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REVIEW

CLINICAL GENETICS WILEY

Best practice guidelines on genetic diagnostics of facioscapulohumeral muscular dystrophy: Update of the 2012 guidelines

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Abstract

The gold standard for facioscapulohumeral muscular dystrophy (FSHD) genetic diagnostic procedures was published in 2012. With the increasing complexity of the genetics of FSHD1 and 2, the increase of genetic testing centers, and the start of clinical trials for FSHD, it is crucial to provide an update on our knowledge of the genetic features of the FSHD loci and renew the international consensus on the molecular testing recommendations. To this end, members of the FSHD European Trial Network summarized the evidence presented during the 2022 ENMC meeting on Genetic diagnosis, clinical outcome measures, and biomarkers. The working group additionally invited genetic and clinical experts from the USA, India, Japan, Australia, South-Africa, and Brazil to provide a global perspective. Six virtual meetings were organized to reach consensus on the minimal requirements for genetic confirmation of FSHD1 and FSHD2. Here, we present the clinical and genetic features of FSHD, specific features of FSHD1 and FSHD2, pros and cons of established and new technologies (Southern blot in combination with either linear or pulsed-field gel electrophoresis, molecular combing, optical genome mapping, FSHD2 methylation analysis and FSHD2 genotyping), the possibilities and challenges of prenatal testing, including pre-implantation genetic testing, and the minimal requirements and recommendations for genetic confirmation of FSHD1 and FSHD2. This consensus is expected to contribute to current clinical management and trial-readiness for FSHD.

For affiliations refer to page 12

KEYWORDS
facioscapulohumeral muscular dystrophy, genetic diagnosis, genotype phenotype correlation, guidelines, molecular diagnostic techniques, outcome assessment, trial readiness, worldwide consensus

1 | GENERAL INTRODUCTION

During the 268th European Neuromuscular Centre workshop—Genetic diagnosis, clinical classification, outcome measures, and biomarkers in facioscapulohumeral muscular dystrophy (FSHD): Relevance for clinical trials in September 2022 in Hoofddorp, the Netherlands,[1] it was concluded that there is a need to update the "gold standard" for genetic diagnostic procedures for FSHD that was published in 2012.[2] With the increasing complexity of the genetics of FSHD1 and 2, it is crucial to renew the consensus on the molecular testing methods. This need is reinforced by the emergence of clinical trials, all of which require a confirmed molecular genetic diagnosis for entry.

To this end, the members of working group 1 (clinical and genetic diagnosis) of the FSHD European Trial Network summarized the evidence discussed during the meeting.[1,3] Six additional virtual meetings were organized between December 2022 and November 2023 to reach consensus. The working group invited genetic and clinical experts from the USA, India, Japan, Australia, South-Africa, and Brazil to reach a global perspective. Final agreement was reached by the ENMC workshop participants during a virtual meeting in December 2023.

2 | CLINICAL AND GENETIC FEATURES OF FSHD

FSHD is the second most common muscular dystrophy in adults.[4] The condition is slowly progressive, and affects primarily muscles of the face, shoulders, and upper arms, often in an asymmetric manner. In addition, many patients have weakness of trunk and leg muscles that sometimes may be the initial manifestations. There is marked variation in clinical manifestations, ranging from a severe early-onset form with higher incidence of nonmuscular features to asymptomatic or paucisymptomatic presentations.[5,6] Specifically, asymptomatic or paucisymptomatic cases refer to individuals who have the genetic marker for FSHD but are not aware of muscle symptoms. However, these individuals do show signs of the disease upon muscle examination.[7] It is important to differentiate these from nonpenetrant cases; individuals that carry the FSHD genetic marker but lack symptoms and signs of muscle involvement upon examination. Although a typical FSHD phenotype allows a prompt diagnosis, the marked variability in manifestations can hamper its recognition, even by experienced neuromuscular physicians. Typical signs are depicted in Figure 1. Any of these signs, alone or in combination, mostly together with a positive family history, should prompt FSHD genetic testing.[8] A negative family history is not uncommon due to de novo mutations, somatic mosaicism, or incomplete penetrance.

The prevalence of FSHD was estimated at 12/100 000 using capture-recapture methodology in the Netherlands.[9] This is higher than estimates of 5/100 000 prior to genetic testing.[9] Considerable numbers of clinically affected individuals remain genetically undiagnosed, suggesting that the prevalence rate of 12/100 000 is conservative.[9]

FSHD is caused by derepression of the embryogenic transcription factor *DUX4* in muscle cells, the expression of which is hypothesized to be toxic, leading to cell death. The *DUX4* gene is located at the distal end of the macrosatellite repeat array D4Z4 in the subtelomeric region on chromosome 4q (sub-band 4q35.2). Single D4Z4 units of the repeat array are 3.3 kilobases (kb) in size, defined by a *Kpn*I restriction site, and based on this, the repeat array on 4q chromosomes starts and ends with partial D4Z4 repeat units. For the sake of clarity, only complete D4Z4 units (U) are reported. Hence, a standard FSHD1 allele with a reported D4Z4 repeat size of 6 units have an actual length between 6 and 7 units (Data S2). The array normally ranges between 8 and >100 U in the European population. In 95% of FSHD patients, the derepression of *DUX4* is caused by a contraction of the D4Z4 repeat array to a size between 1 and 10 U (FSHD type 1, or FSHD1). The repeat contraction is associated with local chromatin relaxation as evidenced by, among others, repeat-size dependent loss of CpG methylation at the contracted allele and a roughly inverse correlation between the size of the pathogenic repeat array and disease severity.[10] There is an overlap between control individuals and FSHD1, especially in the 8–10 U region, where epigenetic changes probably explain the development of FSHD. The D4Z4 repeat array can also be found in the subtelomeric region on chromosome 10 (sub-band 10q26.3), but this repeat array is not permissive to FSHD.[11]

Two main genetic variants of the 4q subtelomere are known, designated 4qA and 4qB, which in both European and Asian control populations are of near-equal frequency (50%). These main variants are subdivided into haplotypes (e.g., 4A161), of which most 4qA haplotypes are permissive to FSHD, while the 4qB haplotypes are not.[12,13] The disease permissiveness of the FSHD locus depends on whether the haplotype harbors a complete *DUX4* gene. On permissive 4qA haplotypes, the *DUX4* gene in the most distal D4Z4 unit contains an extra sequence immediately distal to the D4Z4 repeat array (called pLAM), which provides *DUX4* a mRNA stabilizing polyadenylation signal (PAS) that is used in somatic cells. Functional PAS sequences are absent on 4qB and 10q26 nonpermissive haplotypes.[12]

There is also a second mechanism leading to FSHD (accounting for approximately 5% of patients), which is called FSHD type 2 (FSHD2). FSHD2 is due to pathogenic variants in chromatin modifiers of the D4Z4 repeat array, most often the structural maintenance of chromosomes flexible hinge domain containing 1 (*SMCHD1* gene located on chromosome 18 [sub-band 18p11.32]). FSHD2 is

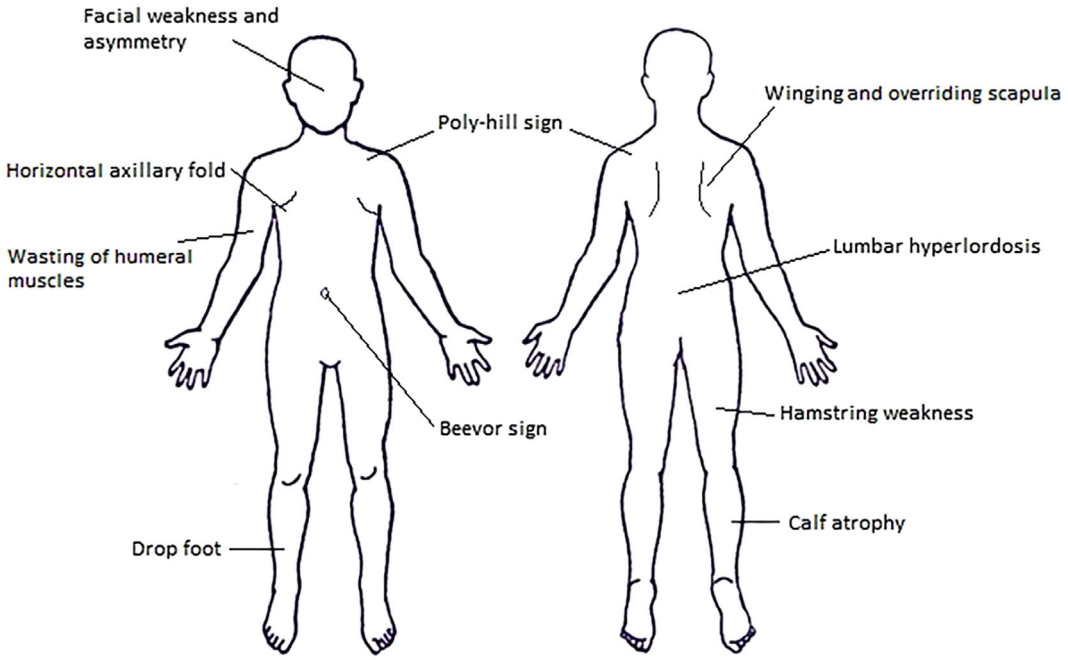

FIGURE 1 Typical signs of facioscapulohumeral muscular dystrophy. These do not occur in a predetermined order and are mostly asymmetric. Reproduced with permission of *BMJ Publishers*.[8] [Colour figure can be viewed at wileyonlinelibrary.com]

associated with partial D4Z4 chromatin relaxation including loss of CpG methylation in the repeat arrays on both chromosomes 4 and 10 in somatic cells.[14] The size of the 4qA repeat array in FSHD2 patients is generally between 8 and 20 U, which is shorter compared to the distribution in the general population. Clinically FSHD1 and FSHD2 are indistinguishable.[15] Like FSHD1, FSHD2 shows a roughly inverse correlation between the size of the shortest permissive 4qA repeat array and disease severity.[10]

3 | FSHD1

3.1 | Threshold FSHD1 (1–10 U) and gray zone FSHD1 (8–10 U)

The first studies on FSHD1 were mainly performed in Europe and the United States, with diagnostic testing based primarily on recognition of a shortened D4Z4 repeat (<10 RU), using Southern Blotting (SB) after Linear Gel Electrophoresis (SB-LGE). Consequently, most of the patients tested have a European genetic background. Initially, the pathogenic repeat array size was determined to be 1–7 U, but this became wider as more patients were studied. Around 1997 the threshold was definitively determined to be 1–10 U in European populations.[16,17] A rough repeat-array-size-dependent severity was reported, with 1–4 U FSHD1 alleles generally seen in the clinically more severe and often early-onset patients, while the 8–10 U alleles carriers were generally less severely affected with more asymptomatic or paucisymptomatic carriers.[7,18–20] However, these thresholds might vary in different ethnic populations.

In parallel, the repeat array size on chromosome 4 in controls was suggested to be >10 U, but the number of tested healthy individuals was limited. Later, it was recognized that 8–10 U repeat arrays on chromosome 4qA are found in approximately 1%–2% of the European control population without any clinical signs or family history of FSHD.[21,22] Based on the frequency of FSHD, these 8–10 U repeat arrays appear to be generally nonpathogenic in the absence of a FSHD family history. This overlap between controls and FSHD1 cases for 8–10 U repeat arrays on 4qA alleles is referred to as the gray zone for FSHD1. The high prevalence of FSHD1-sized alleles among healthy individuals is in line with the presence of nonpenetrant carriers of the FSHD1 allele in relatives of affected individuals, and this phenomenon increases with increased repeat array size (Figure 2).[7] Other factors may influence penetrance and disease progression in this 8–10 U size range. For example, a higher prevalence of females has been reported among the asymptomatic and/or nonpenetrant carriers, highlighting possible gender-related differences in FSHD.[20,23] More recently, gender specific severity differences could not be confirmed[7] and further research is required. An unusual high clinical severity may arise from the combination of borderline FSHD1 alleles with a secondary muscle disorder, this situation is often referred to as "double trouble."[24–28] This scenario could account for patients who exhibit atypical phenotypes or clinical variability within the same family. This might also explain some examples where the 4qA repeat array is even >10 U. Further research is required to understand this phenomenon. One suggested modifier for disease severity are D4Z4 repeat array size-independent differences in CpG methylation, a marker of chromatin compaction.[14] It has been shown that pathogenic variants in FSHD2 genes (see section on FSHD2) may act as modifiers

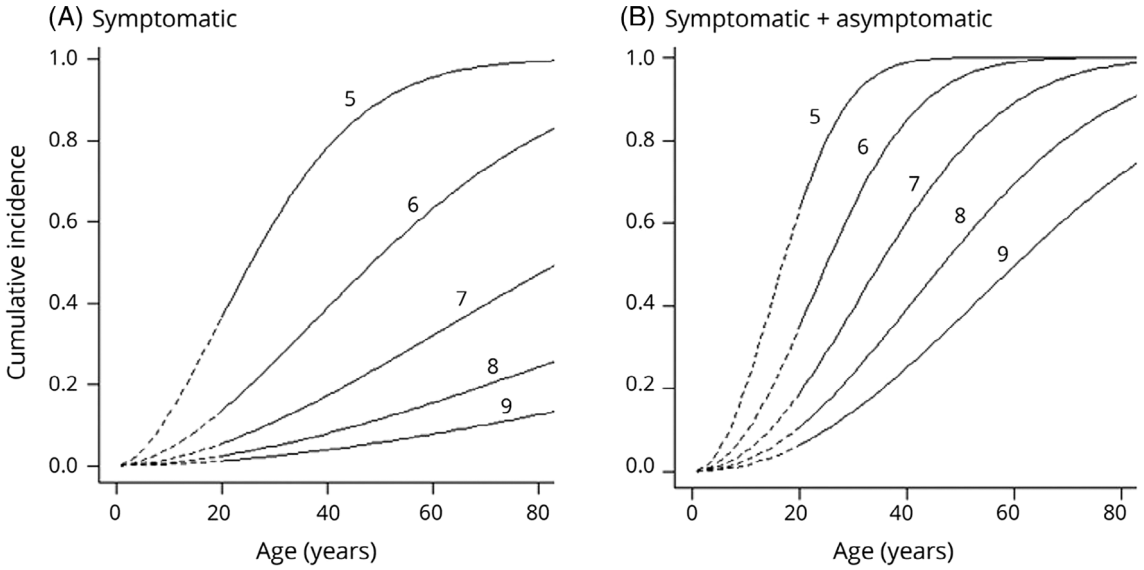

FIGURE 2 Maximum likelihood curves of the penetrance related to FSHD1 allele size (in units). These curves show the likelihood of an FSHD1 allele carrier experiencing symptoms of facioscapulohumeral muscular dystrophy (FSHD) (A: Symptomatic) and of experiencing symptoms or showing signs of FSHD (B: Symptomatic + asymptomatic). Individuals with an FSHD1 allele of 9 U have a likelihood of approximately 10% of reporting symptoms in middle adulthood (category A), whereas the likelihood of reporting symptoms or showing signs (category B) is approximately 50%. This leaves 50% who are still non-penetrant at that age. Reproduced with permission of *Wolters Kluwer Health, Inc.*[7]

of FSHD1, as was shown in families with a gray zone FSHD1 allele.[29] Later, it was shown in a larger cohort of 7–10 U FSHD1 allele carriers that interindividual differences in repeat-size independent D4Z4 methylation correlate with clinical variability even in the absence of a pathogenic *SMCHD1* variant.[10]

3.2 | FSHD1 by a new rearrangement

In 10%–30% of cases, FSHD is caused by a de novo D4Z4 rearrangement in the patient or their parents.[30,31] In about half of these de novo events, the new D4Z4 rearrangements occur in the parental germline, where the FSHD1 allele is undetectable in the somatic cells of the unaffected parent. Alternatively, the rearrangement can occur during the first cell divisions. Consequently, two cell populations arise, one containing the parental-sized alleles and another in which one of the parental 4qA alleles has contracted to FSHD1 size.[31] This phenomenon is termed gonadosomatic mosaicism, as both cell populations are found throughout the body, including the germline. The clinical severity of a mosaic FSHD1 carrier depends on the proportion of affected cells in the muscle and the size of the D4Z4 repeat array. The ratio of affected cells in blood to some extent predicts the clinical severity, suggesting that the proportion of affected cells might be similar between blood and muscle.[20,31] Consequently, a carrier of a mosaic D4Z4 repeat array contraction can also be an asymptomatic or nonpenetrant parent of a de novo FSHD1 patient. The risk of transmitting the FSHD1 allele is smaller for mosaic (affected or unaffected) carriers of an FSHD1 allele than for non-mosaic FSHD1 patients, but if transmitted, the offspring of a mosaic carrier will have the FSHD1 allele in all cells and therefore will most likely be more severely affected than the mosaic parent. In some de novo FSHD1 patients, the FSHD1 allele is found in neither of the parents (usually tested in genomic DNA from white blood cells). Therefore, we assume that the FSHD1 allele arose from a de novo rearrangement in the germline of one of the parents.

To estimate the risk of (recurrent) transmission of an FSHD allele, it is important to determine the D4Z4 rearrangement type (mosaic or non-mosaic) and the proportion of affected cells in case of mosaicism (assuming that the mosaicism is comparable in soma and germline). Determining whether a sample is mosaic for the FSHD1 rearrangement already identified on standard SB can easily be done by using molecular combing (MC), optical genome mapping (OGM), and pulsed-field gel electrophoresis (PFGE) combined with SB (SB-PFGE) if an extra chromosome 4 allele is found and if 2 of the 5 D4Z4 alleles are present in a lower quantity (the details of the different technologies are discussed in later sections). Determination of the proportion of affected cells is possible by comparing the ratio of the mosaic signals. It is recommended to test for mosaicism in all sporadic FSHD patients, given its significant impact on the clinical severity, and impact on genetic counseling and reproductive decision-making.

3.3 | More complex D4Z4 alleles

Probably due to its repetitive nature and its subtelomeric localization, the D4Z4 repeat array is prone to DNA rearrangements. Consequently, multiple complex repeat array structures, some of which are permissive, have evolved over time, which can be difficult to characterize during genetic testing. These are presented in Data S1 and Figure S1, under

the two headings of "S and L alleles," and "Complex D4Z4 alleles." First, at the distal end of the repeat array, two 4qA variants can be distinguished by the size of the most distal partial D4Z4 unit: 4qA-S, for the short partial unit (often referred to as 4qA because it is the most prevalent one) and 4qA-L (for the long partial unit). Both variants are equally pathogenic, and the 4qA-L alleles seem European-specific.[32] The "complex alleles" include D4Z4 proximal extended deletion (DPED) alleles, cis duplications of the D4Z4 repeat array, hybrid alleles, translocations between chromosomes 4 and 10, and rarer 4qA haplotypes. It is essential for any FSHD-diagnostic service to be aware of the potential occurrence of the S/L polymorphism and of complex alleles as they can interfere with accurate diagnosis depending on the applied technology, and together complex alleles account for perhaps 5% of FSHD1 cases in total; mostly being ones which otherwise would give false negative results from initial diagnostic testing. These complex D4Z4 alleles can interfere with accurate diagnosis depending on the applied detection technology and are discussed in Data S1.

3.4 | FSHD1 threshold in different ethnic backgrounds (Europe vs. Asia and Africa)

The 4qA repeat array size in FSHD1 patients in Japan and South-Korea range between 1 and 6 U, suggesting that array sizes of 7–10 U are less pathogenic in these populations than in European and North-American populations.[33,53] As with the difference in clinical severity for 8–10 U FSHD1 allele carriers, epigenetic, and other unknown factors are likely to contribute to these ethnic differences in FSHD susceptibility. It is therefore essential to ascertain a patient's ethnic background prior to genetic testing and document this on the patient data form. For example, a 4qA repeat array of 8 U should be reported as likely pathogenic in a European patient while the pathogenicity is less certain in a Japanese patient. To date, little has been reported on FSHD in patients with a Sub-Saharan African ancestry.[34,35] Population studies on the 4qA/4qB haplotype distribution suggest that most (91%) of the African chromosome 4 alleles are of the 4qA haplotype.[36] Due to this preponderance of 4qA alleles in African ancestry, one would expect more patients with an African background among FSHD populations of Europe and the United States, where genetic testing is readily available. However, the number of identified FSHD patients of African ancestry appears to be very low, suggesting that other, yet unidentified factors, reduce the susceptibility to developing FSHD. Although access to healthcare and diagnostic testing may contribute to the apparent discrepancy, it is unlikely to be the major contributor, and further research is required to elucidate this observation.

4 | FSHD2

4.1 | DNA methylation

In FSHD2, global D4Z4 chromatin relaxation (DNA hypomethylation) is caused by pathogenic heterozygous variants in a chromatin modifier gene, most often *SMCHD1*.[14,37] In addition, D4Z4 chromatin relaxation can also be caused by pathogenic variants in other genes like *DNMT3B* (20q11.21, dominant) or *LRIF1* (1p13.3, recessive).[38,39] The chromatin relaxation at the D4Z4 repeat arrays only lead to FSHD2 in the presence of a permissive 4qA allele. For individuals that carry a pathogenic variant in *SMCHD1* in combination with two 4qB alleles, there will be no DUX4 expression and no FSHD. With the requirement of both a DUX4 expression-permissive allele on chromosome 4qA and a pathogenic variant in one of the FSHD2 genes located on different chromosomes, FSHD2 shows a digenic inheritance pattern.[11] Similar to FSHD1, in FSHD2, there is a linear correlation between CpG methylation and the size of the D4Z4 repeat array and consequently there is a rough inverse correlation between the size of the shortest permissive 4qA repeat array and disease severity.[10] Generally, the size of the shortest 4qA repeat array in FSHD2 patients varies between 8 and 20 U. Consequently, there is an overlap between FSHD1 and FSHD2 in the 8–10 U range, with carriers of both conditions being defined as FSHD1 and FSHD2 and generally exhibiting a severe FSHD phenotype.[29] FSHD2-level hypomethylation in combination with a >20 U 4qA repeat array generally does not lead to clinical manifestation of FSHD or only mild symptoms, but there are exceptions; therefore, this combination does not exclude the genetic diagnosis of FSHD2. Due to the digenic inheritance pattern, one can expect to find many unaffected and asymptomatic carriers of a pathogenic *SMCHD1* variant among family members of FSHD2 patients. These carriers should be traced and informed as they can pass on the pathogenic variant in the FSHD2 gene, with a high risk of FSHD in their offspring when combined with a permissive <20 U allele from the partner. All permissive haplotypes, including the complex alleles discussed in the FSHD1 section (hybrid 4qA, DPED, in *cis* duplication alleles) can also be pathogenic in FSHD2. Unexpectedly, *in cis* duplication alleles, which are relatively rare in unaffected individuals, have been found in about 8% of FSHD2 patients.[40] As the chromatin relaxation happens on both chromosome 4 alleles in FSHD2, a higher severity can be observed in patients who carry two permissive alleles.[32] One FSHD2 family has been described with a possible recessive mode of inheritance in which two in *trans SMCHD1* variants seem to act synergistically on D4Z4, leading to hypomethylation and disease penetrance.[41] The primary method for confirming FSHD2 is to determine global D4Z4 hypomethylation. The threshold for FSHD2 methylation varies depending on the technique used. If the CpG methylation level is indicative of FSHD2, further sequence analysis is advised to pinpoint the causative genetic variant. When the hypomethylation is far below the FSHD2 threshold this result already confirms FSHD2, even without the identification of the responsible genetic variant (Figure 3, Table 1).

On the other hand, there are also examples of patients who have a typical FSHD phenotype but no hypomethylation and are genetically not FSHD2. These patients are difficult to diagnose, but when they carry at least one permissive 4qA allele and have a typical FSHD phenotype we suggest that they should be diagnosed as "clinical FSHD, genetic cause unknown." Further research is required to elucidate the genetic mechanism for these unusual patients.

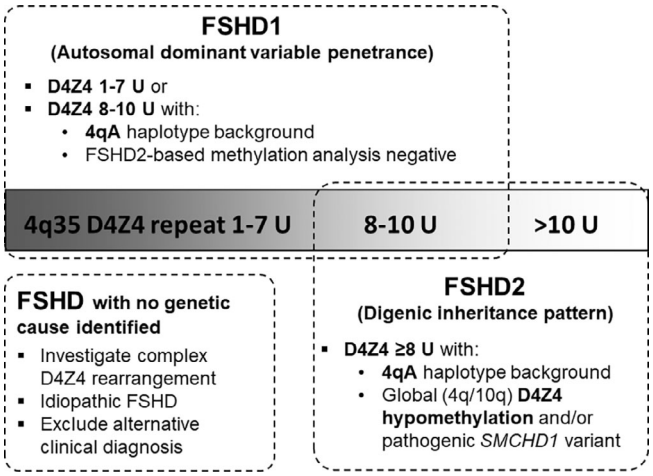

FIGURE 3 Schematic representation of minimal criteria for genetic confirmation of facioscapulohumeral muscular dystrophy.

4.2 | *SMCHD1* sequencing

Several hundred pathogenic *SMCHD1* variants have been identified across the entire 48 exon large *SMCHD1* locus. The FSHD2 variant spectrum includes all types of variants (nonsense, splice site, small deletions or insertions, and missense variants), but missense and splice site variants are most common.[14,37] About 10% of the splice site variants are found in a variant hotspot in intron 25.

Variants previously found in *SMCHD1* in unaffected individuals are listed in the Genome Aggregation Database (gnomAD, https://gnomad.broadinstitute.org/). However, the identification in unaffected individuals does not provide a definitive answer to the possible pathogenicity of these *SMCHD1* variants. Due to the digenic inheritance pattern of FSHD2, pathogenic *SMCHD1* variants will only cause FSHD in about 20% of carriers; individuals with a permissive 4qA allele and mostly with a D4Z4 repeat array size of 8–20 U.[40,42] There are also databases listing reported pathogenic *SMCHD1* variants in FSHD patients, including ClinVar, The Human Gene Mutation Database (HGMD) and the LOVD database https://www.ncbi.nlm.nih.gov/clinvar; https://simple-clinvar.broadinstitute.org/; https://databases.lovd.nl/shared/genes/SMCHD1. However, the number of variants in these databases is determined by the willingness of researchers to submit variants. Although the pathogenicity of missense and splice site variants can be predicted in silico, this is not always accurate, and they are often classified as variants of uncertain significance (VUS). Hence, D4Z4 methylation analysis (on a blood-derived DNA sample) is essential to functionally evaluate *SMCHD1* variants.

4.3 | Next generation sequencing

Panel-based next generation sequencing (NGS) has proven to be an effective manner to identify molecular causes in neuromuscular disorders. It has become a first-tier test in many countries, unless there is high probability of a specific condition or when the genetic cause consists of a repeat array rearrangement.[43] As a result, *SMCHD1* variants of variable pathogenicity are increasingly encountered as so-called "incidental" findings. In a retrospective study of 55 families with limb-girdle weakness, two families with a (likely) pathogenic variant in *SMCHD1* were identified. In both cases, further analysis revealed a permissive 4qA allele changing the diagnosis to FSHD2.[44] At the Santa Lucia Foundation IRCCS, diagnostic NGS revealed 11 novel *SMCHD1* variants in patients with a permissive 4qA allele over the past 5 years. At the Radboud University Medical Centre, a very limited number of *SMCHD1* variants have been identified during that period.[43] D4Z4 methylation analysis and haplotype analysis to confirm FSHD2 should be the recommended next steps.

5 | MINIMAL REQUIREMENTS AND RECOMMENDATIONS FOR DIAGNOSIS IN FSHD

The group of experts agreed on the following minimal criteria for genetic confirmation of FSHD (Figure 3). Details on the overview for diagnostic testing, with an emphasis on the importance for interpretation of assessing whether the clinical and molecular information match in anticipated presentation and severity, are also shown in the table below (Table 1).

6 | TECHNOLOGIES FOR DIAGNOSIS OF FSHD

The genetic analysis of FSHD is based on the determination of the size and haplotype of the D4Z4 repeat arrays on chromosomes 4 and 10. For FSHD1, analysis is focused on D4Z4 repeat arrays between 1 and 10 units (Southern blotting after linear gel electrophoresis; SB-LGE), which requires standard liquid DNA of high quality (>100 kb). This DNA is isolated, usually from white blood cells from EDTA blood, via common methods of DNA isolation either manually or in an automated system. To visualize all four D4Z4 repeat arrays (on chromosomes 4 and 10) and enable the analysis of complex D4Z4 rearrangements and FSHD2, higher molecular weight DNA is required as D4Z4 repeat array fragments can be up to 150 U (500 kb). Analysis of long fragments can be by SB-PFGE (Southern blot after pulsed field gel electrophoresis), OGM or MC. For SB-PFGE and MC, high quality DNA is usually obtained by embedding DNA containing cells in agarose plugs prior to pronase and detergent treatment. This methodology prevents DNA shearing as further DNA isolation steps are not needed. OGM can also be done on DNA in agarose plugs, but is usually performed using an OGM-specific DNA isolation method.

The advent of whole exome (WES) and whole genome (WGS) sequencing technologies around 2010 did not immediately benefit FSHD analysis (which continued using traditional SB-based methods), as the size and haplotype of the D4Z4 repeat array cannot be determined by short read WES- or WGS-like technologies and was traditionally performed by SB-based methods. More recently, alternative

TABLE 1 Table showing the essential and desirable actions in the genetic confirmation of FSHD based on the patient's phenotype.

SB+PFGE[1], MC, and OGM					
Clinical presentation	**Result[23]**	**Action essential**	**Result**	**Diagnosis**	**Additional action Recommended[4]**
Typical/atypical FSHD Severity: moderate to severe	≤7 U 4qA	No further testing needed		FSHD1	If early/childhood onset and >5 U, consider FSHD2 methylation analysis
	8–10 U 4qA	FSHD2 methylation analysis, or SMCHD1 analysis (or panel)	Hypomethylation, and/or pathogenic variant in SMCHD1[5]	FSHD1,2	-
			Normal methylation	FSHD1	Test to exclude alternative or additional genetic cause
	>10 U 4qA	FSHD2 methylation analysis, or SMCHD1 analysis (or panel) and check for DPED allele or cis dupliciation	Hypomethylation, and/or pathogenic variant in SMCHD1[5]	FSHD2 (>10 U)	-
			DPED ≤10 U 4qA allele[6]	FSHD1	-
			Cis-duplication allele	Probably FSHD1	Segregation analysis, if possible, to confirm pathogenicity
			Normal methylation No DPED or cis-duplication	No FSHD	Test for alternative diagnosis
Typical FSHD Severity: mild	≤7 U 4qA	Analyze for possible mosaicism		FSHD1 (possible mosaic)	-
	8–10 U 4qA	No further testing needed		FSHD1	Segregation analysis, if possible, to confirm pathogenicity
	>10 U 4qA	FSHD2 methylation analysis, or SMCHD1 analysis (or panel) and check for DPED allele or cis duplication	Hypomethylation, and/or pathogenic variant in SMCHD1[5]	FSHD2 (>10 U)	-
			DPED ≤10 U 4qA allele[6]	FSHD1	-
			Cis-duplication allele	Probably FSHD1	Segregation analysis, if possible, to confirm pathogenicity
			Normal methylation No DPED or cis-duplication	No FSHD	Test for alternative diagnosis
Atypical FSHD Severity: mild	≤7 U 4qA	Analyze for possible mosaicism		FSHD1 (possible mosaic)	-
	8–10 U 4qA	FSHD2 methylation analysis, or SMCHD1 analysis (or panel)	Hypomethylation, and/or pathogenic variant in SMCHD1[5]	FSHD1,2	-
			Normal methylation	Probably FSHD1	Segregation analysis, if possible, to confirm pathogenicity
	>10 U 4qA	Check for DPED allele or cis duplication	DPED ≤10 U 4qA allele[6]	FSHD1	-
			Cis-duplication allele	Probably FSHD1	Segregation analysis, if possible, to confirm pathogenicity
			Normal methylation No DPED or cis-duplication	No FSHD	Test for alternative diagnosis

(Continues)

TABLE 1 (Continued)

SB + LGE							
Clinical presentation	**Result[2]**	**1st Action essential**	**Result[23]**	**2nd Action essential**	**Result**	**Diagnosis**	**Additional action recommended[4]**
SB + LGE							
Clinical presentation	**Result[2]**	**1st Action essential**	**Result[23]**	**2nd Action essential**	**Result**	**Diagnosis**	**Additional action recommended[4]**
Typical/atypical FSHD Severity: moderate to severe	≤7 U 4q	Not required (assuming ≤7 U 4qA)		-		FSHD1	If early/childhood onset and >5 U, consider FSHD2 methylation analysis
	8–10 U 4q	4qA/4qB haplotyping	8–10 U 4qA	FSHD2 methylation analysis, or SMCHD1 analysis (or panel)	Hypomethylation, and/or pathogenic variant in SMCHD1[5]	FSHD1,2	-
					Normal methylation	FSHD1	Test to exclude alternative or additional genetic cause. Consider checking for DPED ≤10 U allele or cis duplication.
	>10 U 4q	Go to 2nd action essential		FSHD2 methylation analysis, or SMCHD1 analysis (or panel)	Hypomethylation, and/or pathogenic variant in SMCHD1[5]	FSHD2 (>10 U)	4qA/4qB haplotyping by SSLP or distal 4qA PCR
					Normal methylation	Possible complex haplotype	Check for DPED ≤10 U allele, or cis duplication
						No FSHD	Test for alternative diagnosis
Typical FSHD Severity: mild	≤7 U 4q	Not required (assuming ≤7 U 4qA)		-		FSHD1 (possible mosaic)	Check for mosaicism
	8-10 U 4q	4qA/4qB haplotyping	8–10 U 4qA	No further testing needed		FSHD1	Segregation analysis, if possible, to confirm pathogenicity
	>10 U 4q	Go to 2nd action essential		FSHD2 methylation analysis, or SMCHD1 analysis (or panel)	Hypomethylation, and/or pathogenic variant in SMCHD1[5]	FSHD2 (>10 U)	4qA/4qB haplotyping by SSLP or distal 4qA PCR
					Normal methylation	Possible complex haplotype	Check for DPED ≤10 U allele, or cis duplication
						No FSHD	Test for alternative diagnosis

TABLE 1 (Continued)

SB + LGE							
Clinical presentation	**Result[2]**	**1st Action essential**	**Result[23]**	**2nd Action essential**	**Result**	**Diagnosis**	**Additional action recommended[4]**
Atypical FSHD Severity: mild	≤7 U 4q	4qA/4qB haplotyping	≤7 U 4qA	No further testing needed		FSHD1 (possible mosaic)	Check for mosaicism
	8–10 U 4q	4qA/4qB haplotyping	8–10 U 4qA	FSHD2 methylation analysis, or SMCHD1 analysis (or panel)	Hypomethylation, and/or pathogenic variant in SMCHD1[5]	FSHD2 (8–10 U)	-
					Normal methylation	Probably FSHD1	Segregation analysis, if possible, to confirm pathogenicity
	>10 U 4q	Not required, exclusion FSHD				No FSHD	Test for alternative diagnosis

All technologies				
Clinical presentation	**Result**	**Action essential**	**Result**	**Diagnosis**
1st degree relative(s) has genetically confirmed FSHD1	FSHD1 allele that matches relative	No further testing needed		FSHD1
	>10 U 4q	No further testing needed		Not likely to be FSHD

Note: The top table is similar for the SB + PFGE, MC, and OGM and the middle for SB-LGE only and the bottom for all technologies. The separation of FSHD1 allele sizes (≤7 U; 8–10 U and >10 U 4qA) is based on individuals with a European genetic background.

Abbreviations: LGE, linear gel electrophoresis; MC, molecular combing; OGM, optical genome mapping; PFGE, pulsed field gel electrophoresis; SB, Southern blot.

[1]The standard SB-PFGE analysis in this table includes 4qA/4qB hybridization.

[2]It is required to do further analysis if genotype–phenotype does not fit.

[3]Absence of any 4qA allele excludes FSHD, unless 10qA ≤3 U is observed.

[4]It is recommended to perform genetic counseling when FSHD is genetically confirmed.

[5]If possible, also consider possible pathogenic variants in other FSHD2 genes.

[6]The identification of an 1–7 U DPED allele confirms FSHD1 and further methylation analysis is not required.

methods have been developed. The most important differences are the capability to visualize all D4Z4 allele sizes (1–150 U), versus only 1–10 U alleles, and to identify now the haplotype and chromosomal background in a single step, versus multiple steps or not at all. Details on the different genetic technologies are described in Data S2. These newer techniques are revolutionizing the ability to solve "difficult cases," particular involving the complex rearrangements listed in Section 5 above and as described in Data S1.

7 | PRENATAL TESTING AND PRE-IMPLANTATION GENETIC TESTING

Advances in genetics, prenatal diagnosis, and medically assisted procreation have increased the options for couples at risk of transmitting a genetic condition. Next to adoption, current options are pre-implantation genetic diagnosis (PGT), which allows implantation of embryos without the causative genotype, and prenatal genetic diagnosis, which provides information on the fetal genotype before the birth. Explaining genetic heterogeneity, incomplete penetrance, inter/intra-familial clinical variability (age of onset, progression rate of muscle weakness, etc.) and unpredictable severity of FSHD disease is crucial when counseling couples.[45,46] In many countries, pre-test genetic counseling is mandatory for pre-implantation and prenatal testing. Several other countries are reluctant to offer pre-implantation and prenatal testing due to these limitations. The best decision is made if couples are well-informed about their options.

Both pre-implantation and prenatal tests present limitations. In families affected by FSHD1, assessing the size of the D4Z4 repeat array is adequate to determine the risk for an embryo or fetus of developing FSHD. Yet, this assessment alone cannot precisely predict the onset, penetrance, or disease progression in most patients, particularly those with an 8–10 U FSHD1 allele. For FSHD2 families, supplementary genetic analyses are required to establish the diagnosis (D4Z4 methylation, sequencing of *SMCHD1*, etc.). The identification of the exact genotype segregating with the disorder is an essential pre-requisite for prenatal and pre-implantation genetic diagnosis. This is generally not yet available for FSHD2. Furthermore, couples should be informed about the influence of pregnancy, delivery, and the postpartum period on FSHD symptoms in the mother and vice versa. A discussion of these and other issues with a gynecologist at a preconception clinic should be considered. This is discussed in a recent review by Vincenten et al.[47]

7.1 | Pre-implantation genetic testing

Pre-implantation genetic testing for monogenic disorders (PGT-M) is a procedure conducted before pregnancy to carefully select embryos free from specific genetic disorders. PGT-M is primarily employed in familial cases to prevent the hereditary transmission of monogenic disorders. For most conditions, PGT involves the detection of the genetic variant(s) responsible for the disease (direct testing), followed by confirmation through indirect tests like segregation analysis. However, in the case of FSHD1, direct testing for D4Z4 repeat array size and haplotype is not feasible using DNA extracted from embryo biopsy, as a large number of cells (1 million cells, or >30 μg DNA) is required for all previously discussed technologies.

Therefore, PGT for FSHD patients relies exclusively on indirect testing, which is further complicated by the subtelomeric location of the D4Z4 repeat array. This location poses challenges in selecting an appropriate number of genetic markers (Short Tandem Repeats—STRs and Single Nucleotide Polymorphisms—SNPs) as they can only be typed proximal to D4Z4, not distal. Among the markers within the 4q35 locus, four microsatellite markers (D4S2390, D4S1652, D4S2930, and D4S1523) have been identified as suitable for PGT-M protocols.

The distance between these STR markers and the D4Z4 locus, along with the possibility of uninformative results, highlights the potential for recombination to negatively impact diagnostic accuracy. As a result, there is an estimated 5% risk of misdiagnosis in FSHD PGT, underscoring the critical role of the set-up stage in evaluating the feasibility of PGT. Consequently, follow up by prenatal diagnosis is advisable to confirm the success of PGT and rule out any potential recombination events.[45,48] This is, however, associated with a small risk of miscarriage or preterm birth, which might make the decision for parents who have otherwise "successfully" undergone PGD challenging.

7.2 | Prenatal genetic diagnosis

Prenatal genetic diagnosis (PND) for FSHD can be performed with direct and indirect tests, using fetal DNA extracted from chorionic villus sampling (CVS) or amniotic fluid (AF). Due to the large amount of DNA required for direct analysis, cell culture is necessary to test for FSHD after amniocentesis. Chorionic villi can be analyzed immediately if a sufficient number of cells have been sampled. The earlier time of diagnosis allowed by CVS (10–13 weeks) compared with AF (generally 15–17 weeks) makes CVS preferable for couples with a high genetic risk. As already discussed, direct testing should be confirmed by indirect analyses (STRs and/or SNPs analysis) to exclude contamination by maternal DNA.[45] Regardless of the results, post-test genetic counseling should be performed in all cases.

7.3 | PND and PGT advice for specific scenarios

In our discussion, the following scenarios were found most challenging. Below we present the consensus recommendations.

7.3.1 | Advice for 8–10 U cases

PND and PGT should consider both the ability to assess the causative genetic variations (not always known) and the actionability of this

genetic information, and in particular, its inability to predict the clinical consequences. Particularly in patients with 8–10 U, accurate prediction of the phenotype, and in particular whether an affected son or daughter would develop symptoms or not, is impossible despite a reliable genetic diagnosis, because of the incomplete penetrance and variable expressivity of the disease. Analyzing phenotype–genotype relationships in other family members has only limited value (Figure 2).

7.3.2 | Possibilities for FSHD2 (with known gene mutation)

For FSHD2, the presence of disease depends on the pathogenic variant in a chromatin modifier (*SMCHD1*), the presence of a permissive allele and the size of the D4Z4 repeat array on this allele. In FSHD2 families with a known pathogenic variant in the chromatin modifier gene, direct testing for the presence of this variant should be the first step for PGT or PND. Prior testing of both parents will provide information on the D4Z4 alleles that may be present in the fetus. This information may allow one to predict the risk of FSHD in the fetus/embryonic cells. When the pathogenic variant is absent, the fetus/embryonic cells have no risk to develop FSHD2. In the presence of the pathogenic variant, further genotyping is required to determine the haplotype and parental origin (and thereby repeat array size) of the inherited alleles.

8 | THE CHALLENGES OF THE CLINICAL VARIABILITY IN GENETIC COUNSELING CALL FOR COMPREHENSIVE CLINICAL INFORMATION PROVIDED WITH GENETIC TEST APPLICATION

The clinical and genetic heterogeneity of FSHD pose challenges to the diagnostic process and the genotype–phenotype correlation.[7,49,50] The dedicated FSHD scores (FSHD clinical severity score, FSHD evaluation scale) standardize recognition of clinical signs.[48,51] In many familial cases, genetic testing is requested by a neurologist or genetic counselor. In less straightforward cases, a multidisciplinary approach can assist in the selection of the most appropriate genetic test.[7,45] This will prevent inappropriate tests and promote a clinical differential diagnosis for patients without a suggestive FSHD phenotype.

8.1 | Necessity of comprehensive clinical information provided with genetic test application

The 1%–2% prevalence of 8–10 U 4qA alleles in the European population may lead to a false positive diagnosis. Genetic testing for FSHD in the absence of a positive family history of FSHD should therefore be requested with caution, preferably only in individuals with one or more typical features. In these cases, genetic counseling represents a bridge between neurologists and geneticists or between clinicians and molecular genetic testing laboratories, ensuring the complete evaluation of patients and familial phenotypes. This might be of great value, especially if the clinician is less familiar with the incomplete penetrance and large intrafamilial variability. An extensive investigation of family history supports the estimation of the mode of inheritance. A clinical information form is recommended to collect the relevant clinical features (Data S3).

Pre-test and post-test genetic counseling should preferably be performed by a geneticist or neurologist experienced in FSHD. In particular, pre-test genetic counseling should address the following clinical and molecular issues: (I—clinical) pathological phenotype that suggest FSHD diagnosis, (II—clinical) pathological phenotypes in family members that suggested FSHD inheritance, (III—molecular) the technologies that will be applied to reveal the patient's genotype, with limitations (sensitivity and specificity) and timing of each test, (IV—molecular) presentation of the final report, incidental findings, and possibility of inconclusive results.[46] During post-test genetic counseling, geneticists, and neurologists explain results of analyses and anticipate the clinical implications. Furthermore, they can assess whether the molecular result match the clinical picture or not. If for example, the severity is much greater or much milder than would be expected for the molecular result, this should be an indicator that further investigation should be considered. The genetic centers are open to discussion about this. A list of the genetic testing centers is available in Data S4.

In different countries, different medical specialties will perform the role of counselor. Ideally, post-test evaluation is multidisciplinary, providing a complete explanation to the patient, with both neurological (confirmation of diagnosis, characteristics of clinical management and prognosis), and genetic (family and recurrence risk, procreative choices) issues. Finally, there are further psychological issues to evaluate. In particular, the assessment of recurrence risk should be accompanied by the explanation of available reproductive choices.

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The authors declare no conflict of interest.

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Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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