, in which a heterozygous severe *F8* mutation is combined with an extremely skewed XIP. Cases 10, 15, and 29 exhibited FVIII:C levels of < 1, 1.5 and 1.2 IU dL^{-1}, respectively, and XIP_{PBL} of 99.9%, 100%, and 97.3%, respectively. Case 7 showed an FVIII:C level of < 1 IU dL^{-1} and mildly skewed XIP_{PBL} (75.1%), but an extremely skewed XIP_{OMC} of 95.3%. In all four cases, the HA phenotype and the skewed XIP results (including the discordant PBL/OMC XIP in case 7) strongly suggest an extreme non-random XCI against $F8^N$ in FVIII-secreting cells (hepatic endothelium) (Tables 1 and S2). Moreover, the gametic phase in case 7 was deduced from the hemophilic father (*AR* $[CAG]_{24}$, *F8**]. Although a *cis* configuration of the *F8* mutation and the Xa was not confirmed in all cases, case 10 expressed predominantly *F8** in *F8* mRNA (Table 1; Fig. S2B).

To explore a possible genetic cause to support X-chromosome skewing in these four symptomatic carriers, *XIST* DNA regions were characterized, and at least one nucleotide variation was observed as compared with reference sequences in each case (Table 1). *XIST*-specific RNA analysis of detected exonic SNPs revealed major expression of one allele, confirming that all cases had XCI skewing (Table 1).

Evidence for phenotypic causation in a female carrier owing to a crossover phasing the preferentially activated X-chromosome and the mutated F8: a proof-of-concept

Female patient case 31 (index case, III.1; Fig. 1) expressed severe HA (FVIII:C < 1 IU dL^{-1}). *F8* genotyping from PBL gDNA from case 31 and all asymptomatic women of the family (case 32, mother II.2; cases 33 and

Table 1 Molecular data of the five symptomatic carriers

Case code	*F8* mutation status	Expressed *F8* allele	MLPA *F8* gene	*AR* STR $(CAG)_n$	XIP (%) PBLs (OMCs)	XIST SNP gDNA (cDNA)
7	Heterozygous c.4388_4391delCTTT	NA	Normal	25/*24*	75.1 (95.3)	rs16992442T>C
10	Heterozygous c.548G>C	c.548C	Normal	*26*/23	99.9	rs16992442T>C (C)
15	Heterozygous c.4241C>A	UD	Normal	29/*27*	100	rs16992442T>C (T)
29	Heterozygous c.4825dupA	UD	Normal	*23*/19	97.3	rs6527C>A (C) rs16992436T>C (T) rs16992443G>T (G)
31	Heterozygous c.325A>G	c.325G	Normal	*29*/25	100 (99)	rs41305409G>C (G)

AR, androgen receptor gene (HUMARA); cDNA, copy DNA; gDNA, genomic DNA; MLPA, multiplex ligation-dependent probe amplification; NA, not available; OMC, oral mucosa cell; PBL, peripheral blood leukocyte; SNP, single-nucleotide polymorphism; STR, short tandem repeat $(CAG)_n$; UD, undetermined; XIP, X-chromosome inactivation pattern; *XIST*, X-inactive specific transcript gene. Italic indicates the preferentially active allele in PBLs.

Table 2 Molecular data of the studied members of the index family

Family code	*AR* STR $(CAG)_n$	*XIST* SNP gDNA (cDNA)	*F8* mutation status (clinical status)	Expressed *F8* allele	FVIII:C (IU dL^{-1})	XIP (%) PBLs (OMCs)
I.1	22/25	G/G (G?G)	Heterozygous (non-symptomatic)	NA	50	53 (ND)
I.2	29 DD	C DD	WT DD (non-affected)	NA	ND	ND
II.1	25	G	WT (non-affected)	NA	ND	ND
II.2	22/*29*	G/C (G>C)	Heterozygous (non-symptomatic)	c.325A	58	85 (60)
II.3	25/*29*	G/C (G>C)	Heterozygous (non-symptomatic)	NA	58	91 (ND)
II.4	25/*29*	G/C (G>C)	Heterozygous (non-symptomatic)	NA	93	78 (86)
III.1	25/*29*	G/C (G>C)	Heterozygous (HA symptomatic)	c.325G	< 1	100 (99)

AR, androgen receptor gene (HUMARA); cDNA, copy DNA; DD, deduced; *F8* mutation, c.325A>G; FVIII:C, factor VIII activity; gDNA, genomic DNA; G?G, uninformative and undetermined *XIST* allele expression; HA, hemophilia A; NA, not available; ND, not done; OMC, oral mucosa cell; PBL, peripheral blood leukocyte; SNP, single-nucleotide polymorphism rs41305409; STR, short tandem repeat $(CAG)_n$; WT, wild-type *F8*; XIP, X-chromosome inactivation pattern; *XIST*, X-inactive specific transcript gene; . Italic indicates the preferentially active allele in PBLs. G>C indicates that the G allele is preferentially expressed in *XIST* cDNA from PBLs (Fig. S2).

34, maternal aunts II.3 and II.4; and case 35, maternal grandmother I.1) revealed a heterozygous nucleotide substitution in *F8* exon 3, c.325A>G, predicting a missense defect, p.N109D (Fig. 1). XCI profiling of all of these carrier family members' $gDNA_{PBL}$ revealed that the X-chromosome bearing the $(CAG)_{n\ =\ 29}$ allele represents the preferentially activated X-chromosome in the index case III.1 (100%, confirmed by an XIP_{OMC} of 99%), in her maternal aunt II.4 (91%), in her mother II.2 (85%, not confirmed, by a XIP_{OMC} of 60%), and in her maternal aunt II.3 (78%, partially confirmed by an XIP_{OMC} of 86%), but random XCI in her maternal grandmother I.1 (52%) (Table 2; Fig. 1; Fig. S3). Considering the perfect concordance observed in the XIP_{PBL} and XIP_{OMC} from the index case as a proxy for the XCI state in the FVIII-producing tissues, the well-documented crossing over phasing *F8** with Xa supports the expression of a severe HA phenotype in this patient (Fig. 1). Additional evidence for extremely skewed XCI in the index case and her mother and maternal aunts was found with a cDNA-based approach assessing monoallelic expression profiles in heterozygotes.

Analysis of *XIST* gDNA from the index case and her female relatives unveiled a heterozygous variant C of a previously reported SNP within exon 1 (rs41305409) (*XIST* NG_016172.1:g.6448G>C), except for her maternal grandmother and her father, who carried the ancestral allele, a homozygous G/G and a hemizygous G, respectively (Table 2; Fig. S3).

XIST-specific RNA analysis of rs41305409 on cDNA samples from the index case and her mother and her maternal aunts revealed major expression of the G allele over the C allele (Table 2; Fig. S3). These data indicated that the C allele is either causatively or physically linked

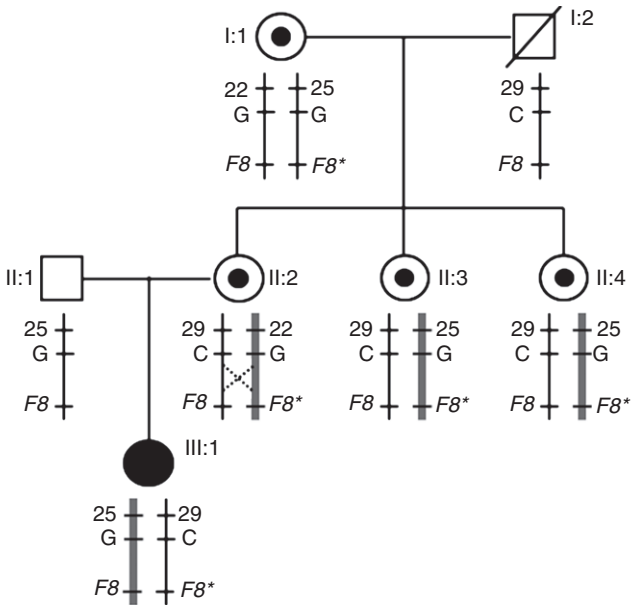

Fig. 1. Index family tree including data on X-chromosome molecular markers. X-chromosome markers (from top to bottom): *AR* $(CAG)_n$ allele on Xq12 (*n* is the repeat number); *XIST* exon 1 single-nucleotide polymophism (SNP) rs41305409 (NG_016172.1:g.6448G>C) on Xq13.2; and *F8* and *F8**, which are normal and missense mutated *F8* exon 3 (p.N109D) on Xq28. The bold line X-chromosome represents the preferentially inactive X-chromosome with an X-chromosome inactivation pattern of > 75%. The X-shaped hatched line indicates a crossover somewhere between the *XIST* and *F8* loci, which, in the II:2 germ cell, set the mutated *F8** in phase with the maternal active X-chromosome bearing the C allele of the *XIST* rs41305409 SNP and the *AR* $(CAG)_{n\ =\ 29}$ allele. It is probable that the phase of the haplotype (*AR* [*n* = 22]; *XIST* SNP[G]; *F8**) in the mother II:2 was set by a crossover in the maternal grandmother I:1 meiosis placed somewhere between Xq12 and Xq28.

to with an X-chromosome inability to become *cis*-inactivated.

Analysis of transcriptional expression of *F8* exon 3 spanning mutation c.325A>G on PBL total cDNA samples revealed expression of a different single allele in both cases, i.e. the index case and her asymptomatic mother, showing preferential expression of the mutated allele in the former and of the wild-type allele in the latter (c.325G and c.325A, respectively) (Fig. S4; Table 2). The nucleotide transition c.325G>A yields the missense defect p.N109D, and is therefore not expected to be involved in allele-specific mRNA decay, as is known for early stop codon mutations [16]. Taking into account that the DNA sequencing detection limit is ~ 20% of the less represented allele, this experiment points to XCI skewing of at least 80% against the normal allele in PBLs, thus conditioning the expression of the $F8^N$ transcript in a phase-dependent configuration that resulted in silencing of the normal allele in the symptomatic carrier index case and silencing of the mutated allele in her asymptomatic mother (Table 2).

DNA analysis of *AR* and *XIST* in the index case's non-hemophilic father showed $(CAG)_{25}$ and rs41305409*G in gametic phase with $F8^N$. This allowed determination of the paternal X-chromosome haplotype in the index case, and the inference, by exclusion, of the maternal haplotype as $(CAG)_{29}$ and rs41305409*C in gametic phase with *F8** (Fig. 1). In parallel, maternal haplotypes of her mother (II.2) (i.e. [$(CAG)_{29}$, rs41305409*C, $F8^N$]/[$(CAG)_{22}$, rs41305409*G, *F8**]) were inferred from the segregation analysis of alleles from the first to the second generation (Fig. 1). Therefore, the index case inherited the chimeric haplotype $(CAG)_{29}$ and rs41305409*C *F8** as a result of a crossover located somewhere between *XIST* on Xq13.2 and *F8* on Xq28 (Fig. 1).

The grandmother of the index case expressed FVIII:C of 50 IU dL^{-1}, random XCI (XIP_{PBL} of 52.8%), and a normal phenotype, typical findings in most heterozygous carriers.

Biphasic correlation between FVIII:C and XCI in HA carriers

Evidence for a correlation between FVIII:C and XIP in heterozygous female carriers of HA-causative mutations has been scarce and controversial in the literature. The classic association study of Orstavik *et al.* [6] did not find a correlation between FVIII:C and XIP_{PBL} in carriers of HA (*n* = 31) and hemophilia B (*n* = 15), whereas Renault *et al.* [7] found a positive correlation in six female carriers from a large family tree affected with HA. To assess the association between FVIII:C and XIP in carriers from a novel perspective, we propose the simplest possible correlation model (linear identity, '/'-shaped function) but including the binary influence of the unknown XCI/*F8** phase by mirroring the XCI values between 0% and 50% to the upper quadrant (between 50% and 100%), resulting in a 'V'-shape. FVIII:C in carriers is influenced by exactly the same source of biological and experimental variation as in non-carriers, except for a defining factor owing to unbalanced XCI in FVIII-secreting cells causing either rising or decreasing *F8** expression. The well-established knowledge on the influence of XCI on the expression of recessive X-linked disorders in heterozygous females indicates that, excluding the FVIII:C variation equally influencing both populations, FVIII:C in non-carriers is average (90.95 IU dL^{-1}, *n* = 20), whereas FVIII:C in HA carriers should result from the biphasic function involving XIP represented by a V-shaped model centered in a half of the FVIII:C average of non-carriers (45.48 IU dL^{-1}) (Fig. 2). A linear model was chosen for simplicity over other non-linear functions (e.g., a parabola). The analysis consisted of multiple comparisons of the differences between observed and expected FVIII:C with the V model against the non-carriers' mean model and two alternative carriers' models: the carriers' mean model (null hypothesis), and the A model, similar to the V model but without biological significance (Fig. 2).

Differences between observed and expected FVIII:C were non-significant as for the V model of carriers and

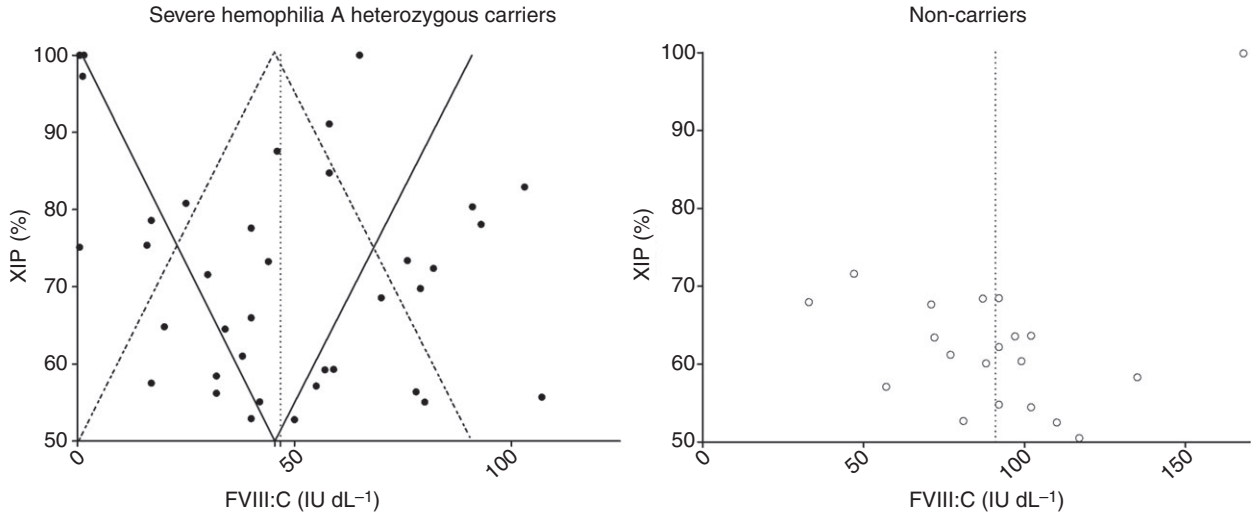

Fig. 2. X-chromosome inactivation pattern (XIP) vs. factor VIII activity (FVIII:C) correlation models: XIP% vs. FVIII:C distribution in heterozygous carriers of severe *F8* mutations (n = 38, left panel) and non-carriers (n = 20, right panel). FVIII:C average values are depicted as vertical hatched lines: 46.79 IU dL^{-1} for carriers and 90.95 IU dL^{-1} for non-carriers. The V model (continuous lines) was made by use of a linear biphasic XIP vs. FVIII:C correlation, taking into account the bipolar influence of XCI by unfolding a value of half the FVIII:C average of non-carriers (45.48 IU dL^{-1}) in two linear branches: a left line to estimate FVIII:C of carriers within the FVIII:C interval of 0–45.48 IU dL^{-1} as $-$ 0.9095 $\times$ XIP% + 90.95; and a right line defined for FVIII:C values of > 45.48 IU dL^{-1} as 0.9095 $\times$ XIP%. The A model (hatched lines) without a biological basis was made by mirroring the V model for the same intervals as the V model, with a left line with FVIII:C levels of 0.9095 $\times$ XIP% $-$ 45.48 and a right line with $-$ 0.9095 $\times$ XIP% + 136.43.

the mean of non-carriers. If the influence of the phase of XCI were excluded it becomes evident that the magnitudes of FVIII:C dispersion are similar. Therefore, that influence cannot be disregarded (Fig. 3). In contrast, highly significant differences were found on comparison of the mean of non-carriers, or the V model of carriers, with the carriers' A model and with the carriers' mean model, indicating that the latter models of XCI involvement should be ruled out (Fig. 3).

Thus, the V model involving XCI_{PBL} cannot be excluded to explain the influence of XCI on FVIII:C in carriers of severe HA mutations.

Because XCI analysis was performed in PBLs and not in FVIII-secreting cells, our study also addressed the usefulness of *AR* methylation-based XCI assay in PBLs for understanding the molecular basis of HA expression in carriers of *F8* mutations.

Two carriers (cases 32 and 13; Table S2) showed FVIII:C of 58 IU dL^{-1} and 65 IU dL^{-1} associated with extremely skewed XIP_{PBL}, and a symptomatic carrier (FVIII:C < 1 IU dL^{-1}) (case 7) showed non-extremely skewed XIP_{PBL} (75.1%), in apparent conflict with the V model. These patients allowed us to examine the correlation with XCI in different tissues, taking into account that, despite the general tendency indicating an XIC correlation in different tissues [17,18], in theory tissue-specific XIPs may result from independent events. Notably, XIP_{OMC} values were 60%, 69%, and 95.3%, respectively, in good agreement with the values predicted by the V model. Further support for the model came from two other carriers expressing extreme FVIII:C of 1 IU dL^{-1} and 93 IU dL^{-1} (cases 31 and 33, respectively; Table S2), the XIP levels in PBLs and OMCs corresponding to extremely and moderately skewed XCI, respectively. Tissue discrepancies in XIP levels in the first two cases and agreement in the last two suggest different prediction scenarios. Discordant XIPs in PBLs and OMCs suggest independent XCI patterns in different tissues (e.g. FVIII-secreting cells), whereas concordant XIPs (particularly concordant skewed XIPs presumably associated with an underlying constitutive genetic cause of skewing) suggest similar XIPs in other tissues.

To compare our series of HA carriers with those lacking an association between skewed XIP and extreme FVIII:C [6], two contingency analyses were performed. Extreme FVIII:C limits of 9.1 IU dL^{-1} and 81.9 IU dL^{-1} were calculated by interpolating the extremely skewed XIP limit (90%) in the V model; and non-stringent extreme FVIII:C limits of 18.2 IU dL^{-1} and 72.8 IU dL^{-1} were calculated by use of the skewed XIP limit (80%) in the V model (Table 3). Our series of carriers (n = 38) showed a significant association (P = 0.0314) between extremely skewed XIP and extreme FVIII:C levels, and no association between moderately skewed XIP and extreme FVIII:C levels (Table 3). Our finding of a modest, but significant, correlation by use of an XIP limit of 90% contrasts with the absence of a correlation between XIP_{PBL} and FVIII:C and FIX:C levels in carriers of hemophilia reported by Orstavik *et al.* [6].

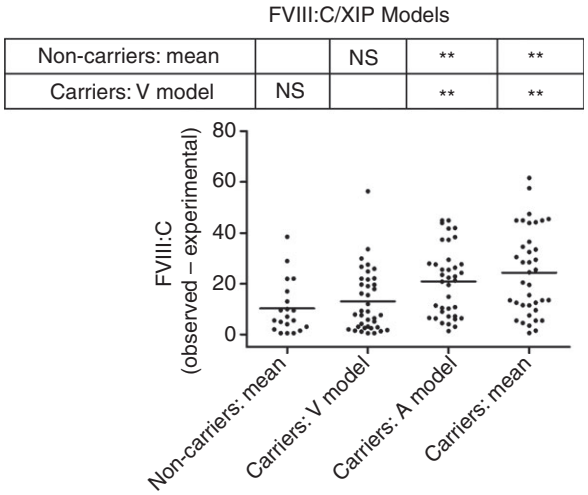

Fig. 3. Statistical analysis of X-chromosome inactivation pattern (XIP) vs. factor VIII activity (FVIII:C) correlation models. Absolute differences between observed and expected FVIII:C levels were obtained by using the observed XIP% calculated in each model (i.e. non-carriers, mean; carriers, V model; carriers, A model; and carriers, mean). The average difference associated with each model is indicated as a horizontal line. Statistical analysis of model differences with the Mann–Whitney test is summarized in the table. *P*-value summary: NS, non-significant; **highly significant differences ($P < 0.01$). Study of differences between the non-carriers mean and other models gave the following results: vs. carrier V model, $P = 0.4717$ (NS); vs. carriers A model, $P = 0.0019$ (**); and vs. carriers mean, $P = 0.0011$ (**). Study of differences between the carriers V model and other models gave the following results: vs. carriers A model, $P = 0.0045$ (**); and vs. carriers mean, $P = 0.0018$ (**). Together, these findings show that the V model of biphasic XIP involvement provides a crucial factor to condition the FVIII:C levels observed in heterozygous carriers of severe *F8* mutations, adding to other sources of variation affecting carrier and non-carrier FVIII:C levels equally.

Discussion

This article reports five cases in which carriers of severe *F8* mutations developed an HA phenotype. In addition, we established the correlation between FVIII:C and XCI_{PBL} in a cohort of carriers with severe *F8* mutations by using non-carriers as study controls to measure the common variability of FVIII:C associated with the null hypothesis (when XCI is not involved).

The FVIII:C values observed in our carriers and non-carriers averaged 48.6 IU dL^{-1} and 91.6 IU dL^{-1}, respectively, in close agreement with the results obtained in other series [19,20]. The *AR* $(CAG)_n$ STR locus was highly polymorphic, and therefore informative in 86%, 87% and 77% (global average of 83%) of HA carriers, non-carriers and normal controls, respectively. Similar rates have been reported in Caucasian and Asiatic populations (90% and 88%, respectively) [13,21].

In women with severe HA, an almost complete qualitative or quantitative reduction in *F8* locus expression is observed. HA cases in women are associated with two

Table 3 Contingency analysis of the factor VIII activity (FVIII:C) vs. X-chromosome inactivation pattern (XIP) biphasic association

	XIP (%)	
FVIII:C class (IU dL^{-1})	≥ 90 (≥ 80)	< 90 (< 80)
Extreme FVIII:C ≤ 9.1 or ≥ 81.9 (≤ 18.2 or ≥ 72.8)	4 (6)	6 (11)
Intermediate FVIII:C 9.1–81.9 (18.2–72.8)	2 (5)	26 (16)

Fisher exact test: XIP ≥ 90%, $P = 0.0314$; XIP ≥ 80% $P = 0.491$ (non-significant).

*F8** alleles, either by homozygosis or by compound heterozygosis [22,23]; an *F8** and skewed XCI [5,7,23–30]; and a hemizygous *F8** associated with a female phenotype caused by a X-chromosome number abnormality (e.g. Turner syndrome 45,X0; or syndrome 46,XY with testicular feminization), or an *F8** and a heterozygous X-chromosome structural aberration mostly involving extremely skewed XCI of the chromosomal marker [31]. In addition, HA phenocopies were also associated with the expression of mild–moderate HA symptoms in women with an autosomal hemorrhagic disease such as VWD or combined FV/FVIII deficiency [32].

Here, we studied a series of patients comprising five heterozygous carriers of severe *F8* mutations with a severe HA phenotype and FVIII:C < 1.5 IU dL^{-1} associated with skewed XCI, providing molecular support for FVIII deficiency (i.e. preferential inactivation of the X-chromosome *cis*-linked to $F8^N$). To the best of our knowledge, this latter *cis* association represents a theoretical molecular requirement that has only been demonstrated in a family [5]. Our index family provided further evidence for the need of preferential inactivation of $F8^N$ for the development of severe HA in a female carrier with a severe *F8* mutation. This case was associated with a crossover between the *XIST* (Xq11.3) and the *F8* (Xq28) loci in the symptomatic girl observed in a recombinant X-chromosome inherited from her asymptomatic carrier mother, setting *F8** in phase with the preferentially activated X-chromosome. Considering interpolated genetic distances, the crossover between *XIST* and *F8* is expected to occur frequently, close to the maximum level of 50% of female meiosis.

As mutations in the promoter of *XIST* have been reported to cause familiar non-random XIC [33,34], and to explore the causes of the extremely skewed XIP observed, we analyzed the regulatory region and *XIST* exons 1–6. Neither the previously reported *XIST* C-43G mutation (NG_016172.1:g.4985C>G) [33,34] nor any other skewing causative mutation was found, in agreement with previous studies [35]. However, our analysis showed five *XIST* SNP variants in a heterozygous state in the group of symptomatic carriers: two in exon 1 (rs6527

NG_016172.1:g.10698G>T and rs41305409 NG_016172.1:g.6448G>C) and three in exon 6 (rs16992442 NG_016172.1:g.31780A>G; rs16992436 NG_016172.1:g.35273A>G; and rs16992443 NG_016172.1:g.31032C>A), all of them showing heterozygosity rates between 0.114 and 0.134 in Caucasians and no clinical significance according to SNP databases. The cDNA analysis of these *XIST* SNPs revealed the preferential expression of one allele in all carriers, confirming the skewed inactivation state in all carriers (as *XIST* RNA is expressed only from the Xi). In particular, the SNP variant rs41305409 was found to be linked to moderately or extremely skewed XCI_{PBL} in the index case and her mother and maternal aunts, all of whom were HA carriers. The cDNA analysis also revealed major expression of the rs41305409*G allele in all carriers, confirming its association with the Xi. All of these findings suggest that the rs41305409*C variant is physically/genetically linked to some inability to undergo *cis* X-inactivation and that it is not causative for the trait. Studies of informative SNPs in *XIST* cDNA allowed other authors to associate a higher or lower probability of inactivation within a family. Bicocchi *et al*. [5] found a *cis* association of the *XIST* SNP (NG_016172.1:g.33772A>G) rs1620574*G allele with the Xa in an Italian family with three heterozygous female carriers expressing severe HA and skewed XCI.

Consistent with the literature, our data show that unaffected individuals, who are expected to show full levels of FVIII:C, also show intermediate levels and even low levels [34].

In addition, we assessed which independent variables may affect the observed correlation between FVIII:C and XCI_{PBL} from carriers with a severe *F8* mutation. Taking into account that Orstavik *et al*. [6] did not find this correlation, which appears to be theoretically unquestionable, we attempted first to dissect the variables affecting FVIII:C, and concluded that, except for XCI, the same set of physiologic and experimental variables qualitatively and quantitatively affect both HA carriers and non-carriers equally (e.g., age, blood group, smoking habit, muscular exercise, menstrual cycle day, and normal plasma pools in laboratories, among many others). The possible causes for the lack of correlation found by Orstavik *et al*. [6] may be associated with the characteristics of the subject carrier population, as they included 31 severe and non-severe *F8* mutations, which may weaken the effect of an eventual XCI skewing, only a few carriers with skewed XCI, and no cases with FVIII:C below 15 IU dL^{-1}, the key subpopulation for demonstrating the FVIII:C–XIP correlation.

Our data revealed a clear correlation between FVIII:C and XIP_{PBL} under the V model in carriers of severe *F8* mutations. Moreover, FVIII:C–XIP_{PBL} correlation analysis in carriers concerns a scarcely addressed and poorly understood issue: does XIP_{PBL} correlate with XIPs present in FVIII-secreting cells? A relevant study of Bittel *et al*. [18] performed this comparison of XCI patterns in multiple tissue biopsies of human females ($n = 26$) by use of the HUMARA assay, and found significant correlations between most tissue pairs. Our results on FVIII:C–XIP correlation in PBLs are consistent with the correlation among XIP values in different tissues investigated by Bittel *et al*. [18].

In conclusion, our data show that the phase between Xa and the mutated *F8* acts as a bivalent molecular switch conditioning FVIII:C and, considering its inverse correlation with HA phenotype, the expression of HA symptoms in carriers of severe *F8* mutations.

Addendum

C. P. Radic and C. D. de Brasi designed and performed research, analyzed data, and wrote the paper. L. C. Rossetti, M. M. Abelleyro, E. Medina-Acosta, and I. B. Larripa analyzed data and gave final approval to the manuscript. T. Tetzlaff discussed and approved statistical methods, and gave final approval to the manuscript. M. Candela, D. Neme, G. Sciuccati, M. Bonduel, and M. de Tezanos Pinto performed clinical evaluation of patients and gave final approval to the manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Distribution of FVIII:C and XIP in HA carriers and non-carriers.

Fig. S2. (A) *F8* analysis of analysis of copy number variations (CNVs) by multiplex ligation-dependent probe amplification (MLPA) with SALSA probemix (P178-B2) on two representative profiles (cases 7 and 15) of symptomatic carriers. Coffalyser.Net analyses show normal profiles in all *F8* probes, ruling out CNVs (i.e. large deletion or duplications) in all five symptomatic HA carriers. (B) Analysis of transcriptional expression of *F8* alleles in

the symptomatic carrier case 10. Relevant genomic DNA (gDNA) and reverse-transcribed RNA (cDNA) sequences of *F8* exon 4 encompassing the *F8* mutation point (squared red arrow). C >G indicates that the C allele is preferentially expressed in *F8* cDNA from peripheral blood leukocytes.

Fig. S3. X-chromosome inactivation (XCI) studies of the index family (shown in Fig. 1 and Table 1).

Fig. S4. Analysis of cDNA expression of *F8* exon 3 in the index family.

Table S1. Oligonucleotide primers for X-chromosome inactivation and *F8* expression studies.

Table S2. Biochemical, molecular and clinical characteristics of the studied population of human females.

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