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Genetic mosaicism in haemophilia: A practical review to help evaluate the risk of transmitting the disease

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Abstract

Approximately 70% of patients with haemophilia exhibit a clear inheritance pattern, while for the remaining 30%, patients are the first to be diagnosed in their family and are considered sporadic cases. In such a setting, the determination of carrier status and the risk estimation of disease transmission to another child are major challenges for genetic counselling. Large studies have suggested that genetic testing reveals 70% of sporadic patients' mothers are carriers. In the remaining 30%, in some apparently non-carrier mothers, the pathogenic variant can be detected as low somatic and gonosomal mosaicism. The significance of mosaic pathogenic variants has thus far been underestimated, since conventional Sanger sequencing and other technology are not sufficiently sensitive. The study of various tissue samples and recent extra-sensitive molecular methods have now made it easier to detect both single-nucleotide variants (SNVs) and copy-number variants (CNVs), at a mosaic level in parents, and to predict the probability of disease recurrence. This review seeks to examine various kinds of mosaicism in haemophilia, including the mechanisms by which they arise and the risk of passing these variants on to the next generation. In addition, we focus on the selection of cell tissues and methods to detect these mosaic variants in the haemophilia setting. Taking into account the high rate of mosaicism in mothers of sporadic cases, we propose a diagnostic flow chart that could facilitate better evaluation of the risk of transmitting haemophilia in genetic and prenatal counselling.

KEYWORDS

F8 gene, F9 gene, germline mosaicism, gonosomal mosaicism, haemophilia A, haemophilia B, somatic mosaicism

1 | INTRODUCTION

Haemophilia, an X-linked inherited bleeding disorder, has been classically characterized by partial or total deficiency of coagulation factor (F)VIII in haemophilia A (OMIM 306700) or FIX in haemophilia B (OMIM 306900). Haemophilia is caused by a spectrum of variants in the *F8* (Xq28) or *F9* (Xq27.1) genes, which are both located on the X chromosome. Both are also associated with a mode of inheritance in which the phenotype is expressed mostly in males that are hemizygous for a gene pathogenic variant. Despite their heterozygous carriership status, recent studies highlight that, among female carriers, 30% demonstrate an increased risk of bleeding, which is similar to the clinical profile of a mild haemophiliac patient.[1,2]

In approximately 70% of haemophilia A and B cases, there is a family history. Thus, the prevalence of sporadic haemophilia is estimated at 30%.

Familial causal variants are always transmitted through the germline. For each pregnancy, a female carrier has a 50% chance of having a son affected by haemophilia and a 50% chance of having a daughter that will carry the altered gene. In contrast, affected males

transmit the pathogenic variant to all of their daughters and none of their sons.

'Sporadic' haemophilia is used to define situations where there is no family history of the disease and when the index case is designated as the first affected sibship in the family. The designation 'sporadic case' is imprecise and confusing, since this includes cases of haemophilia that are due to one of three possibilities: a de novo pathogenic variation that occurs only in the foetus, when the mother is a not a carrier; a carrier mother that has inherited the pathogenic variant but the index case is the first case in the family; or a carrier that has not inherited the pathogenic variant. In this last case, the carrier's genotype will depend on the timing of the onset of the genetic defect (cells fully affected or mosaic).

A de novo variant appears for the first time in one family member, be they male or female, as the result of a genetic error in a cell. The timing of this event, which occurs during embryonic development, leads to two scenarios with different consequences. In the first, the de novo variant is thought to occur during a germline *meiotic* cell division in the egg or sperm cell upon conception, which results in an embryo with a congenital variant that is present in every cell of its body and that is detectable in its leucocytes DNA. This de novo variant is present in the germline cells. In the second scenario, a de novo *mitotic* error may originate in a zygote within the first or later cell divisions, which results in a mosaic embryo with the variant present in a large or low proportion of cells/tissues, depending on when and where the genetic defect takes place. This mosaicism can affect somatic and/or gonadal tissues (Figure 1). In both of these scenarios, the parents of such embryos do not have the pathogenic variant in their cells.

In sporadic haemophilia pedigrees, it is crucial to identify the source of the haemophilia-causing variant in the setting of genetic counselling and risk estimation for future pregnancies, particularly when the mother does not appear to carry the de novo pathogenic variant in her leucocytes DNA. When considering the possibility of low mosaicism in such carriers, the potential recurrence risk is not negligible, requiring additional genetic testing for appropriate genetic counselling.

This review outlines different kinds of mosaicism, including the mechanisms through which they arise and the risk of passing these pathogenic variants on to the next generation. We then focus on the cell tissues and methods that should be selected to detect mosaic variants. Finally, we propose a diagnostic flow chart that could improve evaluation of the risk of transmitting haemophilia in genetic and prenatal counselling.

2 | MOSAICISM: WHAT DOES IT MEAN? WHERE AND WHEN DOES IT HAPPEN?

Mosaicism refers to two or more genotyping distinct populations of cells that are present in one individual, which are caused by postzygotic de novo deleterious genetic events. Usually, mosaicism is only discovered when it leads to inherited conditions in multiple progeny. Mosaicism must not be confused with chimerism, which is caused by the fusion of two different zygotes within a single embryo, rather than a variant. Furthermore, mosaicism should not be confused with X-inactivation, in which all cells (in mammalian females) display the same genotype but repress a different X chromosome copy in different cells.

Depending on the developmental stage in which the genetic defects arise, mosaicisms can be classified as somatic, germline or gonosomal.[3-5] *Somatic mosaicism* results from variants occurring upon mitotic cell divisions, with subsequent clonal expansion of the affected cells. If the variants occur considerably late in the embryonic developmental stage, the phenotype is likely to be confined to a single body region or organ. In this setting, the variant cannot be transmitted to progeny. *Somatic mosaicism* is typically encountered in cancer, when pathogenic variants are responsible for the inactivation of genes that encode tumour suppressors. However, it is also described in skin diseases and venous malformations.[6,7] This local somatic mosaicism has never been reported to cause the disease of haemophilia.

Germline mosaicism (also termed *gonadal mosaicism*) arises through a de novo variant in a parent's germline cell (sperm or egg). The proportion of gametes that are affected depends on the stage at which the error occurs. This means that only some germ cells display the pathogenic variant, which remains clinically 'silent' because it is undetectable in the parent's leucocyte DNA or other somatic tissues. Usually, this form of mosaicism is only discovered when it leads to inherited conditions in multiple progeny.[8,9] Unlike in the parent, the pathogenic variant is detected in all cells of the affected child. Germinal mosaicism in haemophilia appears to be rare, with only very few cases heretofore reported.[10]

If a pathogenic variant occurs very early in embryonic development, it is likely to partially or totally affect one or more tissues/organs that involve both somatic and germ cells. This is also termed *gonosomal mosaicism*, which has been observed in a wide array of Mendelian disorders, including Duchene muscular dystrophy, Marfan syndrome and ornithine transcarbamylase deficiency, as well as haemophilia.[11] In this case, the patient with haemophilia could be unaffected or bear only a mild deficiency, whereas the pathogenic variant might be inherited in the zygote.[12-14] All cells of the developing offspring would bear the pathogenic variant and would result in a de novo severe-to-mild disease phenotype, depending on the type of variant.

The timing of occurrence of a postzygotic variant influences the distribution of mutant cells in the zygote. When the variant occurs before the blastocyst stage, somatic variants are not diagnosed. This is because very few cells are destined to form the embryonic body at this stage. However, when the genetic defect occurs upon the first mitosis of the inner cell mass, and, more specifically, within the epiblast (the precursor of endoderm and ectoderm embryonic tissues, as well as germ cell lineage), the mutated alleles' ratio will depend on when and where the deleterious genetic event occurs. If it occurs during the first mitosis, approximately half of the individual's cells will be affected. Genetic

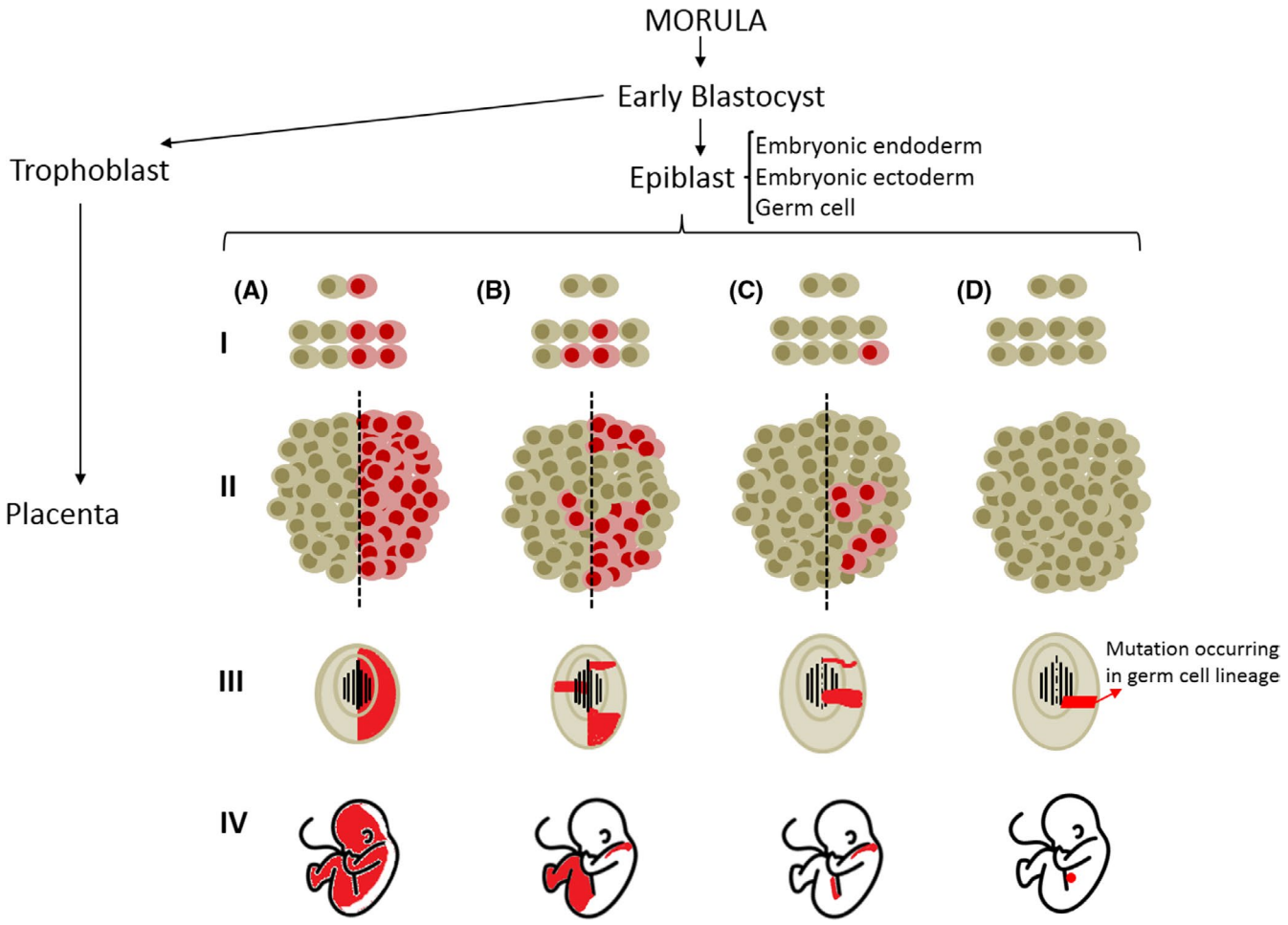


FIGURE 1 Distribution of mosaicism in an individual depends on when it happened. If the mutation occurs during the first epiblast mitosis, half of the individual will be affected (A). If the mutation arises before the left-right determination, mosaicism can affect both individual's sides, including one or both gonads (B). However, if the mutation appears after the left-right determination, mosaicism can be confined to only one side, with only one gonadal likely to be affected (C). Later in the embryological development, if the mutation occurs after differentiation of primordial germ cells, mosaicism is confined to germ cells and absent from somatic tissues

defects taking place before left-right differentiation can affect both sides of the zygote and affect either one or both gonads. Variants that occur after the two embryonic sides are determined can be confined to only one side, so only one gonad is likely to be affected. Finally, variants that arise after the primordial germ cells (PGCs) are determined will be absent from somatic tissues and present in germ cells only (Figure 1).[15]

3 | MOSAICISM IN HAEMOPHILIA AND IMPLICATIONS FOR PATIENTS

Mosaicism is a fairly common event in human genetic disorders: 10%-20% mosaicism has been observed in retinoblastoma,[16] Duchenne muscular dystrophy[17,18] and tuberculosis sclerosis complex.[19]

Most pedigrees have more than one patient with the disease, who passes it down through several generations. In haemophilia, however, around 30% are sporadic, with a single patient within a family, whose causal variant is likely to be de novo.[3,20]

The mother of a new index case could be a non-carrier, a carrier with a de novo variant or a carrier that has inherited the variant from her parents. It has been possible in this last case to identify either a mother or grandmother carrier with an abnormal FVIII:C level or a grandfather with a mild phenotype.[12,21,22] This has been reported on multiple occasions, following extensive analysis in the family of a sporadic haemophilia patient.

Three classes of pathogenic variants in the *F8* gene are responsible for haemophilia A: inversions of intron 1 or intron 22, deletions/duplications and point changes (missense, nonsense and splicing changes). Only deletions/duplications and point changes in the *F9* gene are associated with haemophilia B.

In sporadic haemophilia A, a variant type-specific gender ratio of variant frequencies seems to arise preferentially, with certain types of pathogenic variants.[23-25] It has been hypothesized that, for the most common genetic defect, intron 22 inversion (Inv22), which occurs in approximatively 40% of severe haemophilia A cases, the presence of this pathogenic mechanism would argue against a somatic origin. Previously, this rearrangement was thought to occur

almost exclusively during the meiosis of gametes and predominantly in spermatozoa of the patient's healthy maternal grandfather.[26] This means that, if a pathogenic Inv22 is identified in a 'sporadic patient', 98% of mothers are carriers whose origin is the male germline of the maternal grandparent.[26] This hypothesis was, however, revised in the publications of Oldenburg et al and Lu et al, who demonstrated that the Inv22 pathogenic variant is not restricted to meiotic cell divisions but also occurs upon mitotic cell divisions, either in germ cell precursors or somatic cells.[27,28]

Previous studies suggest that large intragenic deletions/duplications predominantly originate in female germ cells, as well as whole vs. partial gene deletions/duplications, as accounted for by the recombination of two X chromosomes in female meiosis.[25,29] With regard to point changes, the higher degree of methylation in the male germline and the higher number of mitotic cell divisions occurring in spermatogenesis throughout life would explain a predominantly male origin. Indeed, the number of germ cell divisions required to produce sperm is about 265 divisions for men aged 25 and 840 divisions for men aged 50 years. In contrast, the number of successive female cell divisions from zygote to mature egg is only 24, approximately.[30] Moreover, many variants arise at sites that mutate at unusually high rates, such as CpG dinucleotide sites.[31] Methylation has been suggested to occur at a much higher rate in spermatocyte DNA than oocyte DNA, thereby increasing the point variant frequency of paternal, rather than maternal, origin.[32]

Many studies have estimated that most sporadic patients' mothers were carriers, while also reporting that somatic mosaicism in haemophilia may represent a fairly common event with a proportion of about 13%.[3,12,25,33] Studies of large Chinese and Swedish cohorts, in which 257 and 45 sporadic cases with family members of three generations, respectively, demonstrated that most mothers carried the same *haemophilia-causing variant* than their sons, with the majority of them exhibiting the causal variant tracked back to their maternal grandfather (mostly in the case of Inv22) or grandmother (in a smaller proportion).[24,28] These results align with previous studies (Table 1).[3-4,23,34] In de novo sporadic maternal or grandmaternal cases, 1/5 was tested as a mosaic mother in the Swedish and 9/40 in the Chinese studies, using sensitive methods for somatic mosaicism. In the latter study, the authors were surprised to detect a relatively high proportion of de novo Inv22 at mosaic state in the patients' mothers (3/10 mothers tested), as this contrasts with the view that Inv22 exclusively occurred upon the meiosis of gametes, predominantly in spermatozoa.[24,28] Whereas only very few publications of large studies are available in haemophilia B, somatic mosaicism has been regularly reported, suggesting this biological phenomenon is not a rare event in haemophilia B[13-14,22,35] (Table 1).

4 | DETECTION FOR SOMATIC MOSAICISM: WHICH CELL TISSUES AND METHODS TO SELECT?

4.1 | Cell tissues

The detection of mosaicism in human disease has been challenging, as it requires the analysis of a tissue that harbours the causal variant. Most human genetic diagnoses are performed on peripheral blood DNA, due to access and isolation ease. To detect mosaicism, which can greatly vary according to variant timing, cell migration and determination during development, analysis of multiple tissues is requested. Postzygotic variants occurring in embryonic ectodermal tissue can be sampled from buccal brushings or hair root bulbs. In embryonic mesodermal tissue, mosaicism is detected from blood or saliva, while endodermal origin can be detected from urothelial cells that are collected in urine samples. Molecular investigations to detect gonadal mosaicism must involve direct observation of germ cells. For male individuals, sperm samples may be particularly informative for recurrence risk

TABLE 1 Published studies of sporadic cases in haemophilia with mosaicism in the mother

Sporadic cases	Carrier mother	Carrier Maternal GM	Mosaicism in the mother (defined as not carrier in her leucocyte DNA)	References
257[a] HA	185 (72%)	9	9/40 (22.5%)	28
45[a] HA	28 (62%)	5	1/5 (20%)	24
73 HA	57 (78%)	N/A	3/16 (18%)	23
61 HA	None	None	8/61 (13%)	3
29 HA	21 (72%)			33
12 HB	9 (75%)	N/A	N/A	4
66 HA + HB	56 (84%)	2/19 (tested)	N/A	30

Note: The first column indicates the number of 'apparently sporadic cases', *ie* the first case in a family.

The second column represents the number of cases in which a mutation was detected in the mother's DNA.

The third column indicates the carrier status of the maternal grandmother (MGM) when DNA samples are available and tested.

The fourth column indicates the number and percentage of mosaicism detected in mothers who seem not to be carrier with conventional method.

[a]Represents the number of families selected because the DNAs of mothers and maternal grandparents were available for linkage analysis.

assessment, whereas, for females, invasive biopsy of potentially both ovaries is requested.

4.2 | Methods

High-grade mosaicism, at a rate of 70% and higher, usually remains unrecognized and is thus not assessed in clinical practice (Figure 2). In contrast, medium- and low-grade mosaicism probably represents an underreported cause of genetic disorders, due to detection challenges in routine molecular diagnostics. Traditional molecular screening techniques, like single-strand conformation polymorphism analysis, heteroduplex analysis, denaturing high-performance liquid chromatography and Sanger sequencing, all fail to detect low-level somatic mosaicism, as these techniques require detection of a minimum of 10%-20% mutated cells.[3,36,37]

New molecular technologies, such as quantitative polymerase chain reaction (qPCR), next-generation sequencing (NGS), single-base extension assays and droplet digital polymerase chain reaction (ddPCR), have reduced the time and costs involved in genetic testing. However, quantification of mosaicism still requires a significant amount of work, including a personalized focus on each variant. With the advances of these extra-sensitive genotyping techniques, the detection and quantification of the mosaicism level in parents of a sporadic haemophilia case, even with a mosaic rate of only 1%, are rendered easier, in a more routine fashion.

4.3 | Quantitative polymerase chain reaction

qPCR is based on the monitoring and detection of a targeted DNA sequence upon PCR, using either specific fluorescent dyes (eg SYDR green or EvaGreen® dyes) that intercalate with any double-strand DNA or specific fluorescent probes (TaqMan probes). The PCR reaction, prepared in the usual manner, is run in a 'real-time PCR instrument'. After each cycle, the fluorescence intensity is measured with a detector. At the reaction end, it is possible to determine how much DNA has been amplified, and the intensity is proportional to the PCR product amount that has been generated by amplification. Using a calibration curve to construct artificial mosaics with a dilution series of 100%, 75%, 50%, 25% and 0% of the mutant allele with normal DNA, this method was used by Abelleyro et al to estimate the mosaicism of a *F8*-promoter deletion in a family affected by haemophilia A.[21]

4.4 | Next-generation sequencing

NGS technologies have revealed that somatic and gonosomal mosaicisms play a greater role in human genetic disorders than previously recognized.[5,8,37] Compared with traditional sequencing methods, digital NGS technology allows sequence variants to be quantified, which enables the detection of low-level mosaic variants. Indeed, the Sanger sequencing method, which conveys an *average* signal from the mutant, in relation to the wild-type DNA pool that is present in a sample, produces false-negative results when the amount of the mutant allele present is too low. In contrast, NGS technology using a preamplification step has the ability to directly count the mutant and wild-type alleles within the sample, since *each* DNA molecule is sequenced and analysed. These sequencing reads are aligned to a reference genome, in order to generate aligned binary sequence alignment/map (BAM) files.

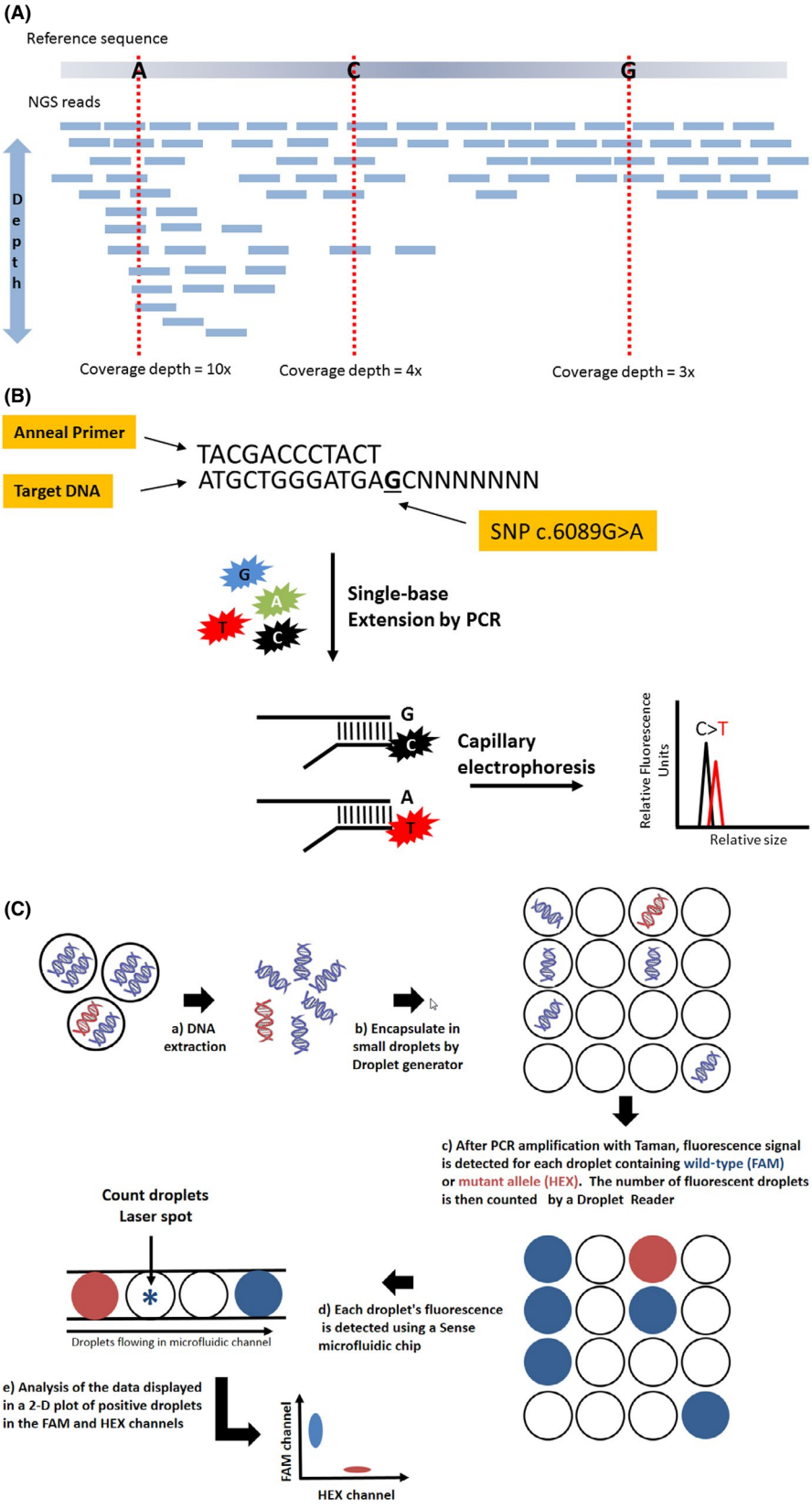
Essentially, in NGS technology, sequencing coverage corresponds to the average number of reads that cover known reference bases. With regard to detecting variants (SNVs and rearrangements) in a molecular diagnosis, guidelines often recommend between 10× to 30× depth of coverage (Figure 2A). Increasing the mosaicism level's quantitative precision and accuracy requires a higher DNA molecule number covering a given base position. Consequently, a customized DNA probe library, which reduces the capturing template from the candidate exon listings, could detect a higher coverage (>500-1000-fold) of targeted exons of interest and appreciate the mosaicism percentage.

4.5 | Single-base extension assays

The single-base extension assays (SNaPshot) method was used by Lu et al to detect mosaicisms of the *F8* gene point or small deletion/insertion variants, following the recommendation of the manufacturer's instructions (Applied Biosystems).[28] In this method, the variant/wild allele is first amplified by common primers. After purification, the amplification product is employed as a template for the second PCR round, using a specific unlabelled primer that anneals to the PCR product template immediately (5' to the position of variant). The reaction contains only dideoxynucleotide (ddNTP) terminators. Upon the reaction, the polymerase can extend the primers by only one base, which is complimentary to the template strand at the site of interest. As the ddNTPs are labelled with different fluorescent dyes, the primer is extended by a fluorescently labelled ddNTP terminator that can be separated, sized and detected by capillary electrophoresis. In this case, the genotype was read at the fluorescent intensity peak and analysed using the GeneMapper 4.0 software (Figure 2B). Two different fluorescent peaks were detected in the carriers, versus only one in the patient and normal control. Mosaicisms were estimated based on the ability to differentiate two DNA template sets from artificially mixed variant/wild DNA, in different ratios (2%, 3%, 5%, 10% and 15%).

4.6 | Droplet digital polymerase chain reaction

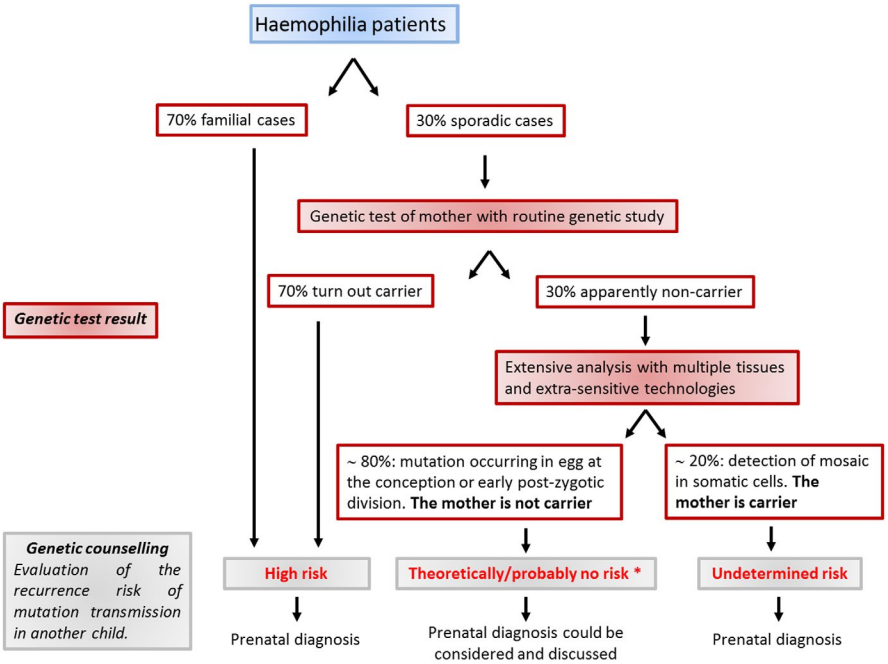
This powerful approach is able to quantify genomic mosaicism at levels as low as 0.1%.[38] By combining high-throughput microfluidics with quantitative (digital) fluorescent readouts, using either TaqMan-style probes or an intercalating fluorescent EvaGreen



dye, ddPCR assays achieve unprecedented sensitivity and accuracy levels in PCR-based detection. The ddPCR technique can be used for analysing genomic variants, from the smallest (SNVs) to the largest (CNVs), by utilizing a water-oil emulsion droplet technology that compartmentalizes the PCR reaction solution into 15 000-20 000 droplets. After the droplets are generated, PCR amplification occurs in each droplet using fluorescent dyes, such as FAM or VIC, and PCR primers. Digital droplet PCR improves

FIGURE 2 Personalized assays for detection of mosaicism. A, Next-generation sequencing (NGS). In NGS technology, the sequencing coverage describes the average number of reads that align to, or 'cover', known reference bases. The coverage depth estimates how many times each base of a reference sequence has been sequenced. Guidelines recommend a depth of coverage for each base from 10× to 30×. In the example shown here, nucleotides dATP, dCTP and dGTP are read 10×, 4× and 3×, respectively. To detect and quantify mosaicism, the increasing coverage depth of the variant must be higher (>500-1000-fold). B, Single-base extension assays or SNaPshot. In this technology, after a first PCR to amplify the target DNA, a designed unlabelled primer with its 3' end directly flanking the SNP binds to a complementary template in the presence of labelled ddNTPs and polymerase. The example shown here illustrates the c.6089G > A (p.Ser2030Asn) in *F8* exon 19. The Taq polymerase extends the primer by one nucleotide, adding a single-labelled ddCTP or ddTTP complementary to the polymorphic base located in the target DNA. The fluorescence colour readout reports which base/bases was/were added using specified software. Quantification of mosaicism is obtained based on artificially mixed mutant/wild DNA in different ratios. C, Droplet digital PCR (ddPCR). The ddPCR technique is based on water-oil emulsion droplet technology, which permits the precise quantification of rare target nucleic acids in a sample. This method improves mosaicism detection by segregating wild-type and mutant alleles into individual droplets. After DNA extraction (a), droplets are formed in a water-oil emulsion to form the partitions that separate the template DNA molecules. These droplets are obtained using an automated system that blends oil samples into a cartridge and generates droplets of nanolitre size by a microfluidic system and vacuum generation (b). The emulsion is transferred to a thermocycler for PCR. Fluorescently labelled probes specific for the wild-type or mutant allele can anneal and are degraded by DNA polymerase, releasing fluorophores and resulting in a fluorescent droplet (c). The number of positive fluorescent droplets is then detected by a droplet reader. The system separates and aligns each droplet, each of which is analysed by two lasers to detect FAM or HEX fluorescence relative to each droplet (d). The fluorescence is processed into a two-dimensional scatter plot display. Software is used to draw appropriate gates for each droplet endpoint cluster, and the number of droplets within each gate is counted (e)

FIGURE 3 Risk assessment of transmitting haemophilia, taking into account the high mosaicism rate in mothers of sporadic cases. In accordance with international guidelines, when a pregnancy is at risk of producing a child with haemophilia, parents are offered a prenatal diagnosis, preferably before 12 wk of pregnancy. *It is not possible to confirm whether a mutation arises at conception or very early in the zygote, within its first few cell divisions. Thus, guidelines recommend that the mother must be considered a 'carrier', and prenatal diagnosis must be proposed and discussed



mosaicism detection by segregating wild-type and variant alleles into individual droplets. Fluorescently labelled probes that are specific to the variant allele can anneal and are degraded by DNA polymerase, which releases fluorophores and results in a fluorescent droplet. The number of positive fluorescent droplets is then detected by a droplet reader and analysed by software that enables the absolute amount of the target genomic region of interest to be precisely counted (Figure 2C).

In Mårtensson's study, TaqMan systems were designed for four haemophilia-causing variants. The sensitivity for each TaqMan assay was determined by analysing a dilution series with 100%, 50%, 10%, 1%, 0.1%, 0.01% and 0% causal variant alleles, which were created by dilution of an index case (100% variant allele) with a healthy male (100% wild allele). The results clearly showed that one of the five non-carrier mothers, who had different pathogenic variants, was a mosaic individual.[24]

5 | KEY MESSAGES FOR APPROPRIATE GENETIC COUNSELLING AND PRENATAL DIAGNOSIS IN SPORADIC HAEMOPHILIA FAMILIES

Nearly 70% of mothers of sporadic haemophilia patients exhibit a carrier status, which means the pathogenic variant can be identified in DNA extracted from all tissues, such as leucocytes, buccal brushings, hair root bulbs, urothelial cells and germ cells[28] (Figure 3).

Based on the four studies that are summarized in Table 1, it is impossible to detect the causal variant in the maternal DNA (leucocytes or other tissues) in about 80% of the remaining 30%. These de novo causal variants occur in the germline eggs of a patient's mother upon conception or during the early embryonic development stage. In this case, the mother is theoretically and probably not a carrier.

However, studies using extra-sensitive genotyping techniques, which are also presented in Table 1, have demonstrated that around 20% of apparently non-carrier mothers are actually carriers with an undetermined germline cell proportion that bears the pathogenic variant. Two possibilities may arise from this: either the genetic defect is confined solely to the germline cells (*germline* or *gonadal mosaicism*) or the genetic defect is present in several tissues, including leucocytes and germline cells (*gonosomal mosaicism*).

The detection of mosaicism is mainly performed in the context of research projects and is not routinely applied in molecular genetic laboratories, since this is expensive and time-consuming. However, detection of mosaicism status with recent extra-sensitive technologies, using multiple tissues that are relatively accessible (such as blood, hair follicles, buccal brushings, urine samples), is very informative for clinicians. This is also informative for carriers and their families, in order to quantify the risk in prenatal counselling, while specifying that zero risk does not exist. Indeed, as it is impossible to confirm whether a variant arises at conception or very early in a zygote, within the first few cell divisions, it is also unthinkable to perform invasive biopsy of both ovaries to detect gonadal mosaicism. Thus, guidelines strongly recommend not to state that *the mother of a haemophiliac is not a carrier, even when the causal variant is not identified in her DNA leucocytes or other somatic tissues*.[39] In the case of an undetectable pathogenic variant using multiple tissues, extra-sensitive technologies drastically reduce the chance that the mother is a somatic mosaic carrier. When the mother is theoretically and probably not a carrier, prenatal diagnosis should be discussed and could be considered.

The risks and benefits of prenatal diagnosis, with invasive or non-invasive procedures, must be discussed and offered in new pregnancy cases. Commonly, chorionic villus sampling (CVS) or amniocentesis is proposed. The most common non-invasive prenatal method for detecting foetal gender DNA in maternal plasma is qPCR.[40] Recently, ddPCR technology has been used to identify the Y chromosome at early gestational ages of pregnant women bearing male foetuses.[41,42] In the future, sensitive technologies will enable the prediction of the pathological phenotype of the unborn, which will thus avoid invasive diagnostic procedures for pregnant women, without risk to the foetus.

6 | CONCLUSION

About 80% of sporadic haemophilia cases with a non-carrier mother status are possibly caused by de novo pathogenic variants that occur in the oocyte of the patient's mother at conception or during the early embryonic development stage. However, a high percentage of mosaicism in apparently non-carrier mothers has been demonstrated, which requires significant work, not only to analyse the DNA of multiple tissues, but also to develop variant detection based on sensitive technologies.

As it is impossible to confirm that causal variants arise at conception or very early in zygotes, guidelines recommend not to state that the *mother is not a carrier* and to discuss and propose prenatal diagnosis in subsequent pregnancy.

DISCLOSURES

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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