



The FSHD jigsaw: are we placing the tiles in the right position?

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Purpose of review

Facioscapulohumeral muscular dystrophy (FSHD) is one of the most common myopathies, involving over 870,000 people worldwide and over 20 FSHD national registries. Our purpose was to summarize the main objectives of the scientific community on this topic and the moving trajectories of research from the past to the present.

Recent findings

To date, research is mainly oriented toward deciphering the molecular and pathogenetic basis of the disease by investigating DUX4-mediated muscle alterations. Accordingly, FSHD drug development has been escalating in the last years in an attempt to silence DUX4 or to block its downstream effectors. Breakthroughs in the field include the awareness that new biomarkers and outcome measures are required for tracking disease progression and patient stratification. The need to develop personalized therapeutic strategies is also crucial according to the phenotypic variability observed in FSHD subjects.

Summary

We analysed 121 literature reports published between 2021 and 2023 to assess the most recent advances in FSHD clinical and molecular research.

Keywords

biomarkers, clinical phenotype, DUX4, epigenetics, facioscapulohumeral muscular dystrophy, magnetic resonance imaging, therapeutic approaches

INTRODUCTION

Facioscapulohumeral muscular dystrophy (FSHD) (MIM 158900), one of the most common myopathies, has been considered a fully penetrant autosomal dominant disease associated with deletions of integral copies of the tandemly arrayed D4Z4 repeat at chromosome 4q35 [1]. FSHD is the only human disease causally linked to Copy Number Variation of macrosatellite deoxyribonucleic acid (DNA) elements [2]. At present, the pathogenesis of FSHD1, accounting for about 95% of cases, is explained by a model that involves the loss of epigenetic silencing and aberrant expression of *DUX4*, a retrogene embedded within the D4Z4 array [3]. In rarer FSHD2 cases, *DUX4* expression is associated with global CpG reduced methylation of the D4Z4 array that is usually caused by defects in genes encoding for proteins involved in epigenetic suppression, that is, the structural maintenance of chromosomes flexible hinge domain containing 1 gene (*SMCHD1*) [4], the methyltransferase 3B gene (*DNMT3B*) [5] and the ligand-dependent nuclear receptor interacting factor 1 gene (*LRIF1*) [6,7],

Although the etiology of the disease has been attributed to gain-of-toxic function stemming from the aberrant expression of *DUX4*, the exact pathogenic mechanisms involved in muscle wasting remain to be elucidated. This is because clinical and epidemiological data do not mirror the idea of a Mendelian disease in terms of penetrance and inter/

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KEY POINTS

- Magnetic resonance imaging-based Imaging and circulating biomarkers are investigated to identify specific outcome measures in FSHD
- Different potential interventional approaches have been studied relying on drugs targeting DUX4 regulation and production, or DUX4-signaling effector genes
- Clinical research suffers from the lack of studies assessing the natural history of FSHD in the last years
- The wide clinical variability observed in carriers of D4Z4 reduced allele suggests the existence of significant subgroups within the FSHD population.
- Patient heterogeneity may account, at least partly, for conflicting results across clinical studies; a standardised clinical evaluation is needed to support patient stratification.

intra familiar phenotypic variability [8–14]. Several intersections are observed between molecular data and various clinical phenotypes, including healthy people carrying the same molecular signature as affected individuals [15–17]. Therefore, it is getting clearer that the number of D4Z4 repeats at 4q35 or the *DUX4* misexpression do not *per se* fully characterize FSHD, and increasing efforts should be implemented to elucidate additional molecular or clinical features which could help patient stratification and promote the understanding of disease pathogenesis.

Here we examine the publications regarding FSHD over the last 18 months. The analysis was accomplished using search engines including Pubmed/Medline, Web of Science and Scopus, and

generic keywords such as FSHD, 4q35 locus, FSHD treatment, FSHD models or more specific terms like DUX4, 4q35 methylation, epigenetics.

RESULTS

Overview of FSHD literature reports

Using the term FSHD, we selected 121 literature reports from July 2021 to January 2023. We subdivided them based on their type: reviews, meeting reports or research articles, and on the examined topics: clinical and epidemiological reports, clinical trial results, basic molecular research and therapeutics development. Figure 1A summarizes the number and the distribution of the selected papers: the large majority of publications regard basic research and clinical studies, 35.04% and 38.46%, respectively; reviews constitute 13.2% of all reports. Research on new potential therapeutic approaches occupies 3.42% and 10.26% of the whole.

In this time window, clinical research was mainly oriented on defining outcome measures to trace disease progression, such as improving muscle imaging by magnetic resonance imaging (MRI) or developing specific outcome measures. Efforts also aimed at exploring imaging and molecular biomarkers for their diagnostic and prognostic potential. Basic molecular research in the field was almost completely centered on DUX4 function and the evaluation of new genomic sequencing methods that could support the diagnostic process, including CpG methylation analyses. Figure 1B shows the literature trajectories over the years. From 1985 FSHD basic research has moved from the genetic and linkage analysis of 4q35 locus to the development of the DUX4-centered model, while the

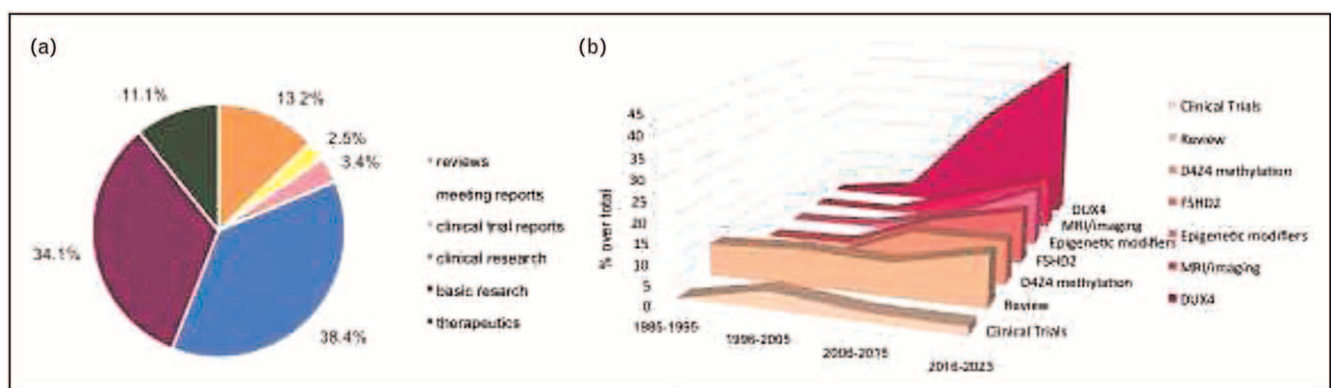


FIGURE 1. Main topics and trajectories of FSHD literature reports in the last 18 months. A. Pie chart representing the distribution of the selected papers as a percentage over the total ($n = 121$) based on the keywords reported in figure legend; B FSHD research trajectories over time. Data are plotted as a percentage of the total at different time points. Analysis was performed by considering 10-year timeframes between the first publication and today. Keywords are reported in the figure legend. FSHD, facioscapulohumeral muscular dystrophy.

clinical approaches left away epidemiology and classical genotype/phenotype correlation in favor of patient stratification. based on MRI and proteomics. The percentage of promising clinical trials over the years remained unchanged.

Clinical studies: are we looking in the right and same directions?

Over the last decade, the FSHD clinical field has focused on identifying disease biomarkers. This is because improved knowledge of the molecular basis of FSHD and results achieved in preclinical studies

have fostered the development of new therapeutic strategies. Table 1 summarizes the main reports focused on prospective and retrospective clinical studies, with particular attention to the type of enrollment and the methodology applied for the clinical data collection, which might have a crucial role in the analysis. In the time window of this review, only a few studies investigated FSHD natural history and clinical variability, whereas the application of patient-reported outcome measures has expanded [18–20]. Two studies, one perspective regarding the US FSHD population and the other retrospective on Chinese cases, analyzed several

Table 1. Synopsis of the analysis and comparison of the major literature reports focused on clinical research in FSHD.

| Article | Type | Participants | Follow up duration | Data collection duration | Outcome measures | Clinical evaluation during follow-up | Study enrollment |
|--|---------------------------|--|------------------------------|--------------------------|---|---|------------------------------|
| Katz <i>et al.</i> Brain. 2021 [18] | prospective | DRA <i>n</i> = 578 | 9 years average | 2002–2019 | Progression to wheelchair use | Self-reporting annual surveys | Registry based ^a |
| Wang <i>et al.</i> , Lancet Reg Health West Pac. 2021 [19] | prospective | DRA <i>n</i> = 1744; FSHD1 <i>n</i> = 971 | 10.5 years average | 2001–2020 | Clinical evaluation-muscle function | Yearly follow-up by telephone or video with neurologist | Clinician based ^b |
| Dijkstra <i>et al.</i> , Neurology. 2021 [20] | prospective | FSHD1 <i>n</i> = 20 children | 2 years | 2018–2020 | Clinical evaluation-muscle function | Clinicians | Clinician based ^c |
| Teeselink S <i>et al.</i> , J Neurol. 2022 [22] | prospective | FSHD1 <i>n</i> = 88; FSHD2 <i>n</i> = 4 | 5 years | 2014–2020 | Respiratory function testing- clinicians | Clinicians, twice over five years. | Clinician based ^d |
| Eichinger <i>et al.</i> Muscle Nerve. 2021 [25] | prospective | FSHD <i>n</i> = 434; DMD <i>n</i> = 271; LGMD <i>n</i> = 69 | preCovid/postCovid infection | May 2020 | COVID-19 Impact Survey-self reported | NO | Registry based ^e |
| Lewis <i>et al.</i> J Neuromuscul Dis. 2022 [26] | prospective | FSHD <i>n</i> = 613; DMD <i>n</i> = 53; LGMD <i>n</i> = 99 | preCovid/postCovid infection | February-March 2021 | Improved COVID-19 Impact Survey-self reported | NO | Registry based ^e |
| Kelly <i>et al.</i> , Muscle Nerve. 2022 [21] | retrospective | FSHD1 <i>n</i> = 86; FSHD2 <i>n</i> = 1 | / | / | Clinical evaluation-comorbidities self-reported | / | Clinician based ^f |
| Morse <i>et al.</i> , PLoS One 2022 [23] | retrospective | FSHD <i>n</i> = 12; BMD <i>n</i> = 15; LGMD <i>n</i> = 12 | / | / | Sleep assessment-self reported | / | Clinician based ^g |
| Hoffmann <i>et al.</i> , Muscle Nerve 2022 [24] | retrospective | FSHD1 <i>n</i> = 352; FSHD2 <i>n</i> = 50; Unsure <i>n</i> = 249 | / | / | Sleep assessment-self reported | / | Registry based ^h |
| Sanson <i>et al.</i> , Orphanet J Rare Dis. 2022 [27] | retrospective/comparative | FSHD1 <i>n</i> = 281 | / | / | Clinical evaluation-Clinician based and self-reported | / | Registry based ⁱ |

^aenrolled by The Registry, <https://www.urmc.rochester.edu/neurology/national-registry.aspx>.

^bCCEF Categories; CSS and ACSS scores.

^cMFM- MRC; NeuroQol fatigue; Kidscreen questionnaire.

^dMFM- MRC; NeuroQol fatigue; Ricci score.

^eenrolled via patient registries and patient advocacy groups.

^fenrolled at Mayo Clinic (USA).

^genrolled at The Neuromuscular Centre-Winsford, UK.

^henrolled via Facioscapulohumeral Muscular Dystrophy Society-FSHDS.

ⁱFrench National Registry of FSHD www.fshd.fr.

BMD, Becker muscular dystrophy; DMD, Duchenne muscular dystrophy; DRA, D4Z4 reduced allele; FSHD, facioscapulohumeral muscular dystrophy; LGMD, limb girdle muscular dystrophy.

parameters to define predictors of disease progression. Both studies established that disease duration is the most important predictor. However, the US study found that 27.3% of participants became wheelchair dependent [18], whereas a significantly lower percentage (8.9%) was reported in the Chinese cohort [19]. Both studies reported a faster rate of progression to wheelchair use in females. Besides the US and China studies on hundreds of participants, other studies on smaller cohorts confirmed the significant clinical variability among early-onset FSHD patients [20], including a wide spectrum of extramuscular manifestations, highlighting the need for careful monitoring of systemic symptoms by physicians [21]. A 5-year follow-up on respiratory function in FSHD patients showed that 44.6% presented a restrictive ventilatory pattern at baseline. However, only one-third developed a progressive respiratory dysfunction measured by forced vital capacity [22]. Two studies using self-reported measures, the Pittsburgh Sleep Quality Index and the Epworth Sleepiness Scale, respectively, found reduced sleep quality and excessive daytime sleepiness in FSHD patients and suggested that pain has a negative impact on sleep quality [23,24]. Two questionnaires, the coronavirus disease 2019 (COVID-19) Impact Survey and the Perceived Stress Scale, documented that the rates and the clinical outcome of COVID-19 infection in these patients were similar to the general population [25,26].

Remarkably, a French study assessing the concordance between a self-report questionnaire and a clinical evaluation form in FSHD found a good agreement only for demographic, diagnosis- and care-related items [27]. Instead, no concordance was found for muscle function-related features emphasizing that surveys alone should be used cautiously and complemented by objective medical data.

Imaging and circulating biomarkers: diagnostic and prognostic potential

Great efforts have been directed towards identifying FSHD biomarkers in clinical research over the last 18 months, mainly imaging biomarkers, especially muscle MRI [28–32]. In a 1-year longitudinal study on 32 patients with FSHD, MRI-derived leg fat fraction was revealed to be a valid biomarker of disease progression [28]. A subsequent study documented that whole-body muscle MRI, especially scores for fat replacement and atrophy score, could be a potential biomarker of disease staging also in pediatric FSHD [30]. Recently in a longitudinal study on seventeen FSHD patients, a whole-body musculoskeletal MRI protocol was developed, and quantitative muscle measurements showed a strong correlation

with functional outcome measures, including timed up-and-go (TUG) and FSHD-TUG [31]. A retrospective study demonstrated that muscle MRI could be a powerful diagnostic tool in differentiating FSHD from other inherited or acquired myopathies based on selective involvement of trapezius, sparing of subscapularis and iliopsoas, and asymmetric involvement of upper and lower limb muscles [32].

Tissue and circulating markers received little attention compared to image biomarkers, and no reliable biomarkers have been identified. Several groups found alterations in the circulating levels of several pro-inflammatory and regulatory cytokines (IL6) [33,34] or miRNAs that is, (miR-206) [35–38] and unsuccessfully investigated for the presence of disease-specific antibodies [39]. These molecules showed no disease specificity weakening their relevance for FSHD.

Molecular basis of the disease: the DUX4 effect

The DUX4-driven model for FSHD assumes that DUX4 stochastic expression during muscle development triggers a toxic signaling cascade leading to muscle degeneration [40]. Banerji and Zammit [41] evaluated the role of DUX4 in FSHD pathogenesis by meta-comparing published data about the DUX4-signaling cascade. Their analysis indicated that the expression of DUX4 target gene is associated with muscle that exhibits inflammation. Instead, they connected the PAX7 signaling with persistent degeneration of FSHD skeletal muscle, even without overt inflammation. These analyses suggest that DUX4, PAX7, and their interactions might be just two of the pieces of the FSHD puzzle.

The Zammit group further proved that the double homeobox 4 centromeric (*DUX4C*) is not just a pseudogene. Still, it is endogenously expressed at the protein level in normal and FSHD myotubes and in protein extracts of FSHD muscle biopsies [42,43]. The hDUX4C protein seems to be associated with muscle regeneration. This implies that DUX4 and DUX4c in regenerating FSHD muscle cells might have antagonistic roles and that caution should be exerted with therapeutic agents aiming for DUX4 suppression as they might also repress the highly similar DUX4c and interfere with its physiological role [44,45].

New preclinical models for FSHD

Currently, the preclinical animal models for FSHD are prevalently represented by mice over-expressing high levels of DUX4. Because of the divergence of mDux and hDUX4, murine models have limitations

that might explain their failure to recapitulate features of disease progression. Nip and colleagues [46] studied the porcine DUXC, showing that pDUXC and hDUX4 activate a highly similar early embryonic program in pig muscle cells. The same group also reported [47] the expression of two isoforms of DUXC mRNA in canine testis, showing that the canonical canine DUXC protein activates a cell signaling cascade similar to hDUX4 and mDux. Guo et colleagues [48] proposed using iPSC-derived, induced iMyoblasts as an attractive model in several neuromuscular diseases. iMyoblast can be readily produced from FSHD patient iPSCs, and their selection protocol can recover a peculiar population of muscular cells with unique properties among other cellular models.

Diagnosis and epigenetics in FSHD

Genetic diagnosis of FSHD is conventionally based on (i) determination of the D4Z4 repeat length and estimate of the repeat copy number of each array by Southern blotting, followed by (ii) confirmation of the presence of the 4qA allele, which constitutes a permissive haplotype. In cases without D4Z4 repeat contraction, *SMCHD1* gene and related epigenetic suppressor genes or D4Z4 CpG methylation are investigated. A nearly identical region complicates all this at the 10q26 subtelomere [49] and the numerous divergent D4Z4 arrays scattered throughout the genome favoring recombination events that may interfere with the capacity to interpret molecular results and generate a proper genotype-phenotype correlation. Furthermore, D4Z4 alleles with 4 to 8 repeats with 4qA can be found in approximately 2% of the general population [15].

In itself, the D4Z4 array structure impedes direct testing in preimplantation genetic diagnosis [50], and the highly recombinogenic nature of the 4q and 10q subtelomeres obstacles the use of alternative markers for PGD [51]. More recently, molecular combing [52–54] and optical mapping techniques (OGM) [55,56] have emerged to estimate the size of the array. In particular (OGM) has been applied to FSHD [55] for its ability to enumerate the repeats of the D4Z4 array on single long molecules of DNA. These methods have the potential to investigate structural variation associated with D4Z4 repeats throughout the entire genome, adding new capacity for interpreting uncommon molecular findings and phenotypes, even though they are technically and cost-effectively demanding, and none of them provides detailed information about the methylation status of the D4Z4 locus, a hallmark of FSHD.

Recently, Caputo and colleagues [57,58] developed a protocol for methylation analysis of specific

CpG residues using iMachine Learning (ML) algorithms to classify FSHD cases. Hiramuki *et al.*, [59[¶]] applied long-read sequencing through a Nanopore CRISPR/Cas9-targeted resequencing to diagnose FSHD by simultaneous detection of D4Z4 repeat length and methylation status at the nucleotide level in FSHD patients. Erdmann *et al.* [60] developed a methylation-based diagnostic workflow comprising a haplotype and high-throughput methylation profile analyses (FSHD-MPA). FSHD-MPA determines the average global methylation level of the D4Z4 repeat array and the regional methylation of the most distal repeat unit by combining bisulfite conversion with next-generation sequencing and a bioinformatics pipeline and uses these as diagnostic parameters.

Although the D4Z4 CpG methylation has been extensively studied [61–66], the heterogeneity in methods and cohorts used in the different studies prevented a definite assessment of its contribution to the FSHD expression and severity. Indeed, Hiramuki *et al.* [59[¶]] recently observed that the hypomethylation in the contracted D4Z4 in FSHD1 is moderately correlated with patient phenotypes, while the Jones group [67] propose that the epigenetic status of the D4Z4 arrays can readily distinguish between healthy, FSHD1 and FSHD2 phenotypes. It can be anticipated that long-read sequencing applied to well clinically characterized cohorts will properly establish the clinical significance of reduced D4Z4 methylation.

Clinical trials and new potential therapeutics

Over the decades, several therapeutic approaches have been attempted for FSHD. These included the administration of corticosteroids, beta2-adrenergic agonists, myostatin inhibitors, and oral supplementation with macronutrients and antioxidants to maintain muscle homeostasis and induce muscle hypertrophy, reviewed in [68]. Unfortunately, none of those nontargeted interventions achieved the primary outcome measures postulated in the study.

Therefore, more targeted approaches have been envisaged. Most of these attempts rely on drugs targeting DUX4 regulation and production or DUX4-signaling effector genes. They include: (i) epigenetic silencing of the D4Z4 repeats; (ii) blocking *DUX4* mRNA production; (iii) targeting downstream pathways triggered by *DUX4* expression as summarized by [68–72]. The main drug-based interventional studies applied to FSHD patients are summarized in Table 2. Notably, three out of six are still active, and two out of three are now recruiting FSHD patients.

Table 2. Description and comparison of drug based FSHD clinical trials started over years.

| Drug/ ClinicalTrials.gov Identifier | Action | Responsible | Phase | Status | Start | End | Participants (n) |
|--|--|----------------------------|--|---|--------------------------------------|--------------------------------------|-------------------------|
| Losmapimod/ NCT05397470 | P38 MAPK inhibitor | Fulcrum Therapeutics | PHASE 3 | Recruiting | Jun 2022 | Est. 2024 | 230 |
| RO7204239/ GYM329/ NCT05548556 | antimycostatin antibody | Hoffmann-La Roche | PHASE2 | Recruiting | Feb 2023 | Est. 2025 | 48 |
| Losmapimod/ NCT04264442 NCT04004000 | P38 MAPK inhibitor | Fulcrum Therapeutics | PHASE2 | Active- Not Recruiting | 2022 2019 | Est. 2025 2024 | 76 14 |
| Testosterone+ Somatropin/ NCT03123913 | Hormone | University of Rochester | PHASE1 | Completed | Feb 2019 | Feb 2023 | 20 |
| Losmapimod/ NCT04003974 | P38 MAPK inhibitor | Fulcrum Therapeutics | PHASE2 | Completed | Aug 2019 | Jan 2021 | 80 |
| ATYR1940 Resolaris/ NCT02603562 NCT02836418 NCT02531217 NCT02579239 NCT02239224 | T-cells activation inhibitor | aTyr Pharma, Inc. | PHASE1/2 PHASE1/2 PHASE1/2 PHASE2 PHASE1/2 | Completed | 2016 2016 2015 2015 2014 | 2017 2017 2017 2017 2015 | 8 8 9 18 20 |
| ACE-083/ NCT02927080 NCT03943290 | Activins and myostatin inhibitor | Acceleron Pharma Inc. | PHASE2 | Terminated Not achieving secondary endpoints | 2020 2019 | 2022 2020 | 95 62 |

Est., estimated date; Part, Participants.

LOSMAPIMOD: developed by Fulcrum Therapeutics selectively inhibits the p38 α / β mitogen-activated protein kinases (MAPKs), which are reported as modulators of DUX4 expression and mediators of inflammation [73,74]. A Phase 3 clinical trial using Losmapimod (ClinicalTrials.gov Identifier: [NCT04264442](#)) started in June 2022. Mellion *et al.* [73] summarized the results of Phase 1 trial supporting the advancing of Losmapimod into Phase 2. The drug was well tolerated, with no serious adverse effects. Data reported in June 2021 from the Phase IIb trial (ClinicalTrials.gov Identifier: [NCT04003974](#)), showed only a partial beneficial effect after 48 weeks of administration. The study's primary endpoint, the reduction of DUX4 mRNA and protein levels, was not met. It is to note that since p38 and MAPKs are known to regulate early stages of myogenesis, p38 inhibition may have adverse long-term effects in skeletal muscle, raising doubts on the likelihood of long-term benefit. These aspects should be carefully investigated in the next steps of the clinical trials.

ACE-083: Delivered by Acceleron Pharma [75–77], it mimics the ligand trap follistatin, and can bind both myostatin and activins. Although ACE-083 demonstrated a statistically significant increase in mean total muscle volume, which was the trial's (ClinicalTrials.gov Identifier: [NCT02927080](#)) functional endpoint, no significant improvements in functional tests were observed. As a result, Acceleron has recently decided to stop the clinical trial of ACE-083 in FSHD.

DUX4 silencing strategies: Several attempts at silencing DUX4 with small molecules, antisense therapeutics, genome editing techniques [78–85] or endogenous mi-RNAs are reported [86]. These attempts were performed *in vitro* and *in vivo* using cell lines or animal models overexpressing DUX4. This is because detecting DUX4 mRNA/protein in primary FSHD patient-derived muscle cells or muscle biopsies is difficult. In two different works [84,85], authors show that administering an octa-guanidine dendrimer-conjugated phosphorodiamidate morpholino oligomer targeting DUX4–3'UTR, inhibits DUX4 expression and improves muscle functionality in DUX4 overexpressing mice [85]. In the same murine model, delivering a gapmer antisense oligonucleotide targeting DUX4-Orf also effectively knocked down DUX4 and alleviated muscle pathology, although with limited improvement in muscle mass and function [84].

Saad and colleagues [86] circumvented difficulties in delivering oligo-based RNAi systems by detecting a naturally DUX4 targeting human miRNA. They identified *miR-675* as a *DUX4* regulator, which directly targets *DUX4* mRNA, inhibiting its function. The delivery of *miR-675* by AAV-based gene therapy in scAAV6.CMV.DUX4-FL mice decreased DUX4-associated histopathology alterations.

CONCLUSIONS

People living with FSHD experience progressive muscle weakness and atrophy that affect mobility,

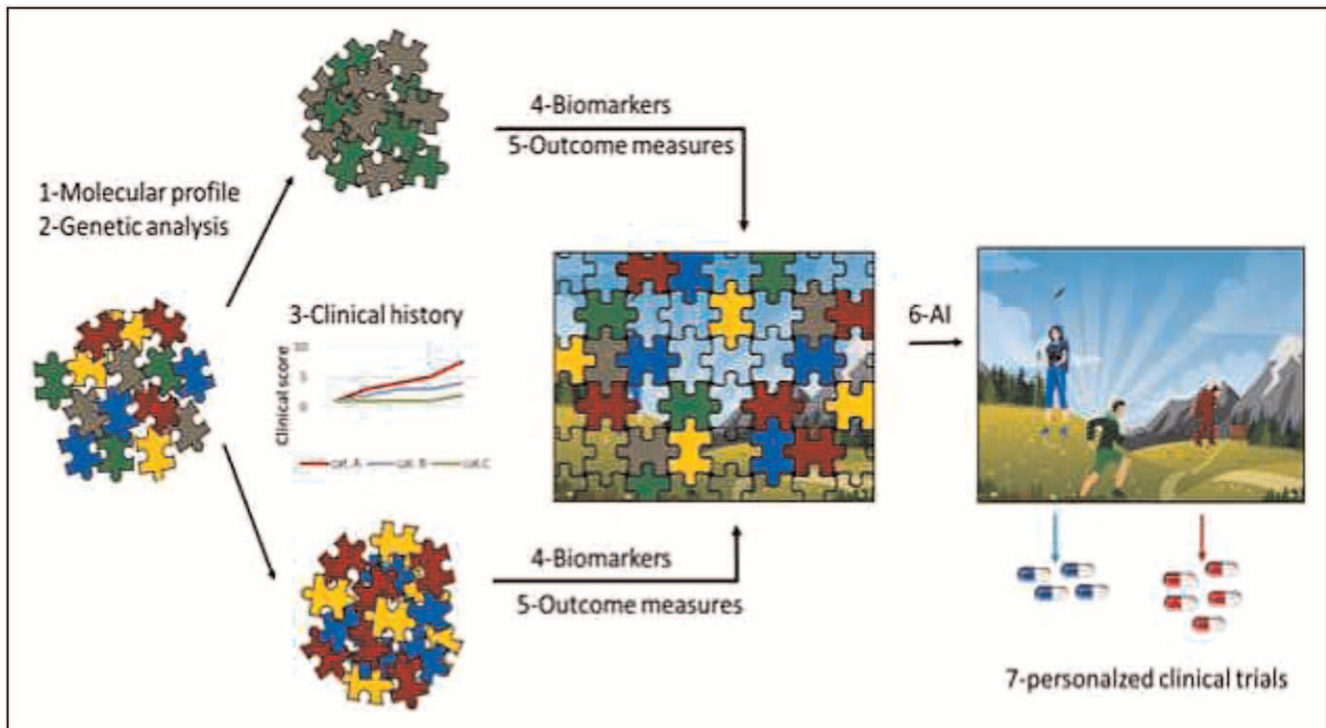


FIGURE 2. Solving the FSHD puzzle requires a multistep and multimodal approach. The wide phenotypic spectra observed in carriers of D4Z4 reduced allele suggest the existence of clinical subgroups within the FSHD population. Patients' stratification relies on the combination of genomic, anamnestic, and standardised clinical data for defining disease natural history associated with the distinct phenotypes. These steps are necessary to develop appropriate outcome measures and biomarkers and to proceed toward personalized trial readiness for FSHD patients. FSHD, facioscapulohumeral muscular dystrophy.

determine general disability, and impair social participation. We are still far from the optimal management of patients, and several responses are needed to address their actual demands. The review of publications in the past 18 months shows that despite the diverse approaches to tackling FSHD, diagnosis is often partial, and the individual prognosis remains uncertain. This lack of knowledge hinders designing therapeutics based on a specific target. At the same time, the demand for interventions is increasing as more people with FSHD age. It is a time for changes. We must elaborate new systems for data analysis that consider the clinical and genetic complexity of FSHD, support patient stratification and lay the basis for defining the disease's natural history associated with the distinct phenotypes.

A multidimensional platform exploiting advanced computational tools for systematically investigating FSHD cases might serve this goal (Fig. 2).

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Conflicts of interest

There are no conflicts of interest.

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Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

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