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Bridging past and future: the evolution of genetic diagnosis in FSHD and the role of emerging technologies in a globalized framework

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ABSTRACT

Facioscapulohumeral muscular dystrophy (FSHD) is a neuromuscular disorder characterized by marked clinical and molecular heterogeneity. Over more than two decades, molecular diagnosis has relied on Southern blotting to resolve the complex D4Z4 macrosatellite at chromosome 4q35. While this approach remains a reference standard, its technical constraints and the need for complementary layers of information have become more evident as FSHD diagnostics is increasingly required to support patient stratification, genotype-phenotype correlation, and clinical trial readiness. In this context, the review traces the evolution of FSHD diagnostics from classical molecular approaches to modern genome-scale technologies that enable direct characterization of the D4Z4 locus, improve interpretation of borderline and atypical cases, and support integrated diagnostic workflows. Beyond technical innovation, the review highlights the growing need for harmonized diagnostic

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algorithms, international collaboration, and federated data infrastructures to support consistent interpretation across populations and healthcare systems. It further emphasizes how emerging requirements for molecular stratification in clinical trials, together with persistent global disparities in access to genetic testing, are reshaping priorities in FSHD diagnostics, positioning FSHD as a model for how rare disease diagnostics can integrate classical expertise with next-generation technologies to support clinical care, trial readiness, and more equitable access to diagnosis worldwide.

1. Introduction

Facioscapulohumeral muscular dystrophy (FSHD) represents a unique condition within neuromuscular genetics, where clinical heterogeneity, structural genomic complexity and epigenetic dysregulation both challenge and enhance diagnostic practice. Since its first clinical description more than 150 years ago, the disease is now considered one of the most common muscular dystrophies in adulthood, with a prevalence estimated between 1:8000 and 1:20,000 across several populations [1]. However, epidemiological studies suggest that the true burden of disease may be substantially higher, owing to underdiagnosis, atypical presentations, and variable penetrance among carriers of the pathogenic contracted D4Z4 arrays on chromosome 4 (i.e., D4Z4 reduced alleles "DRAs"). This discrepancy between the number of genetic carriers and those who receive a clinical diagnosis already highlights a key paradox of FSHD: a disorder with a widely accepted *DUX4*-centered pathogenic model, yet still not straightforward to diagnose [2]. From a patient perspective, this diagnostic complexity often translates into prolonged uncertainty, fragmented diagnostic pathways, and inconsistent interpretations across centers, thus entailing a significant psychological burden.

Part of what makes FSHD unique lies in the coexistence of classical molecular diagnostic tools alongside the rapidly growing availability of advanced genomic technologies. Historically, Southern blotting has been the cornerstone of FSHD diagnosis, detecting pathogenic contractions of the D4Z4 macrosatellite on chromosome 4q35 and distinguishing between homologous arrays on chromosomes 4 and 10 by restriction enzyme sensitivity patterns [3]. The robustness of the technique and its ability to interrogate long, repetitive 4q35/10q26-D4Z4 sequences have made it crucial for decades [2]. However, its labor-intensive workflows, the requirement for high-quality DNA and large amounts of extracted material, and its limited resolution for complex rearrangements (including mosaicism, 4q/10q translocations, and duplications) underscore important limitations in the evolving context of FSHD diagnostics, where patient stratification is increasingly critical for clinical trials and counseling. The genetic architecture and current model of pathogenesis of FSHD itself amplifies these challenges (reviewed in Belayew et al. 2025) [4]. Both FSHD1 and FSHD2 ultimately converge on a shared pathogenic mechanism: inappropriate expression of the *DUX4* gene [5,6], facilitated by a permissive 4qA haplotype [7], and the consequent expression and toxic presence of *DUX4* [8] in muscle cells. However, detecting *DUX4* expression in muscle tissue remains technically challenging. Its transcription occurs in a highly sporadic and transient manner and is restricted to a very small subset of nuclei within affected muscle [5]. Such low frequency and heterogeneous expression limit the reliability of direct *DUX4* mRNA or

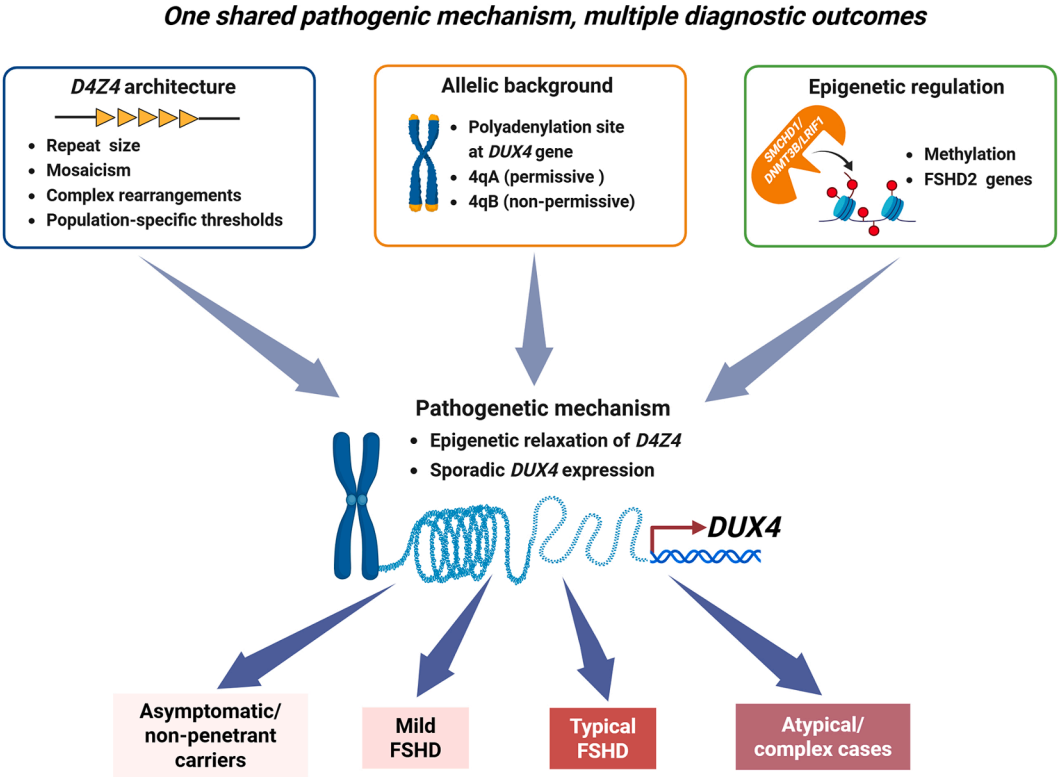

Fig. 1. Pathogenetic complexity in FSHD. FSHD arises from a shared pathogenic mechanism characterized by epigenetic relaxation of the 4q35 D4Z4 locus and sporadic DUX4 expression in skeletal muscle. However, variability in 4q35 D4Z4 repeat architecture, allelic background providing a polyadenylation signal (4qA) or not (4qB) to the DUX4 gene mapped in the distal D4Z4 unit, population-specific differences and epigenetic regulation results in heterogeneous clinical and diagnostic outcomes. These range from typical FSHD to mild or incomplete forms, asymptomatic or non-penetrant carrier states, as well as atypical or complex presentations. This figure has been created with Biorender.

protein detection for diagnostic or disease monitoring purposes. Nevertheless, this model is now widely accepted and supported by extensive molecular evidence showing how D4Z4 contraction or defects in epigenetic regulators such as *SMCHD1, DNMT3B*, or *LRIF1* reduce methylation, remodel chromatin, and enable sporadic bursts of *DUX4* expression capable of disrupting multiple transcriptional and apoptotic pathways [4,9]. Yet even as this conceptual framework has provided a unifying view of FSHD pathophysiology [10], the genotype-phenotype relationship remains highly heterogeneous (Fig. 1). Extensive cohort studies show that individuals carrying the same DRA size, especially within the so-called "gray zone" of 8–10 repeats may present with widely divergent clinical outcomes, ranging from typical FSHD muscle weakness to paucisymptomatic and asymptomatic presentations [11, 12]. This variability complicates not only diagnosis but also genetic counseling, particularly in families showing incomplete penetrance. Against this backdrop, emerging technologies are reshaping the diagnostic landscape, not as direct replacements for established methodologies, but as complementary tools that expand diagnostic sensitivity, accessibility, and efficiency. Pioneering work using molecular combing first enabled the direct visualization of D4Z4 arrays and the identification of complex rearrangements [13,14]. More recently, optical genome mapping enables direct visualization of D4Z4 arrays, sizing, and provides additional structural information, although complex rearrangements may still pose interpretative challenges. Long-read sequencing platforms further extend this capability by simultaneously resolving D4Z4 structure, haplotype, and methylation status. Rather than supplanting classical approaches, these technologies should be viewed as integrated solutions that enhance diagnostic resolution, support interpretation in borderline or complex cases, and hold the potential to reduce turnaround time and overall costs. Their combined use enables a more comprehensive interpretation of genetic and epigenetic features, which is essential for distinguishing FSHD1 from FSHD2 and for contextualizing ambiguous results within a clinically meaningful framework. Likewise, novel methylation assays, including long-read-based methylation calling, are redefining the role of epigenetic biomarkers in diagnosis. However, their clinical utility still requires careful contextualization given the diverse findings and methodological heterogeneity reported across studies [2]. The need for such innovation has become increasingly pressing in a globalized medical landscape. Clinical trials targeting *DUX4* expression or downstream pathways are now underway, and their success depends on precise diagnostic classification, consistent phenotyping, and harmonized inclusion criteria across centers and countries [15]. Recent international guidelines underscore this need by recommending tiered diagnostic algorithms that combine genetic, epigenetic, and phenotypic assessments, while explicitly recognizing a renewed central role for standardized clinical evaluation and genotype-phenotype correlation in guiding genetic characterization and determining when additional, complementary analyses are required, thus reflecting the multidimensional nature of FSHD and the necessity of harmonizing diagnostic approaches across diverse healthcare systems [16]. At the same time, population-based differences in D4Z4 allele distributions and modifying variants emphasize the importance of cross-national data integration, collaborative networks, and federated analytical approaches capable of handling genomic complexity while respecting regulatory constraints on data sharing [17]. Consequently, FSHD stands today as a paradigm for how rare disease diagnostics evolve when classical methodologies meet emerging technologies. It illustrates not only the historical endurance of foundational tools like Southern blotting but also the transformative potential of molecular combing, optical genome mapping, long-read sequencing, and methylation profiling to capture the full spectrum of genomic and epigenomic variations. As therapeutic prospects continue to expand, the diagnostic journey of FSHD, from past to future, offers a compelling model for how precision medicine can integrate structural genomics, epigenetic landscapes, and clinical diversity within an increasingly interconnected global framework. Within this evolving scenario, the purpose of this review is to trace how FSHD has become a diagnostic paradox that bridges traditional methodologies and next-generation technologies, and to assess how this transition is reshaping clinical practice globally. By integrating modern techniques with classical molecular approaches, the review aims to provide a comprehensive overview of the evolution of FSHD diagnostic strategies and their implications for genotype-phenotype interpretation across diverse populations. In parallel, it addresses the emerging need for harmonized diagnostic protocols, international collaboration among expert centers, and federated data-sharing approaches able to support equitable access to future therapeutic interventions targeting the *DUX4* pathway or epigenetic regulatory mechanisms. Ultimately, this perspective illustrates how a multimodal, globally informed diagnostic architecture can better serve both research and clinical care as precision medicine advances for FSHD.

2. Genetics and epigenetics of FSHD

FSHD is primarily driven by changes in the D4Z4 macrosatellite repeat at chromosome 4q35 [18], a complex genomic region composed of 3.3 kb tandemly repeated units, each harboring the *DUX4* open reading frame (ORF) [6,19], with only the most distal repeat unit harboring a complete, functional *DUX4*-ORF [5,10], including exon 3. In healthy individuals, the array typically contains 11–100 repeat units and is embedded in a heavily methylated, heterochromatic environment that prevents *DUX4* expression (reviewed in Belayew et al. 2025) [4]. In FSHD1, contraction of this array to 1–10 units is associated with decreased methylation and local chromatin relaxation, thereby enabling *DUX4* transcription [20]. Beyond repeat size, the allelic background on which the contraction occurs is equally essential for pathogenicity. The 4qter region is characterized by two major alleles, 4qA and 4qB, which occur at comparable frequencies in the general population. However, only 4qA alleles are defined "permissive" for FSHD [3,10]. This permissibility is conferred by the presence, within the pLAM sequence distal to the last D4Z4 unit, of a functional polyadenylation signal (PAS) that stabilizes the *DUX4* transcript and expression [5,10]. By contrast, 4qB alleles, and the nearly identical D4Z4 arrays on chromosome 10q26, lack the PAS and are therefore non-permissive regardless of their repeat size. It should be noted that not all 4qA haplotypes are permissive. For example, the 4qA166 allele lacks a functional polyadenylation signal and is therefore considered non-permissive for pathogenic DUX4 expression [21]. The identification of a contracted D4Z4 array on a permissive 4qA allele, together with the resulting epigenetic relaxation of the locus, represents the cornerstone for defining a genetically permissive FSHD configuration and provides the basis upon which additional genetic and epigenetic modifiers shape disease pathophysiology (reviewed in Belayew et al. 2025) [4].

In most European cohorts, FSHD1 is classically associated with contraction of the D4Z4 array on a permissive 4qA allele to 1–10 repeat units, with smaller arrays generally correlating with earlier disease onset and greater clinical severity [16,22]. However, the upper part of this range requires cautious interpretation. Permissive alleles measuring 8–10 repeat units are detected in approximately 1–2% of control populations and are typically associated with lower penetrance [23,24]. The overlap between unaffected individuals and genetically confirmed FSHD1 cases within this repeat-size range has led to the definition of an FSHD1 "gray zone," reflecting the substantial degree of non-penetrance observed with larger D4Z4 arrays [16]. Consistent with this concept, non-penetrant heterozygous carriers are frequently identified among relatives of affected individuals. Notably, asymptomatic (i.e., individuals without subjective muscle weakness but with detectable FSHD-related signs on clinical examination) or non-penetrant heterozygous carriers (i.e., individuals without both symptoms and clinical signs) are more often female, suggesting that sex-related factors may further modulate disease expression in this context [18,23,25,26]. However, other studies did not observe significant sex-related

differences in penetrance [11,27]. In line with this variability, studies on early-onset FSHD cohorts have shown heterogeneous findings regarding sex distribution, with some studies reporting a predominance of male patients [11,28], while others did not observe a clear male-female skew [27,29]

Importantly, accumulating data indicate that D4Z4 thresholds may differ among different populations. Studies in East Asian cohorts (including Japanese and Korean individuals) have consistently reported a narrower distribution of pathogenic alleles, with most genetically confirmed FSHD cases carrying D4Z4 arrays in the 1–6 units range, and larger alleles being less frequently associated with disease [30,31]. More recent work in a cohort of Indian ancestry further supports this concept, revealing an allele-size distribution intermediate between European and East Asian cohorts, along with a higher proportion of asymptomatic or non-penetrant heterozygous carriers with 4 or 5 units [32]. A sensitive Southern blot-based approach using digoxigenin-labelled p13E-11 and 4qA-specific probes was employed to perform the first molecular characterization of D4Z4 alleles and haplotypes in FSHD patients from Latin America [33], revealing genotype-phenotype correlations comparable to those observed in European cohorts. FSHD has been only rarely reported in individuals of sub-Saharan African ancestry [34,35], despite the high prevalence of the permissive 4qA haplotype [36]. Notably, the few available reports are based on clinically diagnosed cases without genetic confirmation, and some described individuals displayed features atypical for FSHD. While limited access to healthcare and genetic testing may partly explain this discrepancy, additional genetic or epigenetic factors may modulate disease susceptibility in this population. Altogether, these observations suggest that population-specific genetic background is likely to influence both penetrance and expressivity, challenging the universal adoption of European-derived diagnostic cutoffs [16]. However, the interpretation of population-specific genetic background has to be approached with caution in clinical practice, as patient ancestry may be mixed or not formally recorded, and the parent of origin of the D4Z4 repeat array may remain unknown. Moreover, FSHD genetic diagnosis is also complicated by several complex conditions that include mosaicisms, translocations, D4Z4 proximal extended deletion (DPED) alleles and *cis*-rearrangements [37]. Mosaicism is well documented, particularly in apparently sporadic cases, and is due to post-zygotic D4Z4 contractions [38]. In these individuals, disease severity may depend not only on repeat size but also on the proportion of cells carrying the contracted allele [28]. Mosaicism remains clinically relevant because it can hinder segregation patterns and complicate genetic counseling. Additional complexity arises from hybrid 4q/10q configurations and DPED, which result from the high sequence identity between subtelomeric regions or from deletions of sequences immediately proximal to D4Z4 and may escape detection by conventional assays unless additional, locus-specific probes and a dedicated Southern blot are employed [16,39]. *Cis*-duplications of the D4Z4 array have been described in which two or more repeat arrays are present on the same 4qA chromosome [13,40]. These alleles may co-segregate either with a short 4qA allele or with a variant in *SMCHD1* [13,41,42]. In approximately 20% of patients, they segregate with the clinical features of FSHD, suggesting that they may be directly causative of the disease in an autosomal dominant manner, in the absence of a pathogenic variant in an FSHD2-associated gene [13,37,41].

Beyond the D4Z4 repeat contraction and a permissive 4qA allele, the chromatin structure at 4q35 is shaped by a set of epigenetic regulators whose disruption can contribute to *DUX4* expression. This mechanism underlies FSHD2, in which pathogenetic variants in *SMCHD1, DNMT3B* or *LRIF1* and unknown genes and parameters lead to global D4Z4 hypomethylation in combination with a D4Z4 repeat size of 8 to usually 20 repeat units. *SMCHD1* functions as a key chromatin repressor at the D4Z4 locus, and pathogenetic variants are associated with reduced methylation and somatic derepression of *DUX4* transcription [42,43]. *DNMT3B* is responsible for *de novo* methylation, and heterozygous variants have been shown to act as FSHD modifiers by impairing methylation establishment. This mechanism is further supported by recent functional evidence showing that specific *DNMT3B* splice alterations reduce catalytic activity and destabilize repression at D4Z4 [44,45]. *LRIF1* interacts with *SMCHD1* and *HP1* to maintain heterochromatin status. Biallelic variants in *LRIF1* have been shown to disrupt this interaction and facilitate *DUX4* activation, establishing *LRIF1* as an FSHD2 gene [46]. In addition, patients carrying a deletion of chromosome 18p may present with clinical features consistent with FSHD, when the deletion encompasses *SMCHD1*, in association with a permissive 4qA allele [47–50]. The recognition of SMCHD1, DNMT3B, and LRIF1 as key regulators of D4Z4 repression has refined the understanding of FSHD inheritance, particularly in individuals carrying borderline DRAs (8–10 units). In these cases, disease can arise from combination of a borderline DRA with a pathogenic variant in one of the FSHD2 genes, establishing a true digenic mechanism [43,51–54]. Such digenic FSHD2 configurations are frequently associated with greater clinical severity, highlighting the combined effect of the two genetic features on disease expression variability. Clinical data also show that pathogenic variants in FSHD2 genes can modulate disease expression across a broad spectrum of D4Z4 repeat sizes. In some patients, such variants occur in combination with a 4q35A D4Z4 allele larger than 20 units (considered non-pathogenic) and are generally associated with mild FSHD or asymptomatic status, suggesting that the 20-unit threshold should be interpreted with caution and in the context of the clinical phenotype [16,42,47]. In a recent study, variants in FSHD2 genes coexist with short arrays in the 4–7 units range, resulting in more severe presentations [42]. These observations demonstrate that FSHD2 genes can modulate an existing FSHD1 substrate but also can, when sufficiently disrupting D4Z4 repression, independently drive disease across a broad spectrum of repeat sizes on permissive 4qA alleles. Beyond established FSHD2 genes, candidate genes have emerged as additional disease modifiers from whole exome study, including rare variants in chromatin and DNA-methylation regulators such as *CTCF, DNMT1, DNMT3A, EZH2*, and *SUV39H1*, which may modulate penetrance and phenotypic variability through subtle effects on D4Z4 epigenetic stability [42]. Additional evidence has pointed to *FAT1* as a potential genetic modifier in individuals carrying contracted D4Z4 alleles [55]. Notably, *FAT1* loss of function variants have also been reported in patients negative for FSHD1 and FSHD2 genetic testing but presenting with an FSHD-like phenotype [56], suggesting a possible contribution of this gene as a modifier of DUX4 protein expression or toxicity. Furthermore, the relevance of these gene modifiers is highlighted by recent evidence showing that global D4Z4 methylation analysis substantially improves patient stratification, particularly in individuals who carry variants in FSHD2-related genes together with borderline or normal-sized alleles and who present with a wide range of clinical manifestations [42,47]. Such findings demonstrate that disease expression is not determined solely by D4Z4 repeat array size on a 4qA allele, but emerges from the interaction between the underlying repeat architecture and the epigenetic mechanisms that repress it (Fig. 1), a relationship that more accurately reflects the continuum of clinical variability observed in FSHD1 and FSHD2 [57].

3. Evolution of diagnostic strategies in FSHD: from classical to modern technologies

The molecular diagnosis of FSHD has developed under constraints imposed by a disease mechanism that cannot be captured by single-gene or sequence-based approaches alone. Accurate diagnosis has instead relied on methods capable of resolving large D4Z4 repeat arrays, subtelomeric configurations, and epigenetic states that directly influence gene expression. These requirements have limited a straightforward shift toward sequencing-based diagnostics, which explains why classical techniques have remained clinically relevant for decades, even as next-generation technologies have progressively reshaped molecular testing. Within this framework, the evolution of FSHD diagnostics can be viewed across three partially overlapping eras: a classical era dominated by

Southern blot-based approaches; an intermediate era characterized by targeted molecular refinements and the incorporation of methylation assays; and a modern era marked by genomic technologies that aim to provide more comprehensive and scalable solutions (Fig. 2).

3.1. The classical era: Southern blotting as a diagnostic cornerstone

The classical era of FSHD molecular diagnosis is linked to Southern blot-based analysis of the D4Z4 macrosatellite repeat. Following the FSHD linkage to chromosome 4q35, Southern blotting enabled, for the first time, the direct assessment of D4Z4 repeat array size via *Eco*RI digestion and hybridization with the p13E-11 probe that maps just centromeric of the D4Z4 repeat array, providing a practical readout of the underlying copy number variation that defines FSHD1 [18,58]. The subsequent introduction of double-digestion strategies using enzymes such as *Bln*I or *Hind*III represented a critical refinement, allowing discrimination between pathogenic 4q-derived arrays and the homologous, non-pathogenic D4Z4 repeats on chromosome 10q26 [59,60]. Accurate sizing of D4Z4 arrays posed an additional technical challenge, as disease-associated and normal alleles often span tens to hundreds of kilobases. Conventional Linear Gel Electrophoresis (LGE) permits reliable separation of DNA fragments up to approximately 40–50 kb. Moreover, the adoption of pulsed-field gel electrophoresis (PFGE), particularly in combination with high-molecular-weight DNA embedded in agarose plugs, enabled the resolution of much larger fragments, extending into the hundreds of kilobases. This advance proved essential for precise sizing of alleles for resolving high-molecular-weight fragments of both 4q and 10q origin, and for correctly assigning them in family trees, for detecting somatic mosaicism and 4q/10q translocations [59,60]. These approaches were instrumental in defining pathogenic size thresholds and in delineating the region of overlap between normal and disease-associated D4Z4 fragments at the upper end of the FSHD1 range [61].

Nevertheless, Southern blot-based diagnostics remain technically demanding, labor-intensive, require large amounts of high-quality DNA, and are vulnerable to false-negative results in the presence of non-canonical configurations, such as DPED alleles, that impair p13E-11 hybridization [62]. Despite these limitations, Southern blot also offers some practical advantages, including relatively low capital and operational costs and independence from proprietary platforms. Together its ability to directly interrogate repeat size and chromosomal origin explains why it has remained clinically relevant for decades and continues to serve as a reference framework against which newer diagnostic technologies are benchmarked.

3.2. The intermediate era: targeted molecular refinements and the incorporation of methylation assays

3.2.1. Advances in FSHD locus resolution and uncovering the digenic basis of FSHD2

As the limitations of Southern blot-based diagnostics were progressively recognized in clinical practice, a set of complementary approaches emerged with the specific aim of improving resolution at the 4q35 locus and better capturing the structural complexity of the D4Z4 region, while preserving established diagnostic criteria such as repeat array size, chromosomal origin, and allele permissivity. A key advance during this period was the introduction of molecular combing, a technique that enables direct visualization of multiple individual DNA molecules stretched on glass slides at 1 kb resolution, thereby allowing precise characterization of 4q- and 10q-derived D4Z4 alleles through hybridization with locus-specific probes [14]. Unlike Southern blotting, which infers repeat size from fragment migration, molecular combing allowed precise sizing of the D4Z4 array, unambiguous discrimination between chromosome 4- and chromosome 10-derived repeats, and direct identification of the permissive 4qA haplotype [14]. In addition to more precise D4Z4 array sizing and haplotype assignment, this approach has revealed non-canonical configurations that can be missed or remain ambiguous with conventional diagnostics, including somatic mosaicism, deletions affecting the p13E-11 probe region, and *cis*-duplications involving multiple D4Z4 arrays on the same chromosome [13,63,64]. Despite its high diagnostic resolution, the broader adoption of molecular combing has remained limited, largely due to the need for specialized

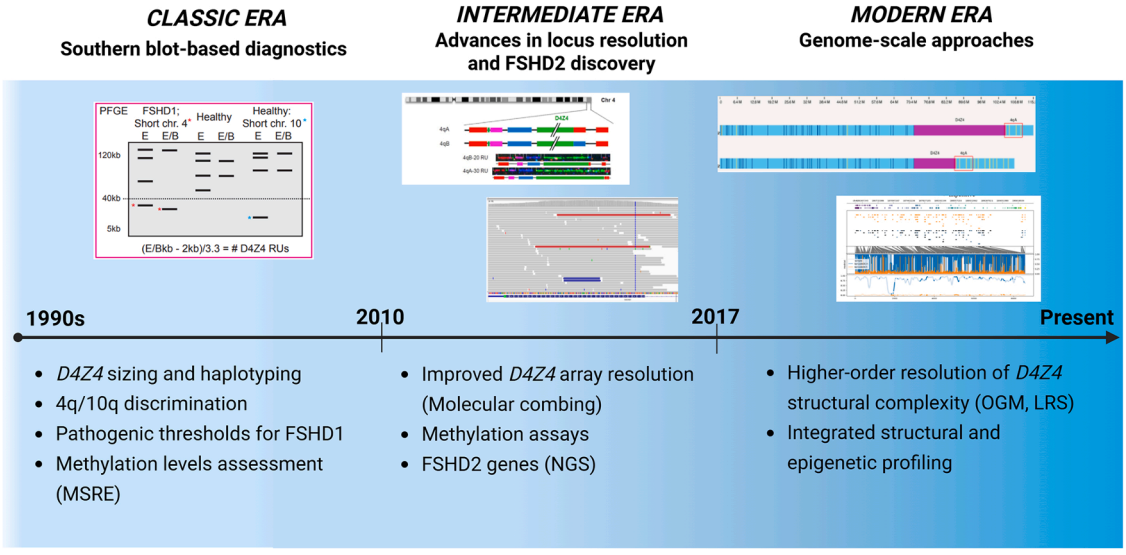

Fig. 2. Timeline of the evolution of molecular diagnostics in FSHD. Over the past three decades, diagnostic approaches to FSHD have progressed through distinct technological eras, each addressing emerging limitations of previous methods. The classical era established Southern blot-based assays for D4Z4 repeat array sizing, 4q/10q discrimination, pathogenic thresholds for FSHD1 and initial evaluation of methylation levels. The intermediate era improved locus resolution, introduced epigenetic assays, and enabled the identification and analysis of FSHD2-associated genes thanks to the advent of NGS. The modern era is characterized by genome-scale approaches that provide higher-resolution analysis of D4Z4 array structural complexity and support integrated structural and epigenetic profiling to improve diagnostic stratification. Overall, this evolution underscores that future diagnostic accuracy will rely on the integration of complementary molecular technologies with standardized clinical assessment and genotype-phenotype correlation, rather than on any single method alone. MSRE: methylation-sensitive restriction enzyme. NGS: Next Generation Sequencing. OGM: Optical Genome Mapping. LRS: Long-Read Sequencing. This figure has been partially adapted by Megalizzi et al. 2024 [17] and has been created with Biorender.

infrastructure and technical expertise, as well as challenges related to standardization and scalability in routine diagnostic settings, restricting its implementation to a small number of specialized laboratories.

This era also marked a conceptual transition in how FSHD genetics was interpreted. Rather than viewing the locus solely through the lens of repeat array contraction, these higher-resolution analyses emphasized the need to consider allele structure, chromosomal context, and rearrangement complexity as integral components of molecular diagnosis. In light of these considerations, molecular combing functioned as a bridge between classical fragment-based approaches and later genome-scale technologies, preserving continuity with established diagnostic criteria while expanding the range of detectable pathogenic configurations.

In parallel with advances in structural and epigenetic analyses, this intermediate phase also coincided with the introduction of sequencing approaches that enabled the discovery of FSHD2 digenic origin. Indeed, besides a shortened D4Z4 repeat array on a 4qA allele allowing for DUX4 protein expression, the discovery of pathogenic variants in *SMCHD1* (involved in DNA methylation/chromatin structure) through next generation sequencing (NGS) provided direct genetic evidence linking defects in epigenetic repression of D4Z4 to disease expression [54]. Subsequent application of NGS in selected cohorts further expanded this framework by implicating additional epigenetic regulators (such as *DNMT3B* and *LRIF1*) involved in D4Z4 silencing [42,45,46]. At the same time, NGS emerged as a powerful diagnostic tool for disentangling complex or atypical FSHD presentations, particularly by resolving genotype-phenotype discordance, uncovering concomitant or modifying genetic variants, or pointing to alternative diagnoses [65m66]. This approach complemented D4Z4 repeat-based diagnostics, reinforcing the concept that accurate molecular diagnosis requires integration of structural, epigenetic, and sequence-level information.

Finally, this era also marked the formal incorporation of D4Z4 methylation analysis into the diagnostic workflow, establishing epigenetic profiling as a complementary tool for interpreting borderline alleles and identifying FSHD2-related configurations.

3.2.2. *Epigenetic profiling in FSHD diagnostics: D4Z4 methylation analysis*

As understanding of FSHD pathogenesis matured, it became increasingly evident that structural characterization of the D4Z4 array alone was insufficient to fully explain the clinical heterogeneity of disease. A unifying feature across both FSHD1 and FSHD2 is the epigenetic relaxation of a D4Z4 repeat array on 4qA, leading to the recognition of DNA methylation as a functional marker of locus repression [67]. Early studies showed that CpG methylation across the D4Z4 array is heterogeneous and influenced by repeat length and chromatin context, highlighting the importance of epigenetic organization in regulating the functional state of the locus [68].

Initial diagnostic approaches relied on Southern blot-based methylation-sensitive restriction analyses, which revealed that FSHD1 is associated with partial hypomethylation of the contracted 4q35 array, whereas FSHD2 displays a more generalized hypomethylation affecting all D4Z4 arrays [67,69]. These observations established DNA methylation as a discriminating feature between FSHD subtypes and laid the groundwork for more quantitative assays. Subsequent bisulfite-based methods enabled higher-resolution analysis of specific D4Z4 regions, with some of them confirming that methylation levels often inversely correlate with repeat size and are particularly informative in individuals carrying borderline arrays [70–72]. Technical refinements to methylation assays have progressively improved their diagnostic utility. Targeted bisulfite sequencing approaches demonstrated that, when correctly designed to distinguish 4q-derived repeats, methylation profiling can reliably differentiate healthy individuals from patients with FSHD1 and FSHD2, even using non-invasive DNA sources [73–76]. In addition, some works showed that selected CpG sites within D4Z4 carry strong discriminative power and machine learning-assisted pipelines have been applied to methylation datasets to improve classification accuracy and support patient prioritization in diagnostic workflows [77,78]. From a clinical perspective, recent large-cohort studies have demonstrated that D4Z4 methylation levels contribute to patient stratification and correlate with disease status across both FSHD1 and FSHD2. High-throughput methylation profiling has shown strong concordance with conventional genetic testing while providing additional resolution in atypical or inconclusive cases, supporting its integration into routine diagnostic practice [27,70,79,80].

Altogether, these studies position D4Z4 methylation analysis as a pivotal component of contemporary FSHD diagnostics. By capturing the epigenetic dimension of the disease, methylation profiling provides a critical layer of interpretation that refines diagnosis, supports genotype-phenotype correlations, and sets the stage for integrated, multi-modal diagnostic strategies [17].

3.3. *The modern era: genome-scale resolution of the FSHD locus*

The most recent phase in FSHD molecular diagnostics is characterized by the advent of novel technologies able to directly resolve the full structural complexity of the D4Z4 locus. Unlike earlier approaches, which relied on indirect inference or targeted interrogation of selected features, optical genome mapping (OGM) and long-read sequencing (LRS) provide a comprehensive view of repeat numbers, chromosomal origins, haplotype configurations, and structural features within a single analytical framework. Although based on distinct technical principles, these technologies share the ability to span hundreds of kilobases, overcoming the intrinsic limitations imposed by the repetitive and subtelomeric nature of the 4q35 region. Together, they mark a transition toward direct, molecule-level characterization of the FSHD locus.

Unlike Southern blot-based approaches, OGM does not infer D4Z4 repeat size indirectly from fragment migration; instead, it visualises individual DNA molecules labelled at sequence-specific motifs and aligned to a reference genome map. This allows accurate quantification of D4Z4 repeat number (typically within ± 1 repeat unit), discrimination between chromosome 4q35 and the homologous 10q26 locus, and determination of 4qA/4qB haplotypes within the same experiment. Multiple validation studies have demonstrated strong concordance between OGM-derived repeat sizing and Southern blot-based analysis, demonstrating its accuracy and high reproducibility for FSHD diagnosis [81–85]. Importantly, OGM has proven particularly informative in complex genetic contexts. The technology reliably detects postzygotic mosaicism, cis-duplications, and other atypical D4Z4 configurations that may be missed or ambiguously interpreted by Southern blotting [81,86]. Several cohorts have shown that low-level mosaic contractions and alleles can be readily identified by OGM, providing molecular explanations for intrafamilial variability and apparently discordant genotypes [84,87]. Nevertheless, recent work has also emphasized the need for cautious interpretation in selected scenarios [37]. Highly complex rearrangements, such as 4q/10q translocations, may still challenge OGM when used alone, thereby requiring complementary analyses. This has led to the proposal of integrated diagnostic strategies in which OGM serves as a first-line approach, complemented by Southern blotting or additional methods when complex structural configurations are suspected [88]. Beyond postnatal diagnostics, OGM has also been successfully applied in prenatal and familial settings, where its ability to rapidly and accurately resolve the D4Z4 locus, combined with linkage-based approaches, offers clear advantages over traditional techniques that require large amounts of high-quality DNA and extended turnaround times [89].

LRS technologies enable direct sequencing across the D4Z4 repeat array, overcoming long-standing technical barriers imposed by repeat length and sequence homology. Early proof-of-concept work demonstrated that Oxford nanopore sequencing could successfully cover the entire D4Z4 array and its flanking regions [90]. Using ultra-long reads, this initial study showed that complete D4Z4 arrays, including the terminal repeat harboring *DUX4* and the pLAM region with the PAS, could be sequenced with high accuracy, providing the first nucleotide-level

view of the pathogenic locus. Recent developments have focused on improving feasibility and diagnostic yield through targeted enrichment strategies, including CRISPR/Cas9-based approaches, which selectively isolate the D4Z4 array prior to sequencing. These refinements have enabled reliable repeat sizing, unambiguous discrimination between 4q and 10q arrays, and accurate assignment of permissive haplotypes within a single assay, with high concordance with Southern blotting and OGM [91–93]. A distinctive advantage of nanopore-based LRS is the ability to detect DNA methylation together with D4Z4 array sizing and haplotyping. Multiple studies have leveraged this property to simultaneously assess D4Z4 repeat structure and CpG methylation patterns, revealing consistent hypomethylation of contracted D4Z4 arrays in FSHD1 and broader hypomethylation profiles affecting both 4q35 and 10q26 arrays in FSHD2. Importantly, these analyses have shown that methylation patterns can be resolved at the level of individual repeats and alleles, capturing heterogeneity that is obscured in bulk methylation assays [91,93,94]. As a key feature of all 4q35 D4Z4 repeat arrays, an asymmetric methylation gradient has been identified, linking genetics, epigenetics and methylation in FSHD. It increases from the proximal to the most distal repeat unit and reaches saturation after approximately 10 repeat units in the absence of pathogenic variants in FSHD2 epigenetic-associated genes. This might rationalize the known threshold in FSHD1 and confirms the validity of methylation within the most distal repeat unit (where the expressed *DUX4* gene maps) as a biomarker for overall disease status. In FSHD2, the slope of the methylation gradient is less steep reaching high distal methylation for a larger number of D4Z4 units explaining the higher threshold of repeat array length in FSHD2. Whole-genome nanopore sequencing has also been shown to enable D4Z4 sizing while also allowing the detection of sequence variants in FSHD2-associated genes [95]. This convergence of structural, epigenetic, and sequence-level data within a single workflow is particularly valuable in complex or atypical cases that are difficult to resolve using classical methods alone. To date, most LRS studies in FSHD have relied on Oxford Nanopore sequencing because its ultra-long reads can span entire D4Z4 arrays. PacBio HiFi sequencing has also been explored [96], although the typical read length obtained (~30 kb) limits full-span reconstruction of long or structurally complex D4Z4 arrays. Recently, dedicated bioinformatic pipelines (e.g., Kivvi) have been proposed to reconstruct D4Z4 alleles, haplotype and methylation levels from PacBio HiFi whole-genome sequencing data, although additional studies are necessary to validate this approach for FSHD testing.

LRS approaches have also been applied in prenatal and familial contexts, where limited DNA availability and the need for rapid, unambiguous allele assignment pose significant challenges. In these settings, nanopore sequencing has demonstrated the ability to accurately resolve repeat number, haplotype configuration, and inheritance patterns, offering an alternative to labor-intensive and time-consuming traditional assays [97,98]. Despite these strengths, LRS remains constrained by practical considerations, including the need for specialized expertise, computational resources, and, in many cases, targeted enrichment to ensure sufficient coverage of the D4Z4 locus, especially for the detection of larger alleles, mosaic configurations or *cis*-duplications.

Altogether, OGM and LRS represent a shift toward direct, long-range characterization of the FSHD locus, providing complementary solutions to challenges that have historically limited molecular diagnosis. From a laboratory perspective, this evolution implies a shift from single-assay diagnostics toward integrated, decision-based workflows, where technology choice is guided by the clinical question rather than availability alone.

4. Emerging need for clinical trials and improved clinical and molecular characterization

The growing number of interventional studies targeting the *DUX4* pathway has brought FSHD into a phase in which clinical trial readiness is tightly linked to molecular precision. Unlike earlier trials based on non-specific anabolic or anti-inflammatory strategies, current therapeutic approaches aim to interfere directly with *DUX4* expression or its downstream consequences, thereby highlighting the importance of robust molecular stratification at trial entry [99]. Several reviews and consensus efforts have emphasized that the intrinsic heterogeneity of FSHD represents a major obstacle for trial design and outcome interpretation. Natural history studies have consistently shown slow and variable disease progression over time, with limited sensitivity of traditional clinical outcome measures within the typical duration of interventional trials [100]. This has reinforced the need to refine inclusion criteria and enrich study cohorts for individuals in whom the pathogenic mechanism is most likely to be active and modifiable. Large international trial-readiness initiatives have highlighted the growing importance of molecular characterization in the design of future FSHD trials [101]. In the context of *DUX4*-targeted therapies, this has brought into focus the limitations of traditional diagnostic definitions when applied to trial enrollment: reliance on D4Z4 repeat array size alone, while historically adequate for clinical diagnosis, may be insufficient to identify biologically homogeneous study populations. Importantly, individuals carrying permissive 4qA alleles within the 8–10 D4Z4 repeat "gray zone" may be genetically classified as FSHD but may not uniformly exhibit molecular features consistent with active *DUX4* derepression, with important implications for patient stratification and outcome interpretation in *DUX4*-targeted clinical trials. Consequently, recent trial-readiness efforts advocate integrating genetic, epigenetic, and functional biomarkers to guide patient selection and stratification, aiming to reduce heterogeneity and improve the interpretability of trial outcomes [15,102]. On the other end, from the patient community's perspective, the increasing selectivity of trial eligibility criteria underscores the importance of transparency and clear communication around trial access and decision-making processes [103].

As FSHD clinical trials move toward pathway-targeted interventions, the identification and validation of robust biomarkers have become key components of trial readiness, supporting patient stratification, assessment of disease activity, and evaluation of therapeutic response beyond conventional clinical endpoints [99]. Among them, imaging biomarkers have emerged as a particularly valuable component of trial readiness in FSHD, providing objective and reproducible measures of muscle involvement that complement genetic and molecular stratification [104]. Muscle MRI studies have consistently shown that patterns of fat replacement, STIR hyperintensity, and selective muscle sparing reflect disease activity and progression, often preceding detectable changes in strength or functional outcomes [105,106]. Quantitative MRI approaches, including fat fraction mapping and longitudinal assessments, have demonstrated sensitivity to change over relatively short time frames, an essential requirement for interventional trials [106–108]. In addition, advanced imaging strategies, such as whole-body MRI and machine learning-based pattern recognition have improved the ability to capture disease heterogeneity and to support patients' stratification, particularly in atypical or early-stage cases [107,108–111]. Together, these data support muscle MRI as a robust, non-invasive biomarker to monitor disease activity and progression in FSHD clinical trials. Transcriptomic and proteomic biomarkers have been extensively explored as complementary tools to capture downstream consequences of *DUX4* activity and disease-related molecular states in FSHD. Transcriptomic studies in muscle biopsies have consistently identified disease-associated gene expression signatures, including activation of *DUX4* target genes and repression of *PAX7*-regulated transcriptional targets, which together reflect key pathogenic processes despite the sporadic expression of *DUX4* in affected muscle tissue [112,113].

Proteomic approaches have further refined this picture by revealing alterations that are not fully predicted at the transcript level. Analyses of muscle tissue, cultured myogenic cells, and interstitial fluids have uncovered consistent alterations in pathways related to mitochondrial function, oxidative stress, inflammation, and protein turnover,

highlighting post-transcriptional mechanisms as important contributors to FSHD pathology [114–116]. Additional studies have also explored circulating proteins and cytokines as minimally invasive biomarkers, identifying candidates associated with disease activity or severity [117–120].

Together, transcriptomic and proteomic data support a model in which molecular biomarkers capture complementary aspects of FSHD biology, reinforcing their potential value for patient stratification and for monitoring molecular responses when interpreted alongside genetic and imaging-based measures [112,116]. However, transcriptomic or proteomic analyses generally rely on muscle biopsy. But the rare and stochastic *DUX4* expression and its rapid protein turnover in FSHD muscles complicate the selection of a spot for biopsy, even though MRI-guided approaches could target zones of inflammation corresponding to active DUX4 expression [112]. Moreover, the invasive nature of this procedure limits its use for repeated measurements in longitudinal studies. Notably, some proteomic studies have explored less invasive biological samples such as blood samples, potentially offering alternative strategies for biomarker monitoring.

5. Ethical and counseling considerations in a complex disease

The expanding molecular characterization of FSHD has brought substantial benefits for diagnosis and trial readiness, but it has also amplified ethical and counseling challenges that are deeply shaped by the marked clinical variability of the disease. Age at onset, rate of progression, and lifetime disease burden vary widely, even among individuals carrying similar genetic configurations, complicating prognostic discussions and reproductive decision-making [16,121].

This variability directly affects reproductive counselling. In FSHD1, offspring of an affected individual carrying a pathogenic D4Z4 contraction have a 50% probability of inheriting the permissive allele, although disease expression remains variable. In contrast, in digenic configurations involving a permissive D4Z4 allele and a pathogenic variant in an FSHD2-associated gene, transmission risk depends on the independent segregation of the two genetic components. For FSHD1, options such as prenatal diagnosis and preimplantation genetic testing (PGT) are technically feasible, yet their application requires careful consideration. The presence of reduced penetrance, sex-related factors, ethnicity and a gray-zone repeat range means that identifying a permissive D4Z4 allele does not allow direct prediction of disease severity or functional outcome in offspring. Although sex-related differences in disease expression have been reported, fetal sex alone cannot be used to predict clinical outcome or guide decision-making in PND/PGT. Counseling therefore must explicitly address uncertainty, avoiding deterministic interpretations while supporting informed and autonomous choices [121]. For FSHD2, the situation is even more complex, as digenic inheritance and the contribution of epigenetic modifiers limit the reliability of predictive testing and currently constrain the use of PGT to selected scenarios within specialized centers. Considering that relatively few centers worldwide currently provide PGT or PND for FSHD, close collaboration between diagnostic laboratories and centers offering reproductive genetic services is crucial to support appropriate patient counseling and referral. An additional ethical challenge arises from the increasing identification of variants of uncertain significance (VUS) in FSHD2-associated genes and very limited access to functional variant analysis in most diagnostic centers. As sequencing-based approaches become more widely adopted, these findings are likely to increase. The interpretation of such variants requires cautious communication, emphasizing their probabilistic nature and the current limits of knowledge, to avoid unnecessary anxiety or reproductive decisions based on incomplete information [16]. This complexity is further illustrated by unusual but clinically relevant scenarios, such as 18p deletions encompassing *SMCHD1* in combination with a permissive 4qA allele, which have been associated with FSHD phenotype [47,49,50].

Beyond genetics, patient-centered perspectives highlight dimensions of FSHD that molecular data alone cannot capture. Qualitative studies and systematic reviews consistently show that patients experience the disease as unpredictable and often socially visible, particularly due to facial weakness and asymmetry, with significant psychosocial consequences [122,123]. Patient-reported outcome studies further demonstrate that perceived disease burden, fatigue, pain, and loss of independence are central concerns influencing life planning, participation in clinical trials, and attitudes toward emerging therapies [103]. These observations underscore the need for counseling frameworks that integrate molecular findings with lived experience. Genetic counseling in FSHD should therefore be longitudinal and multidisciplinary rather than episodic and laboratory-centered, and explicitly focused on patient values, expectations, and informational preferences [124]. As therapeutic options targeting *DUX4* move closer to clinical application, ethical practice will increasingly depend on aligning genetic complexity with transparent communication and shared decision-making. In this context, harmonized guidelines and patient-informed outcome measures represent not only technical tools, but also ethical instruments to ensure that advances in molecular diagnostics translate into care that is both scientifically robust and responsive to the needs of individuals and families living with FSHD.

6. Harmonization of diagnostic pathways and a global health framework for FSHD

Despite major advances in the molecular understanding of FSHD, their implementation in diagnostic practice remains uneven across centers and healthcare systems. One contributing factor is the organizational separation between laboratories performing molecular analyses and clinical teams responsible for longitudinal patient care. As a result, genetic findings are not always interpreted within a fully integrated clinical and imaging context, which can complicate interpretation in diagnostically challenging scenarios, such as borderline alleles or in the presence of modifier variants. This heterogeneity is further amplified by differences in available technologies (Table 1) across centers. While some reference laboratories have transitioned toward OGM-based workflows, others still rely primarily on Southern blot-based workflows, whereas LRS is currently used mainly for research. In this context, the coexistence of multiple diagnostic platforms underscores the growing need for harmonized, multilayered diagnostic strategies that improve consistency and comparability of results across centers [17,125].

Patient registries have emerged as a critical infrastructure to support such harmonization. Large national initiatives in France, Italy, Netherlands, Japan, United Kingdom have demonstrated how standardized collection of molecular, clinical, and functional data enables robust genotype-phenotype correlations and improves diagnostic interpretation on a broad population level [125–129]. Of major interest for clinical trial readiness, the Australian registry sponsored by the “FSHD Global” patient association also includes a yearly MRI with AI-derived fat infiltration profile for individual muscles that could be used to predict disease progression [130]. Moreover, international initiatives such as Project Mercury aim to contribute to trial readiness by promoting alignment across patient registries, shared data standards, and common stratification frameworks, facilitating comparability and interoperability across countries and clinical networks. Beyond high-volume centers, the creation of network-based infrastructures can be crucial in supporting laboratories and clinics with limited local expertise or access to advanced technologies [17]. Federated learning approaches, discussed in Europe within the European Health Data Space (EHDS), but conceptually applicable beyond regional boundaries, enable collaborative analysis of genomic and clinical data without requiring centralization of sensitive information. In these models, data remain locally stored and governed, while shared analytical frameworks allow collective learning across centers. Such approaches are particularly relevant for rare diseases like FSHD, where distributed expertise and limited case

Table 1

Comparative overview of molecular methods used in FSHD testing. The table summarizes the main diagnostic capabilities of the molecular approaches currently available for the analysis of the D4Z4 locus, FSHD-related genes and methylation analysis. Methods are compared according to their ability to size the D4Z4 repeat array, detect structural configurations (e.g., mosaicism, hybrid alleles, and cis-duplications), determine haplotypes, identify variants in FSHD2-associated genes, and their applicability in clinical contexts such as prenatal diagnosis and preimplantation genetic testing. Capabilities are indicated as ✓ (method supports the analysis) and × (method does not support the analysis). Relative cost estimates are indicative and may vary depending on laboratory implementation. LGE-SB: Linear Gel Electrophoresis Southern Blot; PFGE-SB: Pulsed-Field Gel Electrophoresis Southern Blot; OGM: Optical Genome Mapping; LRS: Long-read sequencing; WES: Whole-Exome Sequencing; WGS: Whole-Genome Sequencing; MSRE: Methylation-Sensitive Restriction Enzyme Assay; BSS, Bisulfite Sequencing; DPED: D4Z4 Proximal Extended Deletion; SSLP: Simple Sequence Length Polymorphism; PGT: Preimplantation Genetic Testing; PND: Prenatal Diagnosis.

Method Attributes	LGE-SB	PFGE-SB	Linkage marker PCR	Molecular combing	OGM	LRS	Targeted NGS/ WES	WGS	MSRE	Targeted BSS
Sizing of D4Z4 repeat <11 U (FSHD1)	✓	✓	✓°	✓	✓	✓	×	×	×	×
Sizing of D4Z4 repeat >11–20 U or larger	×	✓	✓°	✓	✓	✓	×	×	×	×
Detection of mosaicism	✓#	✓#	×	✓	✓	✓	×	×	×	×
Detection of a hybrid D4Z4 array	✓	✓	×	×	×	✓	×	×	×	×
Detection of cis-duplication alleles	×	×	×	✓	✓*	✓	×	×	×	×
Detection of DPED alleles	✓	✓	×	✓	✓	✓	×	×	×	×
4qA/B haplotyping	✓	✓	✓	✓	✓	✓	×	✓	×	✓**
SSLP analysis	×	×	×	×	×	✓	×	✓	×	×
Identification of SNV in coding regions of FSHD2 genes	×	×	×	×	×	✓	✓	✓	×	×
Identification of CNV in coding regions of FSHD2 genes	×	×	×	×	✓	✓	✓	✓	×	×
Methylation analysis	×	×	×	×	×	✓	×	×	✓	✓
Applicable in PND	✓	✓	✓	✓	✓	✓	✓	✓	×	×
Applicable in PGT	×	×	✓	×	×	×	✓	✓	×	×
Relative cost per test	low	low	low	moderate-high	high	high	moderate	moderate	low	low-moderate

* only by manual inspection.
** depending on the method.
\# only for mosaicism>15–20%.
° provides indirect evidence; not a direct measurement of repeat size or haplotype.

numbers at individual sites require cross-center interpretation while respecting regulatory and privacy constraints [131].

Structured disease-specific databases further strengthen this ecosystem by enabling standardized variant annotation, longitudinal outcome tracking, and cross-population comparisons. The integration of clinical outcome measures, such as the FSHD-Health Index, now validated in multiple languages, illustrates how harmonized phenotyping can complement molecular data and facilitate both research and trial readiness on a global scale [101,132–134].

Taken together, these efforts point toward a model in which reference centers do not function as isolated hubs, but rather as interconnected nodes within a global network. In such a framework, harmonized diagnostic standards, federated data infrastructures, and shared registries collectively reduce diagnostic discordance, enhance interpretation of rare variants, and promote equitable access to precision diagnostics for FSHD across diverse healthcare settings. However, the concept of harmonization must be framed within the acknowledgement that large regions of the world still lack access to advanced molecular diagnostic technologies for FSHD. In many countries, including most of Latin America, and Africa, diagnostic capacity remains limited to “classical-era” approaches, which are often available only in a small number of specialized laboratories, resulting in significant logistical, economic, and temporal barriers to diagnosis, often at prohibitive cost. In some regions, however, access to FSHD molecular diagnostics is currently absent altogether, and clinicians and patients often depend on international collaborations or commercial laboratories abroad to obtain a confirmed diagnosis. In these settings, genetic counseling and diagnostic orientation still rely predominantly on clinical expertise, frequently accompanied by uncertainty in the interpretation of borderline or atypical presentations. True global harmonization should therefore be understood as a dynamic and long-term objective rather than a uniform, technology-driven endpoint. In the long term, the goal should be to ensure that every patient, regardless of geographical location, can be characterized using the most appropriate and informative technologies for their specific clinical and genetic context, as determined through expert genetic counseling and case-driven diagnostic decision-making. In the short term, however, harmonization efforts must prioritize equitable access to diagnostic testing itself, ensuring that all patients can obtain a reliable molecular diagnosis, even in the presence of substantial differences in technological availability, healthcare policies, financial resources, and organizational infrastructures across countries.

Within this framework, harmonization should not imply uniformity of methods, but rather comparability of outcomes, achieved through shared interpretative standards, tiered diagnostic algorithms, referral networks, and collaborative support models that allow centers with limited resources to participate meaningfully in global diagnostic and research ecosystems.

7. Conclusions

FSHD exemplifies how the evolution of molecular diagnostics does not necessarily follow a linear replacement of old technologies with new ones, but rather a progressive integration driven by biological complexity. From Southern blot-based approaches to OGM, LRS and epigenetic profiling, the diagnostic trajectory of FSHD reflects the need to resolve repetitive genomic structures, subtelomeric configurations, and regulatory states that cannot be captured by sequence-based methods alone, now further supported by advances in genome assemblies such as the recent telomere-to-telomere (T2T) release. Each technological era has contributed complementary insights, shaping a diagnostic framework that is progressively integrated rather than sequential.

The present review highlights how the increasing depth of molecular characterization has brought clear benefits for diagnosis, trial readiness, and therapeutic development, while simultaneously exposing the limits of isolated or technology-centered approaches. The interpretation of borderline alleles, complex rearrangements, epigenetic modifiers, and

rare genetic variants requires coordinated use of multiple methodologies and careful integration with clinical, imaging, and molecular biomarkers.

While the *DUX4*-centered model remains the most robust, experimentally supported, and widely accepted framework for FSHD pathogenesis and provides a solid explanation for the core pathogenic process, the existence of atypical or complex rearrangements, unusual molecular configurations, and FSHD-like phenotypes supports the notion that additional biological mechanisms may operate alongside this central axis. These mechanisms could act independently of, in parallel with, or downstream of *DUX4*, contributing to disease modulation, progression, and phenotypic variability. The systematic investigation of these exceptional and complex contexts should therefore be viewed not as an alternative to the *DUX4* paradigm, but as an opportunity to identify additional components (genetic, epigenetic, or regulatory) of the disease process that may complement or amplify *DUX4*-mediated pathology. Clarifying these factors may add critical pieces to the pathogenic puzzle of FSHD and could have important implications for therapeutic research, particularly in the development of strategies that address disease modifiers, secondary pathways, or *DUX4*-independent contributors to muscle pathology.

In this context, FSHD underscores the importance of moving beyond single-parameter definitions toward multilayer diagnostic models and can be viewed as a paradigm for rare disease diagnostics in the post-genomic era. Achieving this level of integration cannot rely on individual centers, but requires international collaboration, harmonized diagnostic strategies, and network-based infrastructures. Federated learning approaches and structured disease registries provide scalable mechanisms to support shared interpretation, population-specific analyses, and access to expertise, particularly for rare or complex cases and for centers with limited local resources. More broadly, this evolution reflects a transition from isolated, center- or country-specific diagnostic practices toward a true diagnostic ecosystem, built on collaboration across laboratories, healthcare systems, and countries. Such an ecosystem acknowledges differences in technological availability and health policies, while enabling continuous refinement of genotype-phenotype correlations and ensuring that advances in genomics translate into both scientific progress and equitable access to precision diagnostics for individuals affected by FSHD.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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