Somatic/Germinal Mosaicism of a *F8* Promoter Deletion Confounds Clinical Predictions in a Family with Haemophilia A: Key Role of Genotype Quantitation

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Large *F8* deletions cause 10 to 15% of severe-haemophilia A (HA) cases and associate with the highest clinical/biochemical severity and with significantly augmented risks for developing inhibitors against therapeutic factor VIII (FVIII).[1]

Only 45 to 50% of severe-HA cases present family history of the disease.[2] In the remnant cases (sporadic HA), the mutation origin defines different clinical scenarios in which the risk of recurrence and thus genetic counselling significantly vary. The origin of the causative mutation may be either prezygotic or postzygotic generating a genetic mosaicism affecting, partially or totally, one or more tissue/organs including the gonads. Furthermore, the technical features of the genotyping approach for detecting and measuring an eventual genetic mosaicism critically affect its diagnosis.[3]

The quali-quantitative extent of somatic and germinal mosaicisms is passively assumed to be associated with the phenotypic expression of haemophilia severity and inheritance pattern, respectively.

We present a case of a family affected with HA in which the clinical/biochemical severity and inheritance patterns associate with the observed fraction of mosaic cells bearing a *F8*-promoter deletion.

Case Report

In January 2013, an 8-month-old patient (P, III.1) with multiple bruises was diagnosed with severe HA (FVIII:C < 1%). His mother (M, II.2) (FVIII:C = 40%), with epistaxis and menorrhagia and no apparent family history of the disease, asked for molecular diagnosis (►**Fig. 1A**). P and M showed a *F8*-promoter deletion NM_000132.3: c.-2174_-106del2067 (Del2kb) in hemizygous and heterozygous states, respectively (►**Fig. 1B, C**).

In December 2014, P's maternal grandfather (MGF, I.1) (FVIII:C = 35%) (64 years), with mild but inconsistent bleeding symptoms, and P's maternal aunt (MA, II.3) (FVIII:C = 61%), presenting borderline menstrual cycles (7 days), were genotyped (►**Fig. 1A**). At clinical level, MGF showed a history of mild bleedings: presented bruises in the legs after exercise, at the age of 48 years, he underwent a minor surgical procedure without bleeding; suffered persistent bleeding after an arthroscopy and presented an extended haematoma from the abdomen up to the thigh due to an inguinal hernia. Interestingly, MGF showed the familiar deletion, mimicking a heterozygous state in peripheral blood leukocytes (PB) suggesting the involvement of a somatic mosaicism linked to the haplotype *in phase* with severe HA found in his grandson P (►**Fig. 1A, C**). As interestingly, MA showed only the nonmutated allele revealing, first, her noncarrier status and, second, that MGF is a combined germinal and somatic mosaicisms (►**Fig. 1A, C**).

Samples from urine (UC) and oral mucosa (OMC) cells were collected to expand the molecular analysis of PB and interrogate the Del2kb mosaic involvement in different MGF's tissues. To assess the mosaic cell composition, we performed an original approach based on quantitative real-time polymerase chain reaction (qPCR) (►**Fig. 1D**).

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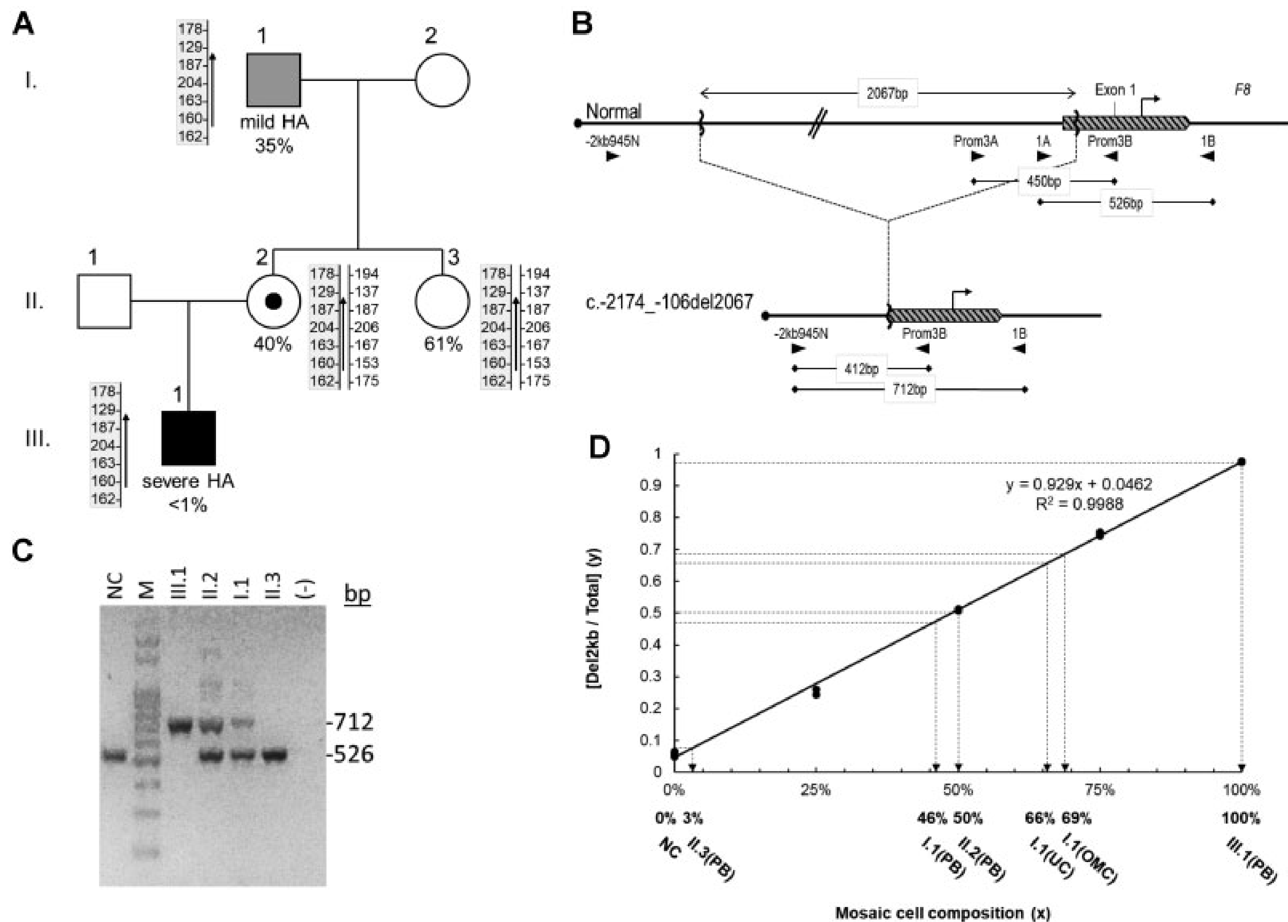

Fig. 1 (A) **Family tree showing the *F8* haplotype-linkage analysis and HA phenotype.** FVIII:C activity levels and clinical severity are indicated. Vertical lines indicate Xq28 STR-haplotypes: extra-*F8* (3′), DXS7423, DXS1073; intra-*F8*, Int25.3, Int22, Int21, Int13; extra-*F8* (5′) DSX1108 STR genotyping (Supplementary Fig. S1 [online only]). Upward black arrow indicates the *F8*. (**B**) ***F8* genotyping approach to characterize the familiar HA-causative mutation.** Scheme of the normal allele and the familiar deletion, NM_000132.3: c.-2174_-106del2067 (Del2kb), showing all relevant molecular size of amplimers of the gap-PCR genotyping approach. Applied primers (closed triangles) Prom3A (5′-AGGGCAAAAGCAGAGAGAC-3′), Prom3B (5′-AATATCTT-TAGCTCCCAGG-3′) (Marchione et al, unpublished), 1A, 1B[12] are part of the updated genotyping scheme of *F8* and -2kb945N (5′-TATAAGAGAAG-CACTGGGAAAGAAAG-3′), which has been designed for amplifying Del2kb allele. (**C**) **Analysis of the Del2kb in the family.** Agarose gel electrophoresis analysis of the gap PCR (primers -2kb945N, 1A and 1B). M: 100 bp ladder marker, (−): negative control (no DNA), and peripheral blood leukocyte's samples from family members (A). An unusual pattern (heterozygous like) is shown in I.1 lane (genetic mosaic MGF patient with mild HA). (**D**) **Mosaic cell composition analysis by qPCR.** qPCR reactions were performed in a Rotor Gene Q instrument (Quiagen) in 25 µL containing 12 µL of Mezcla Real (Biodynamics, Argentina) (with unspecified concentrations of *Taq* DNA Polymerase, dNTPs, $MgCl_2$, Tris-HCl buffer and EvaGreen fluorescent stain), 0.4 µM of each primer and 5 to 20 ng of *Bam*HI-fragmented gDNA. A calibration curve to estimate the mosaic cell composition in MGF tissues was performed by mixing qPCR-dosed PB samples from P and a NC male, using *CTLA4* as reference qPCR product.[13] Two specific targets for Del2kb and normal allele (Prom3) were adjusted using curves Ct versus gDNA concentrations of 0, 5, 10, 15 and 20 ng/µL using samples from P and NC, respectively. Del2kb allele (Del2kb) yields a qPCR product of 412 bp with primers −2kb945N and Prom3B and the normal allele (Prom3), a 450 bp product with Prom3A and Prom3B. The standard curve for estimating Del2kb mosaic cell composition was adjusted by linear regression to five points duplicated achieved with serial mixes of P and NC dosed samples (20 ng of total gDNA input): 0, 25, 50, 75 and 100% of mosaic cells. Del2kb/(Del2kb + Prom3) values calculated from individuals MGF, M, MA and P were interpolated in the linear regression formula $y = 0.929x + 0.0462$. Mosaic cell composition was estimated for MGF's peripheral blood leukocytes (PB), OMCs and UCs. FVIII, factor VIII; gDNA, genomic DNA; HA, haemophilia A; MGF, maternal grandfather; NC, normal control; OMCs, oral mucosa cells; PB, peripheral blood leukocytes; PCR, polymerase chain reaction; qPCR, quantitative real-time PCR; UCs, urine cells

This experiment indicates the mosaic cell composition of PB, UC and OMC samples from MGF estimating 46, 66 and 69% of positive Del2kb cells, respectively (►**Fig. 1D**). PB samples from P, M and MA resulted in 100, 50 and 3% Del2kb cells, respectively, confirming gap-PCR results and the accuracy of the qPCR approach (►**Fig. 1C, D**). Moreover, the difference between qPCR background levels of Del2kb cells from negative samples, MA (noncarrier) 3% and a normal control 0% provides an estimation of the qPCR assay error (►**Fig. 1D**).

Genetic mosaicisms have been reported in many X-linked disorders.[4] In HA, most reported cases involved point mutations and small indels.[5,6] Classical genetic analysis based on clinical observations using Bayesian probabilities converged to the characteristic frequency of 15% (10–20%) of germinal mosaicisms in affected families with sporadic X-linked diseases. In somatic mosaicisms, the percentage of detected patients critically depends on the design of the study and the molecular approach used to investigate the gene defect. Only few cases of mosaicisms of large rearrangements were found

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in haemophilia and all of them were studied by Southern blot analysis, which enables an unbiased mosaic quantification while is hazardous, time-consuming and labour intensive.[7–10] Our qPCR-based approach to measure Del2kb-positive cell composition represents an adaptable tool to interrogate all type of mutations and has proved to be cost-effectively applicable in low–intermediate complexity gene testing laboratories worldwide.

In germinal mosaicisms, the heritability of any disease-causative mutation depends on its percentage of positive gonadal cells. Our tissue screening interrogates all three embryonic layers: ectoderm/OMC, mesoderm/PB, which may be disturbed by eventual immune cells' clonal expansions and endoderm/UC (mainly composed by desquamated cells from bladder and urethra epithelia).

Notably, endoderm-derived UC mosaic level, estimated 66% positive Del2kb cells, or its complementary set of normal cells, 34%, seems to strongly correlate with the resulting FVIII:C activity level, 35%. Germ cells were supposed to derive from epiblast prior the formation of the three embryonic layers.[11] Hence, an average of all three gauged tissues (PB, UC and OMC) may provide an indirect estimation of mosaic germ cells composition predicting a Del2kb mutation inheritance of approximately 60%, which seems to be reflected by the 1:1 ratio observed in the family tree (carrier and noncarrier daughters of MGF). Although these equivalences may represent a statistical coincidence, future international studies evaluating mosaic cell composition in UC versus FVIII:C levels in somatic HA mosaics, and in an average of PB–UC–OMC versus the observed mutational inheritance in germinal mosaicisms will help in confirming or rejecting this sound hypothesis.

We hypothesize that the mitotic postzygotic mutation might occur prior the eight-cell stage in the still undifferentiated embryo, in which all blastomeres are interchangeable to form all cell lineages and tissues potentially composing all of them the somatic–germinal mosaicism.

In conclusion, our results suggest a correlation between phenotype severity and mosaic cell composition, and warn us to be particularly aware in isolated cases of mild haemophilia, in which efforts should be made to detect the origin of the new mutation. Particularly, the eventual existence of a quali-quantitatively determined mosaicism and the unbiased estimation of the recurrence risks will allow taking a consistent global picture, which is crucial to offer an accurate evidence-based genetic counselling.

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Authors' Contribution
All authors confirmed they have contributed to the intellectual content of this article and have met the following three requirements: (1) significant contributions to the conception and design, acquisition of data or analysis and interpretation of data; (2) drafting or revising the article for intellectual content and (3) final approval of the article.

Conflict of Interest
The authors declared no conflict of interest.

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References

1 Marchione VD, Zuccoli JR, Abelleyro MM, et al. A prevalent CTLA4 missense variant significantly associates with inhibitor development in Argentine patients with severe haemophilia A. Haemophilia 2017;23(02):e166–e169
2 Kasper CK, Lin JC. Prevalence of sporadic and familial haemophilia. Haemophilia 2007;13(01):90–92
3 Rohlin A, Wernersson J, Engwall Y, Wiklund L, Björk J, Nordling M. Parallel sequencing used in detection of mosaic mutations: comparison with four diagnostic DNA screening techniques. Hum Mutat 2009;30(06):1012–1020
4 Youssoufian H, Pyeritz RE. Mechanisms and consequences of somatic mosaicism in humans. Nat Rev Genet 2002;3(10):748–758
5 Leuer M, Oldenburg J, Lavergne JM, et al. Somatic mosaicism in hemophilia A: a fairly common event. Am J Hum Genet 2001;69 (01):75–87
6 Ranjan R, Biswas A, Meena A, et al. Importance of investigating somatic and germline mutations in hemophilia A: a preliminary study from All India Institute of Medical Sciences, India. Clin Chim Acta 2008;389(1-2):103–108
7 Levinson B, Lehesjoki AE, de la Chapelle A, Gitschier J. Molecular analysis of hemophilia A mutations in the Finnish population. Am J Hum Genet 1990;46(01):53–62
8 Higuchi M, Kochhan L, Olek K. A somatic mosaic for haemophilia A detected at the DNA level. Mol Biol Med 1988;5(01):23–27
9 Bröcker-Vriends AH, Briët E, Dreesen JC, et al. Somatic origin of inherited haemophilia A. Hum Genet 1990;85(03):288–292
10 Oldenburg J, Rost S, El-Maarri O, et al. De novo factor VIII gene intron 22 inversion in a female carrier presents as a somatic mosaicism. Blood 2000;96(08):2905–2906
11 Barton LJ, LeBlanc MG, Lehmann R. Finding their way: themes in germ cell migration. Curr Opin Cell Biol 2016;42:128–137
12 Williams IJ, Abuzenadah A, Winship PR, et al. Precise carrier diagnosis in families with haemophilia A: use of conformation sensitive gel electrophoresis for mutation screening and polymorphism analysis. Thromb Haemost 1998;79(04):723–726
13 Abelleyro MM, Radic CP, Tetzlaff T, et al. Reliable and cost-effective approach for diagnosis of heterozygous F8/F9 large deletions by quantitative real-time PCR. Haemophilia 2015;21 (03):e247–e251

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