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ASSESSING THE SPECIFICITY OF MOLECULAR TESTING IN FACIOSCAPULOHUMERAL MUSCULAR DYSTROPHY

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Summary

Through the clinical research activity conducted in collaboration with the Italian Clinical Network for facioscapulohmeral muscular dystrophy (FSHD), the objective of my studies has been the phenotypic and molecular characterizaction of FSHD families from the Italian National Registry for FSHD (INRF) to test the predictive significance of molecular variations in FSHD. INRF represents an unique epidemiologic tool allowing the analysis of molecular data integrated with data from clinical examination and anamnestic records in FSHD families. The precise phenotypic classification of patients and families as well as the pattern of inheritance is crucial for the definition of parameters to sub-classify FSHD patients and families. This novel approach is fundamental for good clinical prectice and eventually to identify new susceptibility/causative factors contributing to FSHD.

FSHD, which is considered an autosomal dominant disease, has been associated with reduced numbers (<11, alleles ≤41 kb) of D4Z4 repeats at 4q35. Initially, since the discovery of the molecular defect, a rough and inverse correlation between the residual size of the D4Z4 repeat, the age at onset and the severity of muscular involvement has been reported. Small repeat arrays of 1-3 units appeared to be associated with earlier onset and rapid progression; while wide disease variability was described among carriers of D4Z4 allele with >3 repeats. However 3% of healthy individuals carry D4Z4 reduced alleles (DRA). This fact makes FSHD molecular diagnosis troublesome, especially in presence of atypical phenotypes. Moreover, the observed wide clinical heterogeneity associated with DRA together with the incomplete penetrance has progressively emphasized the concept that the current genetic signature of FSHD alone may not be sufficient to produce disease. Additional molecular factors such as level of D4Z4 methylation or mutations in other genes (i.e. *SMCHD1* gene) have been recently proposed to play a role in disease outcome. Thus, there is the need for clinical practice and research to re-evaluate the significance and the predictive value of DRA.

Our first genotype-phenotype studies on a large cohort of FSHD patients and families recruited from INRF confirmed that DRAs with 1-8 repeats may correspond to various phenotypes and degree of severity in disease expression. Notably, a detailed analysis of carriers of 1-3 DRA, at the lower extreme of the diagnostic spectrum, highlighted a clinical variability also in this subgroup of patients, in spite of the notion that 1-3 DRA are associated with a more homogenous severe phenotype with almost complete penetrance. This argues for

the presence of complex pathogenetic mechanisms involving additional factors beside the short DRA.

In this thesis, we therefore decided to further investigate cases carrying alleles with 9-10 D4Z4 repeats, which reprent the upper extreme of the molecular diagnostic spectrum.

We designed a new Comprehensive Clinical Evaluation Form (CCEF), a clinical tool useful in describing in harmonized manner the phenotypic spectrum observed among FSHD families. The CCEF permit to quantify the motor disability and defines clinical categories by the combination of different features: 1) subjects with typical FSHD phenotype (category A), 2) subjects with muscle weakness limited to scapular girdle or facial muscles (category B), 3) asymptomatic/healthy subjects (category C) and 4) subjects presenting clinical features not consistent with the FSHD (category D). This CCEF can support the precise phenotypic classification of patients and families, the study of the natural history of FSHD, diagnosis, genetic counseling and preparation of clinical trials. This approach also promotes the search of genetic factor(s) contributing to the phenotypic spectrum of FSHD.

Through the phenotipic characterizaction of 228 subjects carrying a 9-10 DRA, we observed that only 53% of probands showed a "complete" FSHD phenotype (category A); whereas 20% of them presented incomplete features of disease (category B). Remarkably, 21% of probands did not fulfill the clinical diagnosis of FSHD, showing additional atypical clinical features (category D). Among FSHD probands, we also confirmed a wide variability of clinical expression, both in term of age at onset and motor disability, ranging from almost asymptomatic carries to severly affected subjects. Importantly, in 75% of families in which D4Z4 alleles with 9-10 repeats segregate, we diagnosed FSHD in one subject, whereas all the other relatives carrying the same 9-10 DRA were found healthy. On the whole our study established that 70% of relatives, carrying the same DRA of the proband, did not display any muscle functional impairment (category C). Among affected relatives, only 8% showed FSHD phenotype (category A).

In a subgroup of patients and families we also investigated other genetic modifiers, including the level of 4q35 methylation and mutations in *SMCHD1* gene, could explain the incomplete penetrance and the wide clinical variability. Our study highlights how a low level of methylation should not be considered predictive of disease outcome in clinical practice and nor even can be used as diagnostic marker.

In conclusion, the systematic and standardized collection of clinical and molecular data of FSHD families from the INRF has allowed to highlight how, to date, the molecular

mechanisms leading to disease are still not clear. More importantly there are no parameters with definite predictive value to support prognosis in this disease characterized by extreme clinical variability of disease expression both in terms of severity and phenotypic features. Moreover we observed that the pattern of inheritance is not always autosomal dominant and there is not a definite knowledge of natural history of disease. This is because there are several pitfalls in the genetic diagnosis of FSHD. Our studies on large cohorts define that the molecular markers proposed for FSHD are not predictive of disease outcome and could be confounding for the clinician wanting to differentiate between the diagnosis of FSHD and a myopathy presenting with FHSD-like features. The future goal of FSHD clinical research will be the selection of patients and families with homogeneous clinical and genetic features, in term of mode of inheritance, regardless of the size of D4Z4 alleles, to provide the appropriate background for molecular studies aimed at dissecting the complex pathogenesis of this disease.

Introduction

Facioscapolohumeral muscular dystrophy: clinical features

The disease was firstly reported in 1862 by Duchenne de Boulogne, who published a picture of an affected patient in his Album de photographies pathologiques. Duchenne described the disease in his famous series of papers in Archives of General Medicine in 1869, which is often cited as the earliest reference of FSHD. In 1885, Landouzy and Dejerine described in detail the clinical features of FSHD, thus also called "Landouzy-Dejerine form of muscular dystrophy", characterized by progressive facial, shoulder girdle and pectoral muscle weakness and atrophy, subsequent involvement of abdominal muscles with lumbar hyperlordosis and anterior leg muscles with steppage gait. Subsequently, in 1982, the thesis of Padberg provided the first modern clinical description of FSHD families. Padberg investigated a group of 107 subjects from 19 families, including 73 subjects displaying clinical signs of FSHD. These studies provided the first evidence for wide clinical variability in FSHD patients, even within the same family.

In 1991 an International Consortium established the clinical, laboratory and genetic criteria for FSHD diagnosis, in absence of a diagnostic DNA test. This work responded for the need of selecting families that could be included in the linkage analysis [Padberg et al. 1991] towards the identification of the FSHD gene. Four main criteria were identified: (1) onset of the disease in facial or shoulder girdle muscles; sparing of the extra-ocular, pharyngeal and lingual muscles and the myocardium; (2) facial weakness in more than 50% of the affected family members; (3) autosomal dominant inheritance in familial cases; and (4) evidence of myopathic disease in EMG and muscle biopsy in at least one affected member. By contrast, (1) involvement of extra-ocular, masticatory, pharyngeal and lingual muscles; (2) regression of symptoms and signs; (3) presence of severe and diffuse contractures; (4) involvement of myocardium with presence of cardiomyopathy; (5) persistently high CK values above five times the upper limit, were considered suggestive of alternative diagnosis.

The FSHD phenotype is characterized by initially restricted distribution of weakness starting with asymptomatic facial weakness followed by weakness of scapular fixator, humeral, truncal and lower extremity muscles. The onset at lower-extremity is often characterized by distal weakness, typically in the anterior leg compartment, presenting with footdrop. Weak abdominal muscles result in a protuberant abdomen and contribute to the lumbar lordosis. Lower abdominal muscles are weaker than upper abdominal muscles, causing strikingly positive Beevor's s sign, a physical finding fairly specific for FSHD [Awerbuch et al., 1990].

A notable distinctive feature of FSHD is that muscle weakness displays asymmetric distribution, which does not correlate with the handedness of the individual [Brouwer et al., 1993]. The creatine kinase (CK) level can be moderately increased or normal. Electromyography (EMG) and histological analysis reveal non-specific myopathic changes associated, in some cases, with neurogenic and/or inflammatory aspects [Lin et al., 1991; Dorobek et al., 2013]. Muscle magnetic resonance imaging (MRI) can detect muscles showing normal MRI signal together with muscles showing abnormalities on T1-weighted MRI sequences, corresponding to areas of fatty fibrous replacement, or areas characterized by increased signal on T2- short tau inversion recovery (T2-STIR) sequences also in muscles not yet replaced by fat tissue, reflecting an increase in tissue water content due to muscle oedema [Tasca et al., 2012]. Ancillary features, such as sensorineural deafness or retinal vasculopathy have been also reported in infantile FSHD forms, but they are not to be considered decisive criteria for FSHD diagnosis [Trevisan et al., 2008 a, b].

In the first clinical descriptions of disease, FSHD showed a fully penetrant autosomal dominant disease with age-dependent penetrance estimated to be >95% by age 20 [Lunt et al., 1989]. However, in contrast with the expected course for a classical autosomal dominant Mendelian disorder, since the first observations of FSHD families it was possible to establish that the chronology of disease progression is unpredictable, and disease expressivity ranges from subjects with very mild muscles weakness, almost unaware of being affected, to wheelchair-dependent patients [Ricci et al., 2014, Annex 1].

The discovery of DNA alterations associated with FSHD

The need for an accurate pre-symptomatic test led to an active search for the identification of the FSHD gene [Lunt et al., 1989]. In 1990 the FSHD locus was assigned to chromosome 4 by positional mapping performed in 10 Dutch families with autosomal dominance inheritance [Wijmenga et al., 1990]. This chromosomal position was confirmed using additional polymorphic markers in other families [Upadhyaya et al., 1990; Upadhyaya et al., 1991]. Subsequently, Wijmenga and coworkers [Wijmenga et al., 1991] reported that D4S139, a Variable Number Tandem Repeat structure (VNTR) locus, was the most closely linked to FSHD. Because D4S139 represents the most telomeric 4q-specific marker, it established the location of the FSHD gene in the subtelomeric region of chromosome 4q. The assignment of the FSHD locus to region 4q35 was definitively established in 1992 by six laboratories, based on the genotyping of 504 affected patients and 559 unaffected subjects from 65 families [Sarfarazi et al., 1992]. Later, Wijmenga and coworkers [Wijmenga et al., 1992] identified a

3.3 kb tandemly repeated sequence (D4Z4) located at the 4q subtelomeric region that could be detected by hybridization of EcoRI digested DNA using the p13E-11 DNA sequence as probe. This study included 11 Dutch families, 6 de novo cases, 29 healthy individuals. One family presenting compound heterozygosity for two D4Z4 alleles smaller than 28 kb was excluded from the study. The authors showed that in healthy individuals the majority (72%) of EcoRI fragments detected by the p13E-11 probe were larger than 28 kb, while in FSHD patients there was an overrepresentation of fragments smaller than 28 kb [Wijmenga et al., 1992]. It was also shown that 5 out of 6 affected individuals with unaffected parents carried a de novo p13E-11 allele smaller than 28 kb. On this basis it was proposed that FSHD is caused by DNA rearrangements of p13E-11 EcoRI alleles. However, 8 healthy individuals presented in the study carried p13E-11 alleles smaller than 28 kb, providing an early clue that D4Z4 allele size alone would be unlikely to explain all cases of FSHD pathogenesis.

The D4Z4 locus

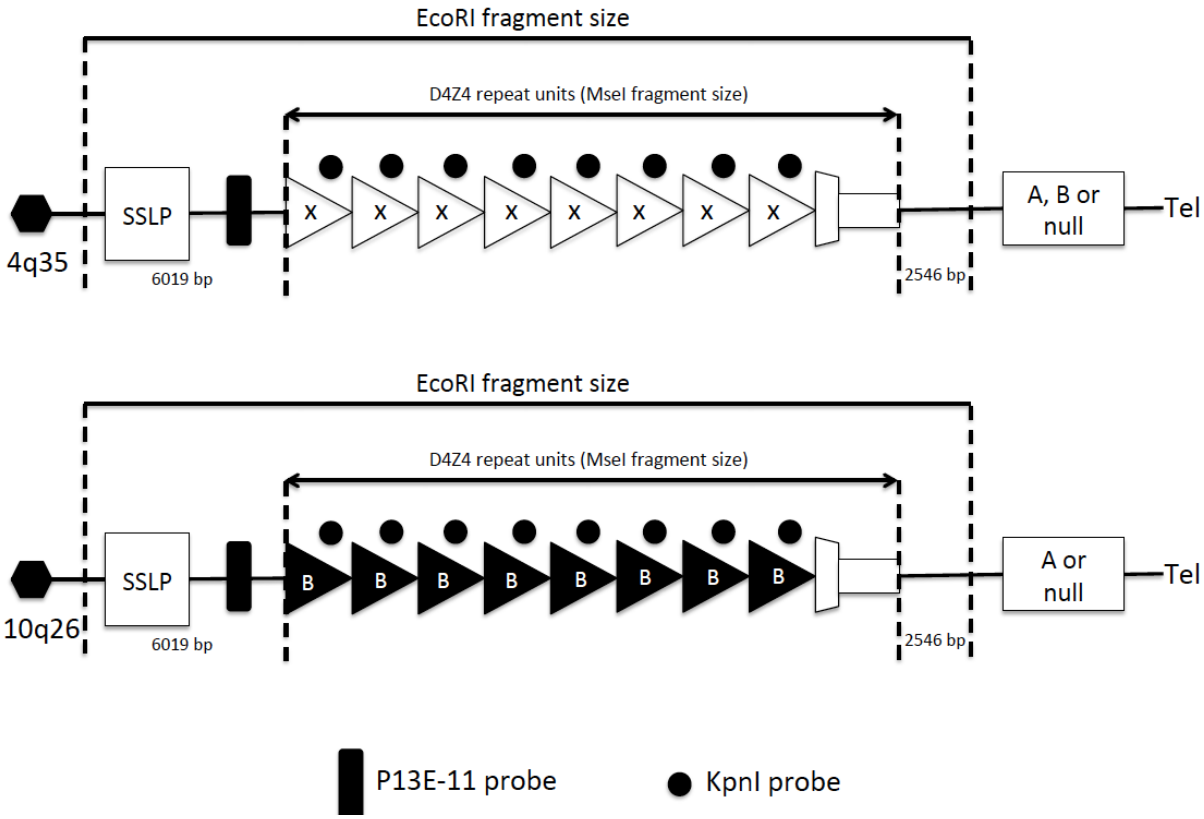
The probe p13E-11 detects a highly polymorphic locus with a VNTR structure constituted by a tandemly arrayed sequence of DNA repetitive elements named D4Z4 [Hewitt et al., 1994]. The variation in size of EcoRI fragments is due to variability in the number of D4Z4 repeats [van Deutekom et al., 1993]. In normal subjects the p13E-11 EcoRI alleles usually range from 40 kb to approximately 300 kb (>10 D4Z4 units), whereas alleles of 35 kb or shorter (≤ 8 D4Z4 units) are present in the majority of either de novo or familial FSHD patients [Upadhyaya et al., 1993; Wijmenga et al., 1994].

The D4Z4 repeats belong to a family of 3.3 repeats scattered within the human genome including chromosome 1 secondary constriction, and the heterochromatin of the acrocentric chromosomes [Winokur et al. 1994]. Importantly, an almost identical D4Z4 array was located at chromosome 10q, with 98% homology between 4q35 and 10q26 regions [Deidda et al., 1995]. The homology between 4q35 and 10q26 is not confined to the 3.3-kb repeats but extends both proximally (42 kb) and distally to include the telomere [van Geel et al., 2002]. Notably, the size of D4Z4 alleles on chromosome 10 overlaps with those on chromosome 4.

The presence of a polymorphism on the D4Z4 copy on chromosome 10, creating a BlnI restriction site, has facilitated the distinction between 4q and 10q D4Z4 alleles by using EcoRI/BlnI double digestion followed by p13E-11 Southern hybridization [Deidda et al., 1996] (Figure 1). This approach has led to the discovery that in 20-30% of the population translocated 4-type repeats reside on chromosome 10q and, viceversa, translocated 10-type

repeats on chromosome 4q [van Deutekom et al., 1996; van Overveld et al., 2000]. De novo reduced allele account for a surprisingly high percentage of FSHD patients (10%–33%) [Zatz et al., 1995, Padberg et al., 1995]. This high incidence can be partly explained by the presence of parental mosaicism for 4q short alleles that has been reported in 19% of de novo cases [Lemmers et al., 2004]. The presence of somatic mosaicism for a rearrangement of D4Z4 was found in as much as 3% of the general population [van der Maarel et al., 2000]. These observations, demonstrate that the D4Z4 array is highly recombinogenic. Notwithstanding, only D4Z4 reduced alleles (DRA) located at 4q have been associated with FSHD regardless of the repeat type composition.

Figure 1: *Schematic representation of the method used to calculate D4Z4 repeat numbers from EcoRI-fragment sizes. Nine and ten D4Z4 repeats (36-41 kb EcoRI fragment size) were defined to be the upper diagnostic range for FSHD. D4Z4 repeat units on chromosomes 4 and 10 can be distinguished because all repeats on 10q contain BlnI restriction sites (B), while all D4Z4 repeats on 4q contain XapI restriction sites (X).*



Genotype-phenotype correlation studies in FSHD

Size of D4Z4 allele and clinical expression

Since the discovery of the FSHD molecular defect, genotype-phenotype studies have conducted in order to evaluate if the size of the EcoRI fragment could be correlated with the clinical manifestations and to assess the impact of the molecular defect on the phenotypic expression.

Lunt and coworkers in 1995 [Lunt et al., 1995b] reported the analysis performed on 14 FSHD families and 25 clinically isolated cases, presumed to be due to new mutation, associated with D4Z4 reduced allele (respectively in two groups the range in allele size was 19-30 and 13-24 Kb). The study revealed a clear correlation between smaller fragment sizes and earlier age at onset. The median age at onset on sporadic cases resulted 6.9 years (range <1-16 years) and on familial cases 18 years (range 8-23). Interestingly, the authors also observed within families a difference between generations in reported onset ages, with onset age becoming younger in successive generations, although it was hypothesized that this trend might be more a reflection of ascertainment bias than a biological anticipation. A similar correlation with fragment size was also observed for age to loss of ambulation in 16 subjects using a wheelchair [Lunt et al., 1995a]. The authors proposed that FSHD families could be divided broadly into three groups, necessarily with some overlap: i) new mutation cases with early onset (range <1-16 years), severe presentation, and small fragment size ≤18 kb; ii) large 'typical' families with median onset age ranging from 8-22 years associated with fragment size is 19-30 kb; iii) small families, often with a later onset presentation (median 15-23 years), or scapulohumeral presentation, in which a 4q35-cosegregating fragment of size 30-38 kb was present and non-penetrance may still be observed above 20 years of age.

The subsequent study of Tawil and coworkers in 1996 [Tawil et al., 1996] confirmed the same results, by examining the genotype in a clinically and genetically well-defined 157 FSHD subjects. In particular, this analysis showed the presence of anticipation and that the size of the deletion and the disease severity were closely related.

Three years later, Ricci et al. [1999], on a cohort of 165 patients with FSHD (range in size 10-27 Kb), further reported the inverse correlation between fragment size and clinical severity. The probability of developing a severe form of disease resulted 100% in the presence of very short fragment (1-2 D4Z4 repeats), decreased to 54% in patients carrying fragments of 16 to 20 kb (3-4 D4Z4 repeats) and dropped to 21% or less in patients carrying

fragment larger than 20 kb (>4 D4Z4 repeats). A severe form of FSHD was defined when it was present: severe weakness of pelvic and proximal leg muscle or both (strength<3 in at least one of these muscle) with inability to stand up from a chair without support or to walk unaided, or wheelchair use.

In the genotype-phenotype correlation study performed by Tonini et al. in 2004 on 238 subjects from 106 unrelated families, it was observed that individuals with larger fragments showed a milder course while those who have the smaller ones were more severely affected. However, when genders are analyzed separately, this correlation was significant for females but not for males.

In 2003, Butz and coworkers conducted a systematic study of 39 unrelated FSHD patients with borderline D4Z4 repeat numbers and 102 healthy controls, in order to identify the molecular diagnostic cut-off point between FSHD cases and the control population and describe the phenotype in patients with borderline D4Z4 repeat numbers. The results indicated that there was not a definite D4Z4 diagnostic cut-off point separating FSHD, FSHD-like myopathies and healthy controls, without the expected correlation of D4Z4 repeat number and clinical severity. Therefore the authors suggested the D4Z4 cut off of 8 repeats [Butz et al., 2003].

In summary, the above studies showed an inverse correlation between the number of D4Z4 repeats and the severity of the disease. Alleles with 1-3 D4Z4 repeats are generally associated with a severe form of disease that presents in childhood, 4-7 D4Z4 repeats with the classical form of FSHD, and 8-10 D4Z4 repeats with a milder disease [Lunt et al., 1995a; Tawil et al., 1996; Ricci et al., 1999]. In addition, D4Z4 alleles between 38-45 kb in size (9-11 D4Z4 repeats) have been described both in normal and affected individuals and are considered as borderline [Butz et al., 2003; Vitelli et al., 1999].

Nevertheless, over the years since the advent of molecular diagnosis for FSHD, a growing number of evidences have emerged to complicate the evaluation of patients, reporting a wide and unexpected variability of FSHD clinical outcomes, also among subjects carrying the same D4Z4 allele, even within the same family [Fitzsimons, 1999; Galluzzi et al., 1999; Felice et al., 2000].

Penetrance of disease in carriers of D4Z4 reduced allele

In pre-molecular era, the first observations performed on large families with clinical diagnosis of FSHD suggested an almost complete penetrance of the disease. However, since the advent of molecular diagnosis for FSHD, subjects carrying DRA without signs of disease

have been reported [Ricci et al., 2014, Annex 1], challenging the notion that DRA alone can cause nearly full disease penetrance. Several such studies are summarized below.

In a previous study on 52 Brazilian families with DRA smaller than 35 kb [Zatz et al., 1998], the estimated penetrance for FSHD allele was found to be 85% for patients until age 30. Furthermore, when the authors considered the sexes separately, the estimated penetrance of the FSHD allele was significantly greater for males (95%) than for females (69%). Interestingly, among 27 families with at least two clinically affected patients it was observed that in 21 families the pattern of inheritance was autosomal dominant (4 of them with incomplete penetrance). Surprisingly, in 3 pedigrees the pattern of inheritance was compatible with the presence of an autosomal recessive trait since there were at least two affected sibs born from asymptomatic parents. These observations suggested that FSHD phenotypes may result from distinct types of mutations in different families.

A study conducted on Italian families [Ricci et al., 1999] reported 7 subjects, aged 20 to 69 years old, with DRA between 21 and 37 kb (4-8 units), without symptoms or signs of FSHD, who were classified as non-penetrant carriers. In this study, unaffected individuals were not observed in families with DRA smaller than 20 kb.

A retrospective analysis conducted on 85 Japanese patients with FSHD and both their parents documented parents with DRA who had no clinical symptoms, confirming an estimated low penetrance of 59% (excluding somatic mosaicism) [Goto et al., 2004].

Tonini and coworkers in 2004 analyzing 238 subjects with DRA <35 kb from 106 unrelated families, observed that about 20% of individuals related to FSHD patients who carried a DRA remained asymptomatic or were minimally affected with a significantly higher proportion of females than males; asymptomatic carriers were found in about 30% of the families.

More recently, Sakellariou and coworkers [Sakellariou et al., 2012] reported clinical and genetic analysis of 133 individuals carrying DRA (71 probands and 62 relatives) from 71 unrelated Greek families, revealing a high percentage (almost 50%) of asymptomatic relatives older than 30 years and carrying DRA. The percentage of unaffected carriers was also lower in males than in females (29% vs 71%). It is also noteworthy that 16 among the 38 multiple-case families (42%) were found to have at least one symptom-free individual, with a greater proportion of asymptomatic or minimally affected gene carriers concentrating in some pedigrees, as previously observed by Tonini and coworkers [Tonini et al., 2004]. A statistically significant association between the genders and the clinical manifestation of the

disease was also observed: among the females the percentage of symptomatic patients was found to be 66.7% whereas among the males it was 86.6%.

Atypical phenotypes associated with D4Z4 reduced alleles: clinical subtypes of FSHD or more complex myopathic conditions?

In the past 20 years, assessment of the D4Z4 array size as diagnostic test for FSHD has led to the identification of phenotypes that differs at various degrees from the original description of disease made by Landouzy-Dejerine. This has provoked a trend towards the expansion of the clinical pattern associated with D4Z4 reduced allele. Several subtypes of FSHD with atypical clinical presentation have been described.

For example, in 2000 van der Kooi and coworkers described six sporadic cases that did not meet most of the diagnostic criteria defined in 1991 but were diagnosed as FSHD because they carried a DRA (range 26 to 38 kb) on 4q. The foot drop was the predominant clinical feature found in three patients; in three others, inability to walk on toes, shoulder pain, and pelvic limb weakness with difficulty in walking were reported, respectively. None of them had facial weakness and only one complained of shoulder weakness. Interestingly, none had a positive family history.

In the same year, Felice and coworkers [2000] described 10 patients out of 14, with facial-sparing scapular myopathy associated with DRA (range 20 to 39 kb). Except for the absence of facial weakness, most patients had clinical and laboratory features otherwise consistent with FSHD. Five patients referred also a positive family history of similar weakness, although DNA analysis was not performed on other family members.

Felice and Moore in 2001 also described four patients, each harboring DRA (range 25 to 34 kb), who presented with atypical phenotypes including facial-sparing scapular myopathy, limb-girdle muscular dystrophy (LGMD) distal myopathy and asymmetric brachial weakness. Only the first two patients had undergone muscle biopsies, which showed unspecific dystrophic features. None of these patients were subjected to other molecular investigations for differential diagnosis. Interestingly, the patient with LGMD phenotype and asymmetric brachial weakness did not report a positive family history for neuromuscular diseases. In this work, the authors concluded that the availability of the DNA test, considered as highly sensible and specific, allowed to establish definitively the diagnosis without the need for the more invasive and less specific muscle biopsy.

Krasnianski and coworkers in 2003 described three patients from a single family (father and two sons) in which a 23 kb DRA segregated. They showed signs consistent of typical FSHD associated with chronic progressive external ophthalmoplegia. The oculomotor impairment was reported as the initial manifestation of disease starting from infancy. The muscle biopsy of the father and one child demonstrated prominent myopathic changes without ragged red fibers or histopathological features of other neuromuscular diseases. The absence of singular or multiple deletions of mitochondrial DNA apparently excluded a coincidental diagnosis of Chronic Progressive External Ophthalmoplegia (CPEO) of mitochondrial origin. On the other hand, the classic FSHD distribution of the muscle weakness had been never described in patients with CPEO. The possibility of oculopharyngeal muscular dystrophy was not investigated. In the same paper [Krasnianski et al., 2003], the authors further described other two familial cases and one sporadic case with facial-sparing FSHD syndrome associated with D4Z4 reduced allele (34 and 30 kb allele respectively).

Cardiac involvement, including hypertrophic cardiomyopathy, conduction defects and arrhythmia, has been reported in subjects carrying a DRA by several reports [Laforêt et al., 1998; Finsterer et al., 2005; Emmrich et al., 2005; Tsuji et al., 2009], although the European Expert Group on FSHD in 1991, that defined the Diagnostic Criteria for FSHD in pre-molecular era [Padberg et al., 1991], defined that "cardiomyopathy is not part of the disease" and "when present it suggests an alternative diagnosis".

Reilich et al. [2010] described five unrelated cases carrying DRA whose biopsies showed signs of vacuolar myopathy with rimmed vacuoles. The atypical clinical features included a form of LGMD phenotype with facial-sparing, a form of distal and proximal weakness, which was associated with dysphagia in one patient and a form of a prevalent asymmetric lower limb distal weakness. Scapular winging or facial weakness was also reported, suggesting the possibility of an overlapping FSHD syndrome. In these cases the family history was negative for neuromuscular disorders or motor impairment, although molecular analysis was not performed in other family members. Only in one family the DNA testing revealed the same DRA (size 35 kb) in the mother and two sisters of the proband affected by distal weakness; these relatives showed a mild facial involvement at clinical examination. The five muscle biopsies of the above unrelated cases showed a pattern of degenerative myopathy with rimmed vacuoles and inflammatory infiltrates. Immunohistochemistry did not detect abnormal desmin, myotilin or alphabeta-crystallin deposits, excluding the diagnosis of myofibrillar myopathies. Electron microscopy revealed autophagic vacuoles containing

myelin-like material and filamentous nuclear inclusions. Interestingly, MRI imaging did not reveal the muscle lower limbs involvement typical of FSHD.

Among other several atypical phenotypes associated with DRA, including the bent spine syndrome, a clinical condition characterized by a stooped posture in the standing position, which is exaggerated in walking or in exercise and disappears in the supine position, sometimes associated with a dropped head. The first reported case [Umapathi et al., 2007] was about a 59-years-old woman with a family history of FSHD presenting with an overlapping condition with camptocormia, scapular winging and mild facial and proximal weakness. Kottlors et al. [2010] described the case of a 65-years-old man complaining of lower back pain and progressive bent spine syndrome, since the age of 60, carrying a 31 kb DRA. The patient recalled that his mother had a similar posture that began at age of 80. The genetic analysis performed on the available family members revealed the presence of DRA in the two daughters, who showed signs of myopathic facies. In one slight weakness of foot extensors was observed. Nonetheless, none in the family presented a typical FHSD phenotype. Jordan and coworkers [2011] reported six sporadic cases carrying a DRA (range 21-34 kb) with prevalent axial weakness. All patients referred late disease onset in fourth-sixth decades. Muscle MRI imaging revealed that in all six patients the most severely affected muscles were the thoracic and lumbar spinal tract together with hamstrings.

The conclusion of some authors is that the extensive use of genetic analysis has expanded the clinical and morphological spectrum of FSHD, and many consider the detection of DRA in a patient sufficient to diagnose FSHD. Interestingly, the atypical phenotypic cases are often sporadic. It may thus be supposed that in these cases the shorter D4Z4 fragment is not per se sufficient to trigger myopathy. Indeed the wide heterogeneity associated with alterations on chromosome 4q35 can suggest that other factors/pathologic conditions influence and modulate the disease expression, such as epigenetic or environmental factors, concomitant inflammatory disease. It may plausible that other genetic and/or environmental factors may participate in the onset of a myopathy that might present clinical features overlapping with FSHD. On the other hand, it must also be considered the possibility that other myopathies might have been misdiagnosed because of the random finding of a DRA in the affected subject.

Several reports of "double trouble" conditions in FSHD families

In 2002, Tonini and coworkers reported two unrelated Brazilian families with members apparently affected by two different forms of muscular dystrophy. In the first one, the 35-

years-old male proband showed LGMD with proximal weakness, elevated CK (16-fold above normal) and a myopathic muscle biopsy. Muscle protein immunohistochemical and immunoblotting analysis revealed a normal pattern for dystrophin, the four sarcoglycans, calpain, dysferlin and telethonin; DNA analysis for caveolin-3 gene was negative. Two of his sisters also complained of muscle weakness. The younger sister, aged 38 years, complained of proximal muscular weakness in upper and lower limbs, had calf hypertrophy, and a serum CK 5-fold above normal but she refused further investigations. The oldest sister, aged 51 years, showed mild clinical signs possibly consistent with FSHD, confirmed through the molecular analysis (30 kb DRA). The DRA was also found in another six relatives: four of them, aged 72, 45, 36 and 22 years, were asymptomatic and two (aged 19 and 16 years) showed only mild facial hyposthenia. Surprisingly the DRA was not detected in the affected proband. In the second family, a 57-years-old male with a typical FSHD phenotype was carrying a 17 kb DRA, which was also present in other affected relatives. However, in a 14-year-old severely affected male cousin, confined to a wheelchair since age 12, but without facial weakness, the small fragment was not found; the patient refused to undergo muscle biopsy. These families illustrate complicated situations that may occur for diagnosis and genetic counseling of neuromuscular disorders. Considering that the prevalence of hereditary neuromuscular disorders is very approximately 1/1000, we estimated that the finding of two families with an additional neuromuscular disorder was about three times higher than expected. Therefore, although the presence of different neuromuscular disorders in the same genealogy could be only a coincidence, we speculated that some epigenetic mechanisms present in particular families might turn individuals more prone to pathological mutations.

However in FSHD, more than in other neuromuscular disorders, several “double trouble” conditions patients are described. In these patients the D4Z4 reduced allele is associated with a well-known pathogenic mutation of other genes, causing complex and overlapping phenotypes. In particular, patients with mitochondrial myopathy/FSHD [Filosto et al., 2008], Becker dystrophy/FSHD [Rudnik-Schöneborn et al., 2008], Duchenne dystrophy/FSHD [Lecky et al., 1991; Korngut et al., 2008], Leber’s hereditary optic neuropathy/FSHD [Chuenkongkaew et al., 2005], LGMD1C with rippling disease/FSHD [Ricci et al., 2012], myotonic dystrophy type 1/FSHD [Masciullo et al., 2013] were reported suggesting the possibility of a synergistic effect of those simultaneous mutations in reaching and in modulating the clinical expression. Besides, the coexistence of facioscapulohumeral

muscular dystrophy and myasthenia gravis in a same patient was also reported [McGonigal et al., 2002].

Faciocapulohumeral muscular dystrophy without D4Z4 repeats contraction on chromosome 4q35 (FSHD2)

A subgroup of FSHD subjects, termed patients with FSHD2 (almost 5%), have no contraction of the D4Z4 repeat on chromosome 4q35 [Tawil et al., 2010], although they result clinically indistinguishable from patients with FSHD associated with D4Z4 reduced allele (also defined FSHD1). In 2010 de Greef and coworkers performed a cross-sectional study on 33 patients with FSHD2 from 27 families, the largest cohort described to date. In the above analysis, FSHD2 patients appeared identical to FSHD1 in clinical presentation. Of the 33 patients with FSHD2, 20 (61%) were male. The average age at symptom onset was 26 years (range 0–60), which is almost 10 years later than in FSHD1. The initial symptom was scapular weakness in 61%, foot dorsiflexor weakness in 27%, facial weakness in 10%, and hip girdle weakness in 3%. A gender differences in disease severity in FSHD2 was not observed. Interestingly, notable difference between FSHD1 and FSHD2 is the mode of inheritance. In fact, the analysis showed that he majority (20/33, 67%) were sporadic, 11 were familial, and in 2 the inheritance pattern was uncertain, suggesting that the familial to sporadic ratio in FSHD2 is inverse to the ratio in FSHD1. Of the familial cases, 3 resulted dominant in inheritance (parent-child pairs) and 2 seemed recessive in inheritance (sibling pairs). It has been suggested that similar epigenetic and molecular mechanisms, also supposed to be pathogenic in FSHD associated with D4Z4 repeat contraction, are involved.

Molecular basis of FSHD: pathogenetic hypothesis

As discussed above, soon after the discovery of D4Z4 repeats on chromosome 4, it was established that the sub-telomere of chromosome 10q is almost identical to that of chromosome 4q and that it also contains a highly homologous and equally polymorphic repeat array. A considerable proportion of individuals in the population carry 4q or 10q chromosome ends with repeat arrays that have apparently been entirely or partially transferred between both chromosomes. However, the observation of linkage of the disease with chromosome 4 and the absence of linkage with chromosome 10 led to the hypothesis that the interplay between D4Z4 and other more proximal elements on chromosome 4 could explain the chromosome 4 specificity of the disease [van der Maarel et al., 2011].

Although it is thought that deletions of D4Z4 are causally related to FSHD, it is not clear how this triggers the disease. It has long been speculated that such deletions may alter the expression of genes located within or nearby the repeats.

FRG1 as possible candidate gene for FSHD

The region immediately proximal to the D4Z4 repeats harbors a number of candidate genes. This FSHD locus includes: i) FSHD-related gene 1 (*FRG1*), which encodes a nucleolar protein involved in RNA biogenesis [van Deutekom et al., 1996b; van Koningsbruggen et al., 2007]; ii) FSHD-related gene 2 (*FRG2*), a predicted transcript with no significant homology to any known protein; iii) adenine nucleotide transporter 1 gene (*ANT1*), a gene involved in apoptosis, lying more distally from the 4qter (5.8 Mb) [Doerner et al., 1997; Bodega et al., 2009].

The overexpression of *FRG1*, *FRG2*, and *ANT1* has been found in some muscles affected by FSHD [Gabellini et al., 2002; Laoudj-Chenivesse et al., 2005]. It has been shown that a transcriptional repressor complex binds D4Z4 unit, so it has been supposed that D4Z4 deletion would trigger the gene overexpression as result of the lack of repression [Gabellini et al., 2002]. Gabellini and coworkers, in 2002, found that, in FSHD muscle, 4q35 genes located upstream of D4Z4 resulted inappropriately overexpressed. In particular, it was shown that an element within D4Z4 specifically bound a multiprotein complex consisting of YY1, HMGB2 and nucleolin proteins [Ginisty et al., 1999]. This multiprotein complex bound D4Z4 *in vitro* and *in vivo* and mediated transcriptional repression of 4q35 genes. The authors hypothesized that deletion of repeated elements in the sub-telomeric region of 4q might act on neighboring genes by derepressing their transcription and thus starting a cascade of events which ultimately lead to FSHD, also explaining the autosomal dominant transmission. This hypothesis results also consistent with the observation that haploinsufficiency of distal 4q does not cause FSHD [Tupler et al., 1996]. Interestingly, the extent of 4q35 gene overexpression in FSHD skeletal muscle resulted inversely related to the number of D4Z4 repeats, suggesting a direct correlation with disease severity. Moreover, the observation that 4q35 gene overexpression is muscle specific could explain the muscular phenotype observed in the disease. Finally, the stochastic variation in gene expression in muscle cells may be responsible of the asymmetric muscle involvement and of the great clinical variability reported between and within families [Tupler and Gabellini, 2004].

Consistently with this hypothesis, interesting data come from the work of Gabellini and coworkers in 2006. In this study, a transgenic mice selectively overexpressing in skeletal muscle the 4q35 *FRG1*, *FRG2* or *ANT1* genes was generated. The authors found that *FRG1* transgenic mice developed a muscular dystrophy; by contrast, *FRG2* and *ANT1* transgenic mice resulted normal. The degree of mice muscle impairment appeared correlated with transgene expression levels: in particular, *FRG1*-low mice showed no evidence of kyphosis, whereas *FRG1*-intermediate and *FRG1*-high mice exhibited mild and severe kyphosis respectively, due to muscle degeneration. Skeletal muscle from *FRG1* mice showed histological and ultrastructural dystrophic features characterized by the increase of fibers size variability, necrosis, nuclear centralizations and connective tissue. In the same study, the authors also found that in muscle cells from *FRG1* transgenic mice and from FSHD patients, specific pre-mRNAs, such as fast skeletal muscle troponin T (Tnnt3) and myotubularin related protein 1 (Mtmr1), underwent aberrant alternative splicing. These genes resulted aberrantly spliced also in myotonic dystrophy patients and animal models [Buj-Bello et al., 2002; Kanadia et al., 2003], but not in muscle cell cultures derived from patients with Duchenne muscular dystrophy and congenital merosin deficient muscular dystrophy 1A [Gabellini et al., 2006]. Nevertheless, several follow-up studies could not reproduce the transcriptionally up-regulation of *FRG1*, *FRG2* and *ANT1* in FSHD muscle [Winokur et al., 2003; Celegato et al., 2006; Osborne et al., 2007]. The use of different techniques and different sources of RNA may partly explain this lack of reproducibility [de Greef et al., 2008].

The role of DUX4 gene

Detailed sequence analysis revealed that the D4Z4 repeat contains the open reading frame (ORF) of a double-homeobox transcription factor, *DUX4* [Hewitt et al., 1994], a 424-aa protein. Homeobox sequences, which are highly conserved during evolution [Gehring et al., 1990], encode homeodomains, that are characteristic domains of some gene regulatory proteins, homeobox proteins, coordinating the expression of sets of genes during development.

The *DUX4* ORF is in a single exon, whereas other members of the double-homeobox family have multiple introns, indicating that *DUX4* was inserted into the genome as a retrotransposed mRNA from an intron containing the *DUX* gene [Leidenroth et al., 2010]. In contrast to the many pseudogenes retrotransposed to our genome, the *DUX4* retrogene maintains a conserved ORF [Clapp et al., 2007]. It was proposed that the contraction of the

D4Z4 array results in the transcription of the *DUX4* retrogene, although the abundance of the *DUX4* mRNA and protein results extremely low [Dixit et al., 2007]. However, the study by Lemmers and coworkers [2010], reporting the requirement of *DUX4* polyadenylation site for develop FSHD, have suggested a new developmental model for the disease that is consistent with the extremely low abundance of the mRNA and protein, providing support for the expression of *DUX4* as a major cause of FSHD. The distal end of the repeat array and flanking pLAM1 sequences are thought to be crucially important for the development of FSHD. This hypothesis has been further corroborated by the finding of a FSHD family in which the disease segregated with a contracted D4Z4 allele of chromosome 10: importantly, the last part of this disease-associated repeat array was replaced by permissive chromosome 4 sequences. The identification of this family in which FSHD segregates with chromosome 10 has suggested the importance of the distal end of the repeat and pLAM1 sequences, apparently precluding a prominent role for other proximal candidate genes on chromosome 4. According to this hypothesis, the major transcript in each unit, the *DUX4* gene, is not stable, probably due to the absence of a polyadenylation signal in internal D4Z4 units. Spliced and unspliced transcripts of the *DUX4* gene in the last unit, however, use a unique 3′ untranslated region (UTR) in the pLAM1 region which is immediately distal to this last unit and which contains a poly(A) signal that presumably stabilizes this distal transcript. Consistently, by transfecting this crucial region in murine C2C12 muscle cells, stable *DUX4* transcripts were identified when the constructs derived from permissive chromosomes with the polyadenylation signal. Therefore, these genetic studies seem to demonstrate the requirement for the polyadenylation site utilized by *DUX4* mRNA and to implicate DUX4 protein as a cause of FSHD. However, as noted above, although *DUX4* mRNA was detected in FSHD muscle, it was still at extremely low abundance. It was supposed that low abundance mRNA in a population of cells could reflect either a small amount of mRNA in all cells or an abundant amount of mRNA in just a few cells. RT-PCR amplification of *DUX4* mRNA in small pools of 100 or 600 differentiated FSHD muscle cells identified relatively abundant transcripts in a subset of the pools [Snider et al., 2010]. Approximately 1 in 1000 FSHD muscle cell nuclei were detected with an abundant amount of *DUX4* mRNA. The *DUX4*-expressing FSHD muscle nuclei had characteristics consistent with *DUX4* induced toxicity, including an aggregation of nuclear *DUX4* protein that occurs coincident with *DUX4*-induced apoptosis. Therefore, the very low abundance of *DUX4* mRNA in FSHD muscle represented relatively abundant amounts of *DUX4* mRNA and protein in a small subset of the nuclei,

probably leading to dysfunction or death of those *DUX4*-expressing nuclei [van der Maarel et al., 2011].

In conclusion, in this unifying pathogenic model of FSHD, the inefficient chromatin-mediated repression, either related to the contraction of the array, may result in the occasional escape from repression in muscle cells, and possibly other somatic cells, with a consequently inappropriate expression of *DUX4* protein [van der Maarel et al, 2011]. On this basis, healthy subjects carrying reduced D4Z4 alleles would be explained by the absence of the 4A(159,161,168)PAS [Lemmers et al., 2010].

Specific haplotypes associated with D4Z4 reduced alleles

Since there are individuals with DRA that do not have clinical signs of FSHD, it has been proposed that additional DNA sequences flanking the D4Z4 repeat array are necessary for disease development. In 2002, a polymorphic bi-allelic segment of 10 kb distal to D4Z4 was identified [van Geel et al., 2002]. Of the two allelic forms, 4A and 4B, only 4A was found to be associated with FSHD [Lemmers et al., 2005]. Additional sequence variations, namely Simple Sequence Length Polymorphisms (SSLP), were found proximal to the D4Z4 repeat. Together with the 4A/4B polymorphisms, these SSLPs generate at least 17 and 8 genetically distinct variants, respectively, at the chromosome 4q and chromosome 10q subtelomeres [Lemmers et al., 2007]. Among these many haplotypes only the common variant 4A161 and the rare variants 4A159 and 4A168 were found associated with D4Z4 reduced alleles in FSHD patients. By contrast, D4Z4 reduced alleles associated with other haplotypes were not detected in the FSHD cases. Finally, a single nucleotide polymorphism (SNP) ATTAAA was found in the pLAM1 sequence of the 4qA alleles that provides a PolyAdenylation Signal (PAS) allowing the expression of the most distal copy of the *DUX4* gene [Lemmers et al., 2010]. Thus, it has been proposed that the combination of (1) a reduction in the number of D4Z4 elements, (2) the presence of the 4qA allele, and (3) the PAS in the pLAM1 sequence together with the (159/161/168) SSLPs represents the molecular signature that defines alleles causally related to FSHD. On this basis it has been hypothesized that this particular chromosomal setting, named 4APAS, causes FSHD through a toxic gain of function attributable to the stabilized distal *DUX4* transcript [Lemmers et al., 2010].

Epigenetics in FSHD

The D4Z4 repeat is GC-rich and contains sequences often residing in heterochromatic domains of the genome [Lyle et al., 1995]. DNA methylation analysis and studies of histone

modifications has supported the hypothesis that the reduction of D4Z4 repeat, that normally is in a relatively closed chromatin configuration, causes a more open chromatin configuration facilitating the transcriptional activity of the repeat and possibly affecting the processing of the different D4Z4 transcripts [Jiang et al., 2003; van Overveld et al., 2003; Zeng et al., 2009]. Chromatin immunoprecipitation studies showed that the D4Z4 repeat is normally occupied by both transcriptionally repressive as well as permissive histone modifications. In FSHD patients chromosomes, it is observed a relative loss of repressive histone modifications; these changes in chromatin structure are restricted to the D4Z4 repeat and do not seem to spread proximally. Chromatin immunoprecipitation studies also identified other chromatin factors that were lost or gained, including HP1γ, the cohesin complex, YY1 (lost) and CTCF (gained) at D4Z4 of disease alleles [Gabellini et al., 2002; Zeng et al., 2009; Ottaviani et al., 2009].

At present, the epigenetic model in FSHD is based on the assumption that methylation or histone modifications, as additional levels of complexity, can help interpreting the complex correlation between genotype and phenotype in FSHD. Because the methylation status of CpG sites could play a critical role in chromatin configuration, D4Z4 methylation status was investigated in FSHD patients. The first study investigated methylation at SmaI, MluI, SacII, and EagI methylation-sensitive restriction sites in blood and skeletal muscle samples of FSHD and normal subjects [Tsien et al, 2001]. The authors observed that D4Z4 was found highly methylated in both normal and FSHD lymphoblasts, as well as in somatic tissues, including skeletal muscle. Nevertheless the study did not discriminate methylation status of the D4Z4 repeat array at chromosome 4 and chromosome 10. Subsequently DNA methylation was examined at two methylation-sensitive restriction sites, BsaAI and FseI, in the most proximal unit of D4Z4 array at 4q35, which was considered representative for the entire array [van Overveld et al, 2003]. Through this approach the D4Z4 methylation level can be assessed on both chromosomes 4, excluding chromosome 10, even though the limitation of this test is due to possibility to analyze D4Z4 methylation status only in individuals carrying standard allele constitution of 4-type repeat units on chromosome 4 and 10-type on chromosome 10 (subjects termed disomic), or on individuals carrying one array of 10-type repeat units at normal sized chromosome 4 (subjects termed monosomic). The authors observed normal level of methylation in healthy subjects, significant hypomethylation at both methylation sensitive sites in FSHD1 patients and similar level of hypomethylation in their non-penetrant relatives, carrying the same D4Z4 reduced allele.

Interestingly, in FSHD2 patients level of D4Z4 methylation on both chromosomes 4 was strongly decreased, while it was equivalent among unrelated individuals affected with muscular dystrophy different from FSHD and healthy controls [van Overveld, 2003]. Further investigations of the methylation status were performed by van Overveld and coworkers in 2005 in 21 monosomic FSHD1 patients and 19 monosomic healthy controls [van Overveld et al., 2005]. The study observed that patients with DRA between 10 and 19 kb (1-3 D4Z4 units) showed very low DNA methylation levels, whereas FSHD patients with DRA with 4-6 D4Z4 units, showed inter-individual variation in both clinical severity and D4Z4 hypomethylation.

It is important to highlight that BsaAI and FseI analysis permit only to quantify methylation at two CpG dinucleotides of the first D4Z4 repeat tof he tandem array. For these reasons more recently a new methodology has been proposed for studying methylation status on D4Z4 repeats. Bisulfite sequencing stands out for its reliability in quantifying the DNA methylation status at a single nucleotide resolution in heterogeneous samples. This methodology depends on the oxidative deamination of cytosines into uracils upon treatment by sodium bisulfite while methylated cytosines remain. Bisulfite-treated samples are then amplified by PCR using sequence specific primers designed to amplify methylated and unmethylated DNA with the same efficiency. The multiple DNA fragments are subcloned and sequenced allowing analysis of the methylation status/CpG/DNA molecule [Gaillard et al., 2014]. Using bisulfite sequencing of DNA from blood and myoblast cells, methylation levels at 74 CpG sites across 3 disparate regions within D4Z4 were measured in FSHD2 patients and controls by Hartweck and coworkers in 2013 [Hartweck et al., 2013]. The authors found that rates of demethylation caused by FSHD2 are not consistent across D4Z4. They identified a focal region of extreme demethylation within a 59 domain, which we named DR1. Other D4Z4 regions, including the *DUX4* ORF, were hypomethylated but to a much lesser extent. More recently, Jones and coworkers [2015] analyzed family cohorts for DNA methylation on the distal pathogenic 4q35 D4Z4 repeat on permissive A-type subtelomeres. They found DNA hypomethylation in FSHD1-affected subjects, hypermethylation in healthy controls, and distinctly intermediate levels of methylation in nonmanifesting subjects.

An other assay that has been used in evaluating the level of DNA methylation is the immunoprecipitation of methylated DNA fragments (MeDIP) followed by quantitative PCR, alghout this approch is less direct and less sensitive [Gaillard et al., 2014]. To investigate the

link between clinical signs of FSHD and DNA methylation, the authors explored 95 cases (37 FSHD1, 29 asymptomatic individuals carrying a shortened D4Z4 array, 9 patients with FSHD2, and 20 controls) by implementing two approaches, the methylated DNA immunoprecipitation and the sodium bisulfite sequencing. Both methods revealed statistically significant differences between asymptomatic carriers or controls and individuals with clinical FSHD, especially in the proximal region of the repeat. Absence of clinical expression in asymptomatic carriers resulted associated with a level of methylation similar to controls.

Collectively, in the last three years the partial loss of D4Z4 methylation in FSHD1 and FSHD2 has been demonstrated by Southern blot analysis using several methylation-sensitive restriction enzymes, by bisulfite sequencing and methylated DNA immunoprecipitation (MeDIP) analysis at D4Z4. These studies have shown that the different approaches revealed similar patterns of D4Z4 methylation, where D4Z4 hypomethylation in FSHD is universal across muscle, fibroblasts and peripheral blood mononuclear cells. Nowadays one of the most commonly used methylation-sensitive restriction site to measure D4Z4 methylation is the FseI site as it is highly predictive to FSHD. The FseI site is located approximately 150 bp upstream of the DUX4 transcriptional start sites in every D4Z4 unit but is most often studied in the most proximal D4Z4 unit, because hypomethylation of the proximal D4Z4 unit has been shown to be representative for the entire array.

The hypothetical unifying pathogenic model

To date, the epigenetic model in FSHD speculates that in autosomal dominant FSHD1 D4Z4 chromatin relaxation and DUX4 expression are caused by a contraction of the D4Z4 repeat array to a size of 1–10 units. In the uncommon form of FSHD (FSHD2), D4Z4 chromatin relaxation occurs in the absence of D4Z4 repeat array contraction. In FSHD1, chromatin relaxation and CpG hypomethylation are restricted to the contracted allele, whereas in FSHD2 chromatin relaxation and CpG hypomethylation occur at the D4Z4 repeat arrays of both copies of chromosome 4 and in the highly homologous repeat arrays on chromosome 10 [van den Boogaard 2016]. The D4Z4 methylation is used as a measure of D4Z4 chromatin relaxation. In particular, it has been considered informative measure of D4Z4 methylation by measuring the methylation of all D4Z4 arrays simultaneously at a unique methylation-sensitive restriction site (FseI) in the D4Z4 unit. It has been observed that D4Z4 methylation level at this site is repeat array size dependent. Moreover, the methylation level at this site is significantly lower in FSHD2 compared with both FSHD1 and controls, and a threshold of

25% was established for FSHD2. The D4Z4 repeat array contraction leads to a less repressive local chromatin structure, marked by CpG hypomethylation in the promoter region of the *DUX4* retrogene embedded in the D4Z4 unit, and a greater probability of aberrant *DUX4* expression in skeletal muscle. D4Z4 chromatin relaxation only results in stable *DUX4* expression when the D4Z4 repeat array contraction occurs in cis with a polymorphic *DUX4* polyadenylation signal (PAS) present on a FSHD-permissive chromosomal background (4A). A similar D4Z4 repeat array is located on the equally common chromosome 4B variant and on chromosome 10, but contractions of the array on these locations typically do not result in stable *DUX4* expression and disease owing to the absence of the *DUX4*-PAS. *DUX4* is a transcription factor normally expressed in the luminal cells of the testis, and its expression in muscle activates germline and early stem cell programs eventually resulting in muscle cell death.

SMCHD1 gene in FSHD1 and FSHD2

In 2012 Lemmers and coworkers firstly identified mutations in *SMCHD1* gene by performing whole-exome sequencing in 12 individuals from 6 unrelated FSHD2 families; all these subjects showed a methylation threshold <25%, on the basis of measurements following cleavage with the methylation-sensitive FseI endonuclease, in an assay that averaged the percentage of D4Z4 methylation on both alleles of chromosomes 4 and 10. The authors observed that individuals with FSHD2 inherited both the hypomethylation trait and the FSHD-permissive chromosome 4 haplotype with the *DUX4* polyadenylation signal, suggesting that two independently segregating loci cause and determine the penetrance of FSHD2. They then confiermed heterozygous out-of-frame deletions, heterozygous splice-site mutations and heterozygous missense mutations in *SMCHD1* in 15 out of 19 FSHD2 families (79%). Because heterozygous *SMCHD1* mutations cosegregated with D4Z4 hypomethylation in families with FSHD2 or occurred de novo in individuals with sporadic hypomethylation and FSHD2, the authors considered *SMCHD1* haploinsufficiency to be a candidate disease mechanism, particularly because many of the mutations were predicted to affect production of the full-length protein [Lemmers et al., 2012]. The *SMCHD1* gene on chromosome 18p consists of 48 exons and encodes for a protein containing a putative ATPase and hinge domain. *SMCHD1* encodes a member of the structural maintenance of chromosomes (SMC) protein superfamily involved in chromatin repression of specific genomic regions including the D4Z4 units. In mice, Smchd1 has been shown to be involved in the establishment and maintenance of DNA methylation of a subset of CpG islands on the inactive X chromosome

(Xi), of repetitive sequences, and of monoallelically expressed autosomal genes [Blewitt et al., 2005]. *SMCHD1* binds to the D4Z4 repeat array in somatic cells, and reduced *SMCHD1* binding to the D4Z4 repeat array has been reported in individuals with FSHD2. In *SMCHD1* mutation carriers, all D4Z4 repeat arrays from chromosomes 4 and 10 are hypomethylated. Together, these data are consistent with a role for *SMCHD1* keeping D4Z4 and DUX4 in a repressive chromatin structure in somatic tissue [Lemmers et al., 2015]. Although the reduced D4Z4 methylation in FSHD2 individuals was found in peripheral blood mononuclear cells (PBMCs), fibroblasts and myoblasts, the expression of *DUX4* was, as in FSHD1, only observed in skeletal muscle biopsies and in differentiated myoblasts (12). In the recent work of Lemmers and coworkers in 2015 [Lemmers et al., 2015], the authors further investigated 41 families with one or more individuals with FSHD2 that have not been analyzed for *SMCHD1* mutations previously and, overall, they reported the results obtained on 60 FSHD2 families. All affected individuals from these families had a phenotype consistent with FSHD and a combined CpG methylation level on chromosomes 4 and 10 D4Z4 that was below 25%, the defined threshold for FSHD2. In 51 of the 60 families, they identified an *SMCHD1* mutation. In total, they reported 83 carriers of an *SMCHD1*mutation with an average D4Z4 methylation of 12.1% and 45 unaffected relatives without mutation and with an average D4Z4 methylation of 46.8%. They also identified 9 families (15%) with a total of 17 affected individuals for which they did not find mutations in SMCHD1 despite an average D4Z4 DNA methylation of 16.5% [Lemmers et al., 2015].

The Italian National Registry for FSHD

The Italian National Registry has been developed since 2007 by Italian Clinical Network for FSHD. The FSHD Italian Network is composed by a diagnostic laboratory (Miogen lab) directed by Prof. Rossella Tupler at the Università di Modena e Reggio Emilia and fourteen Clinical Centers with expertise in diagnosis and management of neuromuscular disorders, networked within the Italian Association of Myology (www.miologia.org) and distributed across all of Italy, from northern to southern regions, including the islands. The neuromuscular Clinical Centers are the following: Department of Neurology, IRCCS Fondazione Ospedale Maggiore Policlinico, University of Milan (Prof. Maurizio Moggio) IRCCS Foundation, C. Besta Neurological Institute at Milan (Dr Lorenzo Maggi), Department of Neuroscience, University of Turin (Prof. Tiziana Mongini), Department of Neurosciences, University of Padova (Prof. Elena Pegoraro), IRCCS S. Camillo at Venice (Prof. Corrado Angelini), IRCCS "C.Modino" Foundation, University of Pavia (Dr Angela

Berardinelli), Department of Neurological Sciences and Vision, University of Verona (Dr. Giuliano Tomelleri), University Hospital "Spedali Civili" of Brescia (Dr. Massimiliano Filosto), Department of Clinical and Experimental Medicine, University of Pisa (Prof. Gabriele Siciliano), Department of Neurology, S. Andrea Hospital, "Sapienza" University of Rome (Prof. Giovanni Antonini), Center for Neuromuscular Disease, University "G. d'Annunzio" of Chieti (Dr. Antonio Di Muzio), Department of Neurological Sciences, "Federico II" University of Naples (Prof. Lucio Santoro), Department of Neurosciences, Psychiatry and Anaesthesiology University of Messina (Prof. Carmelo Rodolico), ASL8 University of Cagliari (Prof. Giovanni Marrosu).

Starting from data collected since 2007 in Italian National Registry for FSHD the following studies are ongoing: 1. investigation the natural history of the disease through the prospective clinical evalution of DRA carriers; 2. classification FSHD patients and families in homogeneous sub-groups on the basis of phenotypic features; 3. research for modifier loci/new genes through whole exome sequencing and candidate gene approach. All these informations can provide key clinical and basic research tools useful to identify prognostic predictors of clinical impairment and measures of outcome, with further relevant repercussions for genetic counseling and possible implications in improving the understanding of the molecular pathogenetic aspects of FSHD. From 2008, a solid clinical network has been established, allowing to collect the majority of the FSHD Italian (to date, 2460 carriers of a DRA within 1-10 repeats from 1273 unrelated families). In the Clinical Centers, FSHD subjects have been recruited and clinically evaluated; the neurological examination has been also extended to all available FSHD family members, also addressing relatives to further diagnostic analysis. The Miogen laboratory have been providing molecular characterization of index cases and their family members. A specific software for data management has been designed. The dedicated website for data management, description of the project and participating groups are available on-line at www.fshd.it.

Data from the Italian National Registry for FSHD: the first results of our researchs

A standardized clinical evaluation of patients affected by facioscapulohumeral muscular dystrophy: the FSHD clinical score [Lamperti et al., 2010]

To define numerically the clinical severity of facioscapulohumeral muscular dystrophy (FSHD), we developed a protocol that quantifies muscle weakness by combining the functional evaluation of six muscle groups affected in this disease. To validate reproducibility of the protocol, 69 patients were recruited. Each patient was evaluated by at

least five neurologists, and an FSHD severity score was given by each examiner. The degree of agreement among clinicians' evaluations was measured by kappa-statistics. Nineteen subjects received a score between 0 and 1, 9 had a score between 2 and 4, 20 received a score between 5 and 10, and 8 had a score between 11 and 15. Of the 13 subjects with D4Z4 alleles within the normal range (ranging from 10 to 150 repeats), 12 obtained a score of 0 and only 1 had a score of 1. Kappa-statistics showed a very high concordance for all muscle groups. The clinical form consists of three parts, named a, b, and c, that examine three aspects of the disease and have been designed to facilitate accurate study of molecularly defined FSHD subjects. Part a investigates the patient's clinical history, focusing on medical conditions and particular habits. Part b evaluates the patient's disability. Part c assesses muscle segmental involvement by using the Medical Research Council (MRC) scale. The evaluation procedure allows to assess the strength and the function of muscular groups belonging to I) face (score from 0 to 2); II) shoulder girdle (score from 0 to 3); III) upper limbs (score from 0 to 2); IV) distal legs (0-2); V) pelvic girdle (score from 0 to 5) VI) abdominal muscles (0-1). More detailed information such as asymmetry of presentation or any observed peculiarity can be added in the section "others". The functional examination of six different groups of muscles, as described in part b of the clinical form integrated with the results of part c, generates the FSHD clinical score. The total score can range from 0, when no signs of muscle weakness are present, to 15, when all muscle groups tested are severely impaired (Figure 1). The FSHD clinical form and the FSHD evaluation scale form, as well as a visual guide, are available online at www.fshd.it. English and Italian versions of the two forms can be downloaded from the website. To date, the Italian Network for FSHD successfully use the FSHD clinical score as a tool in genotype/phenotype correlation and genetic counseling.

FSHD Evaluation Scale *[Lamperti et al., 2010]*

I - Facial weakness

0 - no weakness

1 - moderate weakness; partial ability to do at least one of the following tasks:

- to close eyes
- to protrude lips
- to put out cheeks

2 - severe weakness; unable to do at least one of the following tasks:

- to close eyes

- to protrude lips

- to put out cheeks

II - Scapular girdle involvement

0 - no involvement

1 - mild involvement with no limitation of arm abduction

2 - arm abduction > 45

3 - arm abduction ≤ 45

III - Upper limbs involvement *

0 - no involvement

1 - at least two muscles impaired with MRC >3

2 - at least two muscles impaired with MRC ≤ 3

*The following 4 muscles are assessed on each side: 1.triceps; 2. biceps; 3. Common finger extensors and wrist extensors; 4. long finger flexors and wrist flexors. Only the weaker muscles will be considered for evaluation.

IV - Legs involvement

The ability to walk on tiptoes and heels will be assessed on each side:

0 - no involvement

1 - unable to walk on tiptoes or heels (only one task impaired)

2 - unable to walk on tiptoes and heels (two tasks impaired)

V - Pelvic girdle involvement

0 - no involvement

1 - able to walk and to climb stairs without support but abnormally/ because of posterior leg muscle hypotrophy

2 - able to walk unaided, to climb stairs or to stand up from a chair with support

3 - able to walk unaided but unable to stand up from a chair or to climb stairs without support/ more than 12 seconds

4 - able to walk with support

5 - wheelchair bound

VI - Abdominal muscle involvement

0 - no involvement

1 - presence of Beevor's sign

New insights from compound heterozygotes and implication for prenatal genetic counseling in facioscapulohumeral dystrophy [Scionti, et al., 2012a].

Through the INRF, genotype-phenotype correlations were extensively studied in 11 non-consanguineous families in which two D4Z4-reduced alleles segregate. Overall, 68 subjects carrying D4Z4-reduced alleles were examined including 15 compound heterozygotes. The FSHD score resulted significantly higher in compound heterozygotes than in patients carrying on a single D4Z4-reduced allele. Notably, it was found that 82% of subjects carrying a single D4Z4-reduced 4APAS haplotype were non-penetrant carriers and in 4 families the only FSHD affected subject was the compound heterozygote. In summary, this study has shown that compound heterozygotes display a more severe clinical outcome than subjects carrying a single D4Z4-reduced allele evoking the presence of a dosage effect of D4Z4-reduced alleles. Remarkably, the high frequency of compound heterozygotes and the presence of D4Z4-reduced alleles with the 4APAS pathogenic haplotype in 82% of non-penetrant subjects suggest that carriers of FSHD-sized alleles are more common in the general population than expected on the basis of FSHD prevalence. In fact, 2.7% of cases in the INRF (which contains over 1100 unrelated FSHD patients) were compound heterozygotes carrying two D4Z4-reduced alleles (0,5% were homozygotes for the 4A161 haplotype) and, based on this finding, it has been possible to estimate that the population frequency of the 4A161PAS haplotype associated with a D4Z4-reduced allele could be higher than 1%. These findings challenge the notion that FSHD is a fully-penetrant autosomal dominant disorder, with crucial consequences for genetic counseling and prenatal diagnosis.

Large scale population analysis challenges the current criteria for the molecular diagnosis of fascioscapulohumeral muscular dystrophy [Scionti et al., 2012b]

The reduction in the number of D4Z4 elements combined with the 4A(159/161/168)PAS haplotype (which provides the possibility of expressing *DUX4*) has been used as the genetic signature uniquely associated with FSHD. However, the frequency of compound heterozygotes in patients with FSHD suggested that the frequency of D4Z4-reduced 24-35 kb alleles associated with the 4A161PAS in the Italian population would be >1% [Scionti et al., 2012a]. In order to confirm the high frequency of this signature in the normal population and reevaluate the allele distribution in FSHD patients, it was performed a systematic unbiased clinical and molecular study of 801 normal control subjects from Italy and Brazil and 253

FSHD probands from the INRF. Control subjects were recruited through advertisements from the Italian populations and resulted equally distributed among Northern, Central and Southern regions. DNA from Brazilian controls was provided by Department of Genetics and Evolutive Biology, Institute of Biosciences, University of São Paulo. It was observed that 3% (25 of 801) of normal controls carried D4Z4 alleles of reduced size and 11 (~1.3%) had the supposedly pathogenic 4A161PAS haplotype. The age of all these healthy carriers ranged between 40 and 78 years, an age in which FSHD is considered to be fully penetrant. Only 127 FSHD probands carried the 4A161PAS haplotype associated with alleles having 1-8 D4Z4 repeats. Among the remaining probands, 52 showed reduced alleles associated with the 4A166PAS haplotype previously considered not to be "permissive" for FSHD disease, 13 carried the 4A162PAS, 5 the 4A164PAS, 2 the 4A167PAS, 1 the 4A163PAS, and 3 of them carried reduced D4Z4 alleles with the 4qB polymorphism which lacks both the pLAM1 region and the PAS. Collectively, these data suggested that SSLP allelic variants associated with D4Z4-reduced alleles differed from those previously reported [Lemmers et al., 2007], since the 4A168 "permissive" haplotype associated with FSHD was not found in the study. Interestingly, haplotypes considered not to be "permissive" for FSHD disease were frequent. In particular the 4A166PAS haplotype was reported associated with almost one quarter (23.3%) of D4Z4 reduced alleles detected in FSHD probands. More importantly, 49 of 253 FSHD probands (19%) carried alleles with more than 8 D4Z4 repeats and only 127 (50.1 %) showed D4Z4 reduced alleles associated with the 4A161PAS, the expected molecular signature for FSHD. Therefore, the study shows that the current genetic signature of FSHD is a common polymorphism and only half of FSHD probands carry this molecular signature.

Large scale genotype–phenotype analyses indicate that novel prognostic tools are required for families with facioscapulohumeral muscular dystrophy [Ricci et al., Brain 2013, Annex 2]
The recent study performed by the Italian Clinical Network for FSHD evaluated the degree of motor impairment in a large group of patients affected by facioscapulohumeral muscular dystrophy and their relatives who carry D4Z4 reduced allele (DRA). Clinical assessment was performed in 530 subjects, 163 probands and 367 relatives, from 176 unrelated families according to a standardized clinical score previously validated [Lamperti et al., 2010]. Overall, 32.2% of relatives did not display any muscle functional impairment. This phenotype was influenced by the degree of relation with proband, because 47.1% of second- through fifth-degree relatives were unaffected, while only 27.5% of first-degree family

members did not show motor impairment. The estimated risk of developing motor impairment by age 50 for relatives carrying a DRA with 1-3 repeats or 4-8 repeats was 88.7% and 55% respectively. Male relatives had a mean score significantly higher than females (5.4 vs 4.0, p=0.003). No 4q haplotype was exclusively associated with the presence of disease. In 19 (13%) families in which DRA with 4-8 repeats segregate, the diagnosis of FSHD was reported only in one generation. In 5 pedigrees the pattern of inheritance was compatible with the presence of an autosomal recessive trait since there were at least two affected sibs born from asymptomatic parents. In these cases the lack of autosomal dominant inheritance should prompt us to consider whether the disease develops because of the presence of additional genetic defect(s). This possibility is supported by recent observation that mutations in the SMCHD1 gene segregate independently from the FSHD permissive D4Z4 allele on chromosome 4 in FSHD subjects that do not carry a DRA, also defined as patients with FSHD2 (Lemmers et al., 2012). Therefore searches for secondary FSHD loci should be considered in all cases in which the ratio between affected and unaffected individuals expected for an autosomal dominant disease is not observed and random association between the DRA and FSHD cannot be excluded. For all of these reasons, to define the predictive value of DRA, it is necessary to carry out clinical evaluation and collection of DNA samples of all of the proband's family members, not only in a research setting but also in clinical practice. We believe that broadening the analysis of FSHD families may facilitate genetic counselling of patients and families with FSHD in particular when interpreting the data for prenatal diagnosis.

Aims of the thesis

Through the clinical research activity conducted in collaboration with the Italian Network of FSHD, the objective of my PhD program has been to dissect the phenotypic and molecular characterizaction of FSHD families from the Italian National Registry for FSHD (INRF) to test the predictive significance of molecular variations in FSHD. INRF represents an unique epidemiologic tool to correlate molecular data with clinical examination and anamnestic records in FSHD families. The precise phenotypic classification of patients and families as well as the pattern of inheritance will be crucial to the definition of parameters to sub-classify FSHD patients and families that are fundamental for good clinical prectice and eventually to identify new susceptibility/causative factors contributing to FSHD.

FSHD has been associated with reduced numbers (<11, alleles ≤41 kb) of D4Z4 repeats at 4q35. However 3% of healthy individuals carry D4Z4 reduced alleles (DRA). This fact makes molecular diagnosis in FSHD troublesome, especially in presence of atypical phenotypes. Moreover, the clinical heterogeneity associated with DRA together with the incomplete penetrance emphasize the concept that the current genetic signature of FSHD alone may not be sufficient to produce disease. Additional molecular factors such as level of D4Z4 methylation or mutations in other genes (i.e. *SMCHD1* gene) have been proposed to play a role in disease outcome. Thus there is the need for clinical practice and research to re-evaluate the significance and the predictive value of DRA.

Our first genotype-phenotype study on a large cohort of patients and families (Ricci et al., 2013) established that D4Z4 alleles correspond to various phenotypes and degree of severity of clinical expression.

We therefore decided to investigate patients with 1-3 D4Z4 repeats (1-3 DRA) and cases carrying alleles with 9-10 D4Z4 repeats, which reprent the two extremes of the molecular diagnostic spectrum.

Therefore, the aims of this thesis has been to perfom a large and comprehensive genotype-phenotype correlation study in families in wich a 1-3 and 9-10 DRA segregates, recruited from the Italian National Registry for FSHD, in order to a) carry out a detailed clinical characterization/subclassification of probands and relatives; b) analyse disease penetrance and expression; c) study the mode of inheritance (familial/sporadic cases). We also aimed to test the diagnostic and predictive significance of other possible genetic modifiers in subjects carrying 9-10 DRA, such as the level of D4Z4 4q35 methylation and mutations in *SMCHD1* gene.

Matherials and methods

Recruitment of index cases and relatives carrying D4Z4 reduced allele with 1-3 repeats (1-3 DRA)

The study has been performed on carriers of 1-3 DRA accrued through the INRF by the Italian Clinical Network for FSHD (ICNF) from January 2008 to December 2013. Of 850 index cases from the INRF in December 2013, we identified 114 index cases carrying DRA with 1-3 repeats and fulfilling the clinical diagnostic criteria defined for FSHD. Family studies were conducted in 66 index cases, in which clinical and molecular analysis was extended to all available relatives willing to participate. Screening for 1-3 DRA was

performed in 226 relatives. We defined *de novo* cases as single participant with neither parent carrying DRA; when the DRA was detected in one of the parents and/or other family members (ie, sibs), we classified the participant as familial.We considered participants as not informative when it was not possible to examine their parents and/or other informative family members. Informed consent, according to the Declaration of Helsinki, was obtained from each participant enrolled in the study. The clinical examination was performed using the standardised FSHD clinical protocol with validated interrater reliability [Lamperti et al., 2010]. In order to investigate the earliest signs of disease and to rule out pre- or perinatal events as possible causes of delayed achieving of motor milestones, we designed the Infantile Anamnestic Questionnaire (IAQ) (Annex 3). All data about: (1) pregnancy, (2) birth, (3) the prenatal period and first month of life and (4) psychomotor and language development were collected in a retrospective manner. Items related to each section were scored as normal/altered. We collected anamnestic reports about neurological examinations in the first year of life, together with clinical and instrumental data in the following years, whenever possible, in 80 cases carrying 1–3 DRA [Nikolic et al., 2016, Annex 4].

Study design for definition of a clinical tool for the FSHD phenotypes classification

Through the systematic use of the FSHD Clinical Form [Lamperti et al., 2010; Scionti et al., 2012a; Ricci et al., 2013; Nikolic et al., 2016] we recognized that it assesses the severity of motor impairment by translating disability into a number (FSHD Evaluation Scale), but it does not capture clinical features that may describe various phenotypes. To overcome this limitation we integrated several items including typical and atypical features on the basis of published reports describing the clinical phenotypes observed in carriers of a DRA [reviewed in Ricci et al., 2014]. Typical and atypical clinical features were combined in the new CCEF, which includes the Evaluation Form (CCEF Section 1, Annex 5), the FSHD Evaluation Scale (CCEF Section 2, Annex 5), the Clinical Diagnostic Form (CCEF Section 3, Annex 5) and the Clinical Categories (CCEF Section 4, Annex 5). The definition and the validation of the CCEF were performed in two steps. We first recruited 106 subjects carrying a DRA with 1-9 units (11-38 kb) to test the clinical application of this new tool. The recruitment was based on 452 subjects examined by the Italian Clinical Network for FSHD (ICNF) in two-year time-window (2008-2009). Subjects were summoned by consecutive phone calls following the order of the previous recruitment. We called those near the clinical centers of Modena, Turin and Naples. The latter choice was made to avoid people a long-distance trip. We organized

three meetings dividing the 106 available subjects into three groups on the basis of their geographical location (Northern, Central and Southern Italy). Twelve experienced clinicians of the ICNF were selected according to their geographical location, so that four neurologists examined patients from each one of the three groups. The four selected neurologists used the CCEF to evaluate each subject of a single group independently. The results of this first round of clinical applications were discussed in a subsequent meeting. We revised the emerged critical points, i.e. some difficulties in establishing mild facial weakness, and approved the final version of the CCEF (Annex 5). Then, in a second round the inter-rater reliability in assigning patients to different phenotypic categories by using the new CCEF was tested. Two clinicians, selected by drawing lots, examined additional 56 subjects (Table 1) recruited from the cohort of 452 subjects as described above. The two clinicians administered the functional motor evaluation test of the Evaluation Form (Annex 5, Section 1, parts b and c) to each subject and calculated the FSHD clinical score on the basis of the FSHD Evaluation Scale, previously validated [Lamperti et al., 2010]. Then, the two clinicians completed the Clinical Diagnostic Form (CCEF Section 3) and assigned each subjects to one of the nine clinical subcategories (CCEF Section 4) independently. A tutorial for the clinical assessment is available at www.fshd.it. It takes 20 minutes to collect clinical information and complete the neurological evaluation.

Signed informed consent from patients was obtained before inclusion in the study [Ricci et al., 2016, Annex 6].

Table 1. *Characteristics of the 56 FSHD patients enrolled in the CCEF inter-rater reliability study.*

		Patients	
		Number (n)	**Percentage (%)**
Sex	*Male*	27	48.2
	Female	29	51.8
Age at examination (years)	*14-40*	19	33.9
	41-60	20	35.7
	61-74	17	30.4
FSHD score	*0-5*	28	50.0
	6-10	21	37.5
	11-15	7	12.5

D4Z4 allele size (U)	*1-3*	7	12.5
	4-6	38	67.9
	7-8	8	14.3
	9-10	3	5.4

Recruitment of index cases and relatives carrying 9-10 DRA

The study has been performed on FSHD families accrued through the INRF by the Italian Clinical Network for FSHD (ICNF) in seven-years time-window (2008-2014). All clinical and molecular data have been collected in the INRF database at Miogen Laboratory of University of Modena. Out of 1083 probands from the INRF, we identified 147 index cases carrying 4qA DRA with 9-10 repeats (almost 14%). Clinical and molecular analysis was extended to all available and willing to participated relatives. The phenotipic characterizaction of index cases and relatives was assessed by using the new clinical tool, the CCEF. Informed consent, according to the Declaration of Helsinki, was obtained from each participant enrolled in the study.

Statistical analysis

Assessment of the CCEF inter-rater reliability. The inter-observer reproducibility between the two examiners respect to the four and nine CCEF categories was assessed using the kappa statistics [Fleis, 1981]. Kappa value scores are interpreted as follows: kappa value 1.0 = perfect agreement; kappa value $\geq 0.75 < 1.0$ = excellent; kappa value $> 0.40 < 0.75$ = good; kappa value ≤ 0.40 = poor. The 95% confidence intervals of kappa statistics were calculated using the (biased corrected) bootstrap resampling method [Lee and Fung, 1993].

The genotype-phenoptype correlations analysis was performed using the following statistical tests: Wilcoxon rank-sum and Kruskal-Wallis test, χ^2 test, Wald tests, Kaplan-Meier survival analysis, Student's t-test, univariate analysis of variance. The association between clinical parameters was anlayzed computing *Pearson r* correlation coefficient.

Molecular analysis

Standard molecular test for FSHD

Lymphocytes isolation from whole blood

- 10 ml of fresh blood are collected into a tube containing EDTA as anticoagulant.
- Blood is diluted with an equal volume of 1X PBS.
- 20 ml of the diluted blood are carefully layered over 10 ml of Lympholyte-H in a 50

ml Falcon tube.

- Samples are centrifuged at 800g for 45 minutes at room temperature in a G8 swing-out rotor without brake.
- After centrifugation, it should appear a well-defined lymphocyte layer at the interface. The cells from the interface are removed using a sterile Pasteur pipette, without collecting any of the clear Ficoll layer and transferred into a new centrifuge tube.
- The transferred cells are diluted with 1X PBS up to 30 ml.
- Samples centrifugation at 500g for 5 minutes.
- Cell pellet is washed twice with 10 ml of 1X PBS.

Inclusion of cells in agarose plugs

- Pellet is resuspended gently in 10 ml of 1X PBS.
- Cells are counted with NucleoCounter (Chemometec, Denmark).
- After cell count, cells are spun down and cell pellet is resuspended in PBS to a homogeneous concentration of 2x106 per 40µl. Avoiding formation of air bubbles is recommended.
- An equal volume of 1% Low Melt Agarose in PBS (kept at 50°C) is added to the cell suspension. Cells are mixed gently with a pipette to ensure even dispersion through the agarose and then dispensed immediately into the plug molds (BioRad). Plugs are placed at 4°C in order to allow the agarose plugs to solidify for 20-30 minutes. Each well, in BioRad's disposable plug mold, holds approximately 80 µl of samples and contains 2x106 cells.

DNA isolation in agarose blocks

- Plugs are gently transferred into a 2 ml microfuge tube containing the extraction solution: 1X TE, 1% sarcosyl with EDTA 0,5M (pH 8.0) and Proteinase K (0,5 mg/ml).
- Samples are incubated at 50°C for 48 hours.
- After the Proteinase K treatment, plugs are put on ice for 30 minuts to allow blocks to set.
- After the 30 minutes on ice, the extraction solution is removed and plugs are washed for 5 minutes with distilled sterile water. Washing is repeated for 3 times.
- Plugs are then transferred in a new 2 ml microfuge tube and, after addition of PMSF solution (40µg/ml with TE pH 8.0 – 1ml/plug), are incubated at 50°C for 30 minutes.
- After PMSF treatment, plugs should solidify on ice for 30 minutes, allowing blocks to set.

- PMSF solution is removed and plugs are washed for 5 minutes with distilled sterile water, and washing is repeated for 3 times.
- Plugs are stored in a 0,5M EDTA, pH 8.0 (at least 1 ml per plug), at 4°C.

Restriction endonuclease digestion of DNA in agarose blocks

- EDTA solution is discarded and plugs are washed for 3 times, for 5 minutes with distilled sterile water.
- ½ of plug (corresponding to 1,0x106 cells) is used for each DNA digestion.
- Each plug slice is transferred in a 2 ml microfuge tube and equilibration mix is prepared (appropriate restriction buffer 1X and water). Samples are incubated for at least 1 hour at 37°C.
- After equilibration, the restriction buffer is discarded and digestion mix is prepared (appropriate restriction buffer 1X, restriction enzyme and water):
- EcoRI: 6U/µl (final volume: 200 µl), overnight at 37°C. 1 plug is digested.
- BlnI: 0,25U/µl (final volume: 200 µl), 4 hours at 37°C. 1/2 plug is digested.
- After digestion, plugs are placed on ice for 30 minutes.

Pulsed Field Gel Electrophoresis (PFGE)

- DNA is separated by PFGE on a 1% agarose gel (Megabase agarose, Biorad) . The electrophoresis is performed in 0,5X TBE at 14°C.
- To detect EcoRI, interval is 10-200 kb, switch times increases from 0,74 sec to 17,33 sec at the end of each cycle and it runs for 20 hours and 18 minutes.
- After electrophoresis, the gel is stained with ethidium bromide and blotted to a Nylon+ membrane (ZetaProbe, Biorad).
- Sizes of each chromosome are estimated according to a HMW marker (8-48 kb, Roche).

Isolation of High Molecular weight DNA from frozen blood samples

- Frozen blood in tubes containing EDTA as anticoagulant is thawed.
- To disrupt red blood cells 5 volumes of sterile cold TE 20:5 are added to each blood sample and let it set on ice for 1 hour.
- Samples are centrifuged at 3000 rpm for 10 minutes at 4°C. Supernatant is decanted. Cell pellet is washed with 15 ml of sterile cold TE 20:5 and spun again as previously

described, until the buffy coat becomes white and all red cells have been removed.

- Cell pellet is resuspended in lysis solution: 1/2 blood volume of TE 1X, Sarcosyl 1% and Proteinase K (final concentration 100 µg/ml).
- Samples are incubated over night at 37°C or 2 hours at 55°C.
- After the proteinase K treatment, the solution is transferred into a 15 ml tube. An equal volume of phenol equilibrated with 0,1M Tris-HCl (pH 8.0) is added and mixed by gentle agitation for 15 minutes, at room temperature. To separate the aqueous phase, the sample is centrifuged for 15 minutes at 3000 rpm.
- The 90% of the upper viscous aqueous phase is transferred to a clean centrifuge tube, carefully avoiding protein localized at the aqueous:phenol interface.
- An equal volume of phenol:chloroform:isoamilic alcohol (24:25:1) is added to the aqueous phase. Samples are then mixed for 15 minutes, centrifuged for 15 minutes and aqueous phase is then transferred to a clean tube, as described above.
- An equal volume of chloroform:isoamilic alcohol 25:1 is added. Samples are then mixed for 10 minutes, centrifuged for 10 minutes and the aqueous phase is transferred to a clean tube.
- DNA is precipitated with 5M NaCl at final concentration of 200 mM and 2 volumes of cold ethanol absolute.
- Dried DNA is dissolved in TE 10:1

Digestion of DNA and linear gel electrophoresis (LGE)

- 14 µg of DNA are digested with EcoRI (50U) overnight. Digestion is checked on a 1% agarose gel.
- DNA is precipitated with 5M NaCl at a 200 mM final concentration and 2-3 volume of cold absolute ethanol.
- DNA is washed with ethanol 70% and dried DNA is dissolved in BlnI buffer.
- Half of the DNA is transferred in a clean 1,5 ml microfuge tube and 20U of BlnI enzyme are added to the samples.
- DNA samples are incubated at 37°C for 2 hours.
- Seven micrograms of genomic DNA, digested with EcoRI, double digested with EcoRI/BlnI is separated on a 0,4% agarose gel. The electrophoresis is performed in 1X TAE buffer at 4°C for 48 hours.
- After electrophoresis, the gel is stained with ethidium bromide, photographed under

UV light and blotted to a Nylon membrane (ZetaProbe, Biorad).

• Sizes of each chromosome are estimated according to a HMW marker (8-48 kb, Roche) and 5 kb marker (5-50 kb, Roche).

Southern Blotting

• After staining of the gel with ethidium bromide solution, DNA is nicked by agitating the gel in a tray containing a fair amount of 0,25 M HCl for 10 minutes.

• After 10 minutes, HCl solution is removed through abundance of distilled water.

• Gel is then soaked in 0,5M NaOH solution for 20 minutes. DNA is transferred onto ZetaProbe GT Nylon membrane (BioRad) using 1 liter of 0,5M NaOH, through capillary transfer.

• Capillary transfer is set up as a follow, from bottom to top:

- Pyrex glass dish, containing 1 liter of NaOH solution
- Glass plate
- One sheet of blotting paper (3mm) as a wick
- Agarose gel (top side down)
- ZetaProbe GT membrane cut to the size of the gel and prewetted with NaOH solution
- Four sheets of blotting paper
- A stack of paper towels 10 cm high
- Glass plate
- 1 kg of weight

• DNA is transferred for 18 hours.

• Paper towel and blotting papers are carefully removed.

• Membrane is rinsed in 2X SSC solution.

• Membrane is then dried by blotting onto 3mm paper, at 80°C, for 40 minutes and it can be used for hybridization.

Probe labeling and hybridization

• Membrane is incubated in pre-warmed “Myracle hybridization solution” (Stratagene) for 1 hour at 68°C in a roller bottle.

• Probe p13E-11 (D4F104S1) is labeled with 32P-dATP using High Prime DNA labeling Kit (Roche).

• Radio-active labeled probe is denatured by heating in a boiling water bath for 5 minutes and chilled quickly in ice.

- Probe (1-2X106 cpm/ml) is added to pre-warmed "Myracle hybridization solution".
- Membrane with probe is incubated overnight at 68°C.
- Hybridization solution is removed and membrane is washed quickly with solution 1 (2X SSC with 0,1% SDS) and then incubated for 20 minutes, at room temperature, with the same solution.
- Solution 1 is removed and the rinse solution is replaced with 50 ml of pre-warmed solution 2 (0,5X SSC with 0,1% SDS). Membrane is incubated for 30 minutes at 65°C.
- Solution 2 is removed and the rinse solution is replaced with 100 ml of pre-warmed solution 3 (0,1% SDS). Membrane is incubated for 30 minutes at 68°C.
- Membrane is then removed from the bottle and covered with plastic seal wrap.
- Membrane is placed into a cassette and exposed to X-ray film (Kodak X-Omat LS) at -80°C with an intensifying screen to obtain an autoradiographic image.

FseI methylation assay (Methylation Sensitive Restriction Enzyme analysis, MSRE)

DNA methylation level was analyzed through two approaches: MSRE1 and MSRE2.

MSRE1 allows to assess the level of DNA methylation from the two CpGs positioned in the most proximal D4Z4 repeat in the both 4q chromosomes, excluding chromosome 10q. The limitation of this test is due to possibility to analyze D4Z4 methylation status only in individuals carrying standard allele constitution of 4-type repeat units on chromosome 4 and 10-type on chromosome 10 (disomic), or on individuals carrying one array of 10-type repeat units at normal sized chromosome 4 (monosomic)[van Overveld et al, 2003]. For MSRE1 approach we applied the following method:

a) 10 μg of DNA extracted from isolated lymphocytes are digested for 5 hours with 100U of BglII (NEB). Digestion is checked on 1% agarose gel.

b) DNA is precipitated with 3M NaOAc in a 300 mM final concentration and 2.5 volume of cold absolute ethanol.

c) DNA is washed with ethanol 70% and dried DNA is dissolved with CutSmart buffer (NEB) at 37ºC for 1 hour.

d) Dissolved DNA is subsequently digested with 40U of AvrII (NEB) at 37ºC for 2 hours.

e) Mix is divided in two (5 μg each) separate 1,5 ml microfuge tube.

f) 5 μg are digested with 14U (NEB) of methylation sensitive restriction enzymes FseI (NEB).

g) 5 μg DNA digested with BglII/AvrII/FseI are separated on 0,8% agarose gel. The electrophoresis is performed in 1X TAE buffer for 24 hours.

h) After electrophoresis, the gel is stained with ethidium bromide and blotted to a Nylon membrane (ZetaProbe, Biorad).

i) DNA is hybridized with the radioactive-labeled probe p13E-11.

l) Digested fragments are detected by autoradiography with Typhoon Trio system (GE Healthcare).

The intensity of the band representing was estimated with Phosphor imager and methylated band and unmethylated band acquired a percentage. The percentage representing the intensity of methylated band is extrapolated and considered as a level of methylation on the examined FseI site.

MSRE2 approach analyzes the level of D4Z4 methylation on both chromosomes 4q and both chromosomes 10q. Therefore the subjects without suitable chromosomal profile for MSRE1 analysis can be analyzed with MSER2. For the methylation analysis of the proximal D4Z4 repeats units on both alleles of chromosomes 4q35 and 10q26 (MSRE2), we applied the same protocol, but the DNA was not digested with AvrII, as described at point d).

Anlysis of SMCHD1 gene

Genomic DNA analysis of *SMCHD1* gene was performed by next-generation sequencing (NGS) workflow and sequencing analysis at Telethon Institute of Genetics and Medicine (TIGEM), Naple (see Savarese et al., 2016). A library according to the manufacturer's instructions (HaloPlex Target Enrichment System for Illumina Sequencing; Agilent Technologies, Santa Clara, CA) was drawn up. Bioanalyzer High Sensitivity DNA Assay kit (Agilent Technologies) was used to validate and quantify library preparation. Twenty individual samples were run in a single lane of a HiSeq1000 system (Illumina Inc., San Diego, CA), generating 100-base pair paired end reads. Analysis of NGS data was performed using an in-house pipeline described in Savarese et al., 2014. The variations detected by NGS approach was then tested by amplicon Sanger sequencing.

Results

Clinical expression of facioscapulohumeral muscular dystrophy in carriers of 1-3 DRA

In 66 unrelated index cases, we extended molecular characterisation to parents and/or other relatives and found that 26 probands were familial (39.4%) and 40 probands were de novo

(60.6%). To verify the prevalence of infantile onset, we then subdivided these two groups of patients on the basis of the age at onset, before 10 years and after 10 years of age (table 1B). In 54.5% of index cases carrying 1-3 DRA, the disease onset was in the first decade of life. A higher percentage of these cases with infantile onset reported facial weakness as the most common sign of disease (76.5%), while shoulder girdle weakness at onset resulted most commonly (69%) among patients developing FSHD after 10 years of age. Notably, the extimation of the age-specific cumulative motor impairment incidence in the two cohorts of de novo and familial probands by Kaplan-Meier survival analysis revealed that the risk of developing FSHD before 10 years of age is significantly higher in participants carrying a de novo 1-3 DRA. In particular, the analysis showed that among participants carrying a de novo 1-3 DRA, the risk of developing motor impairment is 65% by age 10 years, 88% by age 15 years and 98% by age 20 years. Among participants carrying a familial 1-3 DRA the risk is 38% by age 10 years, 77% by age 15 years and 88% by age 20 years.

We then tested whether de novo index cases present a more severe disease expression, comparing the degrees of motor disability indicated by the FSHD score. Statistical evaluation failed to detect any significant difference in the mean FSHD score adjusted by age and sex between the two groups (Wald test p value=0.280). Moreover, comparison of the severity of disease expression between index cases (both de novo and familial) with early age at onset (0–10 years; age and sex adjusted mean FSHD score=11.4) versus probands with age at onset over 10 years (age and sex adjusted mean FSHD score=9.5) failed to detect statistically significant more severe FSHD disease in probands with infantile onset (Wald test p value=0.064).

No patients had perinatal onset. Among index cases, 36 (54.5%) showed the first signs by 10 years of age. The large majority of patients with early disease onset (26 out of 36, 72.2%) were de novo; whereas the majority of patients with disease onset after 10 years of age were familial (16, 53.3%). Comparison of the disease severity outcome between index cases with age at onset before and over 10 years of age, failed to detect statistical significance (Wald test p value=0.064). Of 61 index cases, only 17 (27.9%) presented extra-muscular conditions. Relatives carrying 1–3 DRA showed a large. clinical variability ranging from healthy subjects, to patients with severe motor impairment. We gathered anamnestic data
about pregnancy, delivery and birth from all participants who were able to respond to this questionnaire. We interviewed 80 cases carrying 1–3 DRA.

No significant alterations in pregnancy, delivery and birth were reported. There was no report of any floppy infant at birth. In 72 of 80 participants (90%), psychomotor development

milestones were reached appropriately. This analysis shows that children carrying 1-3 DRA do not display signs of muscle weakness prenatally or at birth. Moreover, signs that can possibly be attributed to early onset of muscle weakness are reported only in a small percentage of participants. Therefore we conclude that very early onset is not a frequent feature of FSHD. Thirteen participants suffered from sensorineural deafness (21.3%). In eight cases, it was isolated, with no other recognisable medical condition, and in five cases we detected additional extra-muscular manifestations. In four cases, we observed Coats' retinopathy (6.6%). In one it was found as an isolated condition, whereas in three other cases it was associated with sensorineural deafness or cognitive impairment. Cognitive impairment was reported in six cases (9.8%), and two of these also suffered from epilepsy. All cases with mental retardation showed a very severe form of disease.

Overall, the above results are detailed in Annex 4 [Nikolic et al., BMJ Open 2016].

Comprehensive Clinical Evaluation Form (CCEF)

The CCEF consists of four sections. The first section, the Evaluation Form (Section 1, Annex 5), investigates the subject's clinical history (part a), evaluates the patient's disability (part b) and assesses muscle segmental involvement by using the Medical Research Council (MRC) scale (part c). The other sections include the FSHD Evaluation Scale (Section 2, Annex 5), the Clinical Diagnostic Form (Section 3, Figure 2) and the Clinical Categories (Section 4, Figure 3).

Several items are examined in the Evaluation Form section.

- Family history. Questions such as "did/does any of your relatives have a posture like yours?", "was any of your relatives sleeping with half-open eyes?" are asked to identify subjects with possible muscle weakness suggestive of FSHD.
- Evaluation of age at onset. To obtain a more objective evaluation of age at onset and the type of muscle initially affected, we introduced specific questions, such as "have your relatives ever noticed that you were sleeping with half-open eyes?", "when have you noticed the appearance of winged scapula?", "have you ever noticed thinness of upper arms or a dropped shoulder?", "have you ever noticed asymmetry of the mouth or smile when looking in a mirror or in past photographs from childhood?".
- Functional motor evaluation. For a precise description of the distribution of muscle weakness, the CCEF evaluates: a) the presence of widened palpebral fissures; orbicular oris weakness, horizontal smile; inability to protrude lips, to puff out cheeks, to close eyes and

bury the eyelashes (facial weakness); b) the maximum degree in abducting arms (scapular girdle weakness); c) the ability to climb 4 stair-steps, to stand up from a chair, to rise from the floor, to walk (pelvic girdle weakness); d) the ability to walk on tiptoes and/or heels (distal legs weakness); e) the presence of Beevor's sign (abdominal muscles weakness).

- Evaluation of segmental muscle strength by MRC scale. Fourteen muscle groups are examined. Neck extensors are evaluated as single muscle group; external-rotator muscles of upper limb, triceps, biceps, common finger extensors, wrist extensors, long fingers flexors, wrist flexors, gluteus maximum, iliopsoas, quadriceps, biceps femoris, triceps surae, tibialis anterior are evaluated on both sides.
- Annotation of typical signs. Shoulders with symmetric/asymmetric winging on attempted shoulder abduction or forward flexion, straight clavicles, forward sloping of shoulders at rest, axillary creases reflecting pectoral muscle wasting, sunken or flattened appearance of the chest, "poly-hill sign" with neck, shoulders and arms observed from behind in fullest possible abduction (70–90°), with external rotation of the shoulders, hyperlordosis.
- Annotation of atypical signs. Palpebral ptosis, myotonic phenomenon, muscle rippling, weakness of extra-ocular, masticatory, pharyngeal and lingual muscles, bent spine syndrome, early contractures, pes cavus , dropped head, myoglobinuria and persistently high CK values above the level of 1000 U/L are considered atypical signs [Padberg et al., 1992; Ricci et al., 2014]. The presence of cardiomyopathy and a respiratory restrictive insufficiency at onset or in subjects still walking (FSHD score <12) is also considered an atypical sign [Padberg et al., 1992; Ricci et al., 2014].

The Evaluation Form allows completing the FSHD Evaluation Scale to calculate the FSHD clinical score (Section 2, Annex 5) [Lamperti et al., 2010]. The score considers the regional distribution of muscle weakness and the functionality of: (I) facial muscles (scored from 0 to 2); (II) scapular girdle muscles (scored from 0 to 3); (III) upper limb muscles (scored from 0 to 2); (IV) leg muscles (scored from 0 to 2); (V) pelvic girdle muscles (scored from 0 to 5); and (VI) abdominal muscles (scored from 0 to 1). Overall, the total FSHD score ranges from 0 to 15 and numerically defines the clinical severity of the motor impairment.

All sections of CCEF are used for the assessment and the classification of a patient. Based on the distribution of muscle weakness, scored by the FSHD Evaluation Scale, and the combination of the clinical features suggestive or not of FSHD, summarized in the Clinical Diagnostic Form (CCEF Section 3, Figure 2), it is possible to assign patients to different phenotypic categories (CCEF Section 4, Figure 3). In particular, we assigned 1) subjects with

typical FSHD presenting facial and scapular girdle muscle weakness in category A; 2) subjects with muscle weakness limited to facial or scapular girdle muscles in category B; 3) asymptomatic subjects without motor impairment in category C; 4) subjects with myopathic phenotype presenting other anomalous clinical features not consistent with FSHD in category D.

Figure 2: *CCEF Section 3, Clinical Diagnostic Form.*

	TYPICAL FEATURES	UNCOMMON FEATURES
1. ONSET OF MUSCLE WEAKNESS	□ Facial weakness of orbicularis oculi or oris □ Scapular weakness with alterated ability to abduct arms □ Humeral muscles (biceps/triceps)	□ Distal lower limbs onset with triceps surae weakness □ Distal upper limbs onset □ Pelvic girdle onset
2. AXIAL MUSCLES INVOLVEMENT	□ Hyperlordosis □ Beevor's sign	□ Camptocormia □ Dropped head
3. FACIAL INVOLVEMENT	□ Weakness of Orbicularis oculi (facial score ≥1) □ Weakness of Orbiculari oris (facial score ≥1)	□ Weakness of extra-ocular muscles □ Weakness of masticatory muscles (persistent dysphagia)
4. SCAPULAR GIRDLE INVOLVEMENT	□ Impairment of upper limb abduction with winged scapula or limitation of forward flexion (scapular FSHD score ≥1)	□ Isolated distal upper limb muscle weakness □ Impairment of arms abduction (<90°) without winged scapula at rest and/or on attempted shoulder abduction or forward flexion
5. PELVIC GIRDLE INVOLVEMENT	------	□ Isolated and/or prevailing pelvic girdle muscle weakness
6. LOWER LIMBS INVOLVEMENT	□ Weakness of tibialis anterior muscles weakness	□ Early gastrocnemius and/or soleus atrophy/weakness
7. BLOOD CPK LEVEL (at least two samples 1 month apart)	□ Normal range □ < 4x normal value (<1000 U/L)	□ Value > 4x normal value (>1000 U/L)
8. OTHER SIGNS	□ Shoulders winging on attempted shoulder abduction or forward flexion □ Horizontal clavicles □ Forward sloping of the shoulders at rest □ Sunken or flattened appearance of the chest □ Atrophy of pectoralis muscles □ Orbiculari oris hypokinesia during speech	□ Myotonic phenomenon □ Rippling □ Eyelid ptosis □ Extra-ocular muscle weakness □ Early muscle contractures □ Cardiomyopathy □ Early respiratory insufficiency (Non Invasive Ventilation, NIV; FSHD score <12) □ Pes cavus □ Myoglobinuria

Importantly: Indicate the presence of comorbidities / results of previous injuries / illnesses that can possibly affect the neurological examination:_____

Extra-muscular involvement: □hearing loss, □epilepsy, □retinal involvement, □cognitive impairment

Figure 3: *CCEF Section 4, Clinical Categories*.

CATEGORY A

Category A1
Severe facial weakness (unable **both** to close eyes **and** to protrude lips) **+** impairment of upper limb abduction with winged scapula (scapular FSHD score ≥1) **+** absence of uncommon features

Category A2
Facial weakness (upper **and** lower facial weakness) **+** impairment of upper limb abduction with winged scapula (scapular FSHD score ≥1) **+** absence of uncommon features

Category A3
Facial weakness (upper **or** lower facial weakness) **+** impairment of upper limb abduction with winged scapula (scapular FSHD score ≥1) **+** absence of uncommon features

CATEGORY B

Category B1
Impairment of upper limb abduction with winged scapula (scapular FSHD score ≥1), no facial weakness + absence of uncommon features

Category B2
Facial weakness (facial FSHD score ≥1), no impairment of upper limb abduction + absence of uncommon features

CATEGORY C

Category C1
Subject with presence of at least one typical sign + FSHD score =0

Category C2
Subject without signs of muscle weakness **+** FSHD score =0

CATEGORY D

Category D1
Subject fulfilling criteria of categories A1, A2, A3, B1, B2 **+** at least one uncommon feature

Category D2
-Subject fulfilling criteria of categories C1 or C2 + at least one uncommon feature
-Subject no fulfilling criteria of any of the above categories

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Moreover, in view of our experience on FSHD phenotypes accrued through the past years in INRF [Ricci et al., 2013; Nikolic et al., 2016], we further described additional variants within each category (Figure 3, Figure 4). Patients with typical phenotype were classified in three

subcategories (A1, A2, A3), on the basis of the severity of facial involvement, which seems to discriminate some classical phenotypes (Figure 4A-C). This is because we observed that some infantile forms or more severe phenotypes [Nikolic et al., 2016] are characterized by an early and prominent weakness of orbicularis oculi and oris with facial diplegia and dysartria. Thus, these patients were defined as category A1 to distinguish them from the vast majority of patients in which we observed a milder facial involvement (categories A2 and A3). This distinction should facilitate the identification of a specific clinical group deserving ad hoc studies. Incomplete FSHD phenotype, not presenting a coexisting involvement of facial and scapular girdle muscles without other uncommon features, are considered category B1 or B2 (Figure 4D, E). We identified these categories because, for instance, an isolated scapular girdle muscle weakness can be observed in FSHD relatives, but it can be also related to other myopathic disorders or nerve injuries.

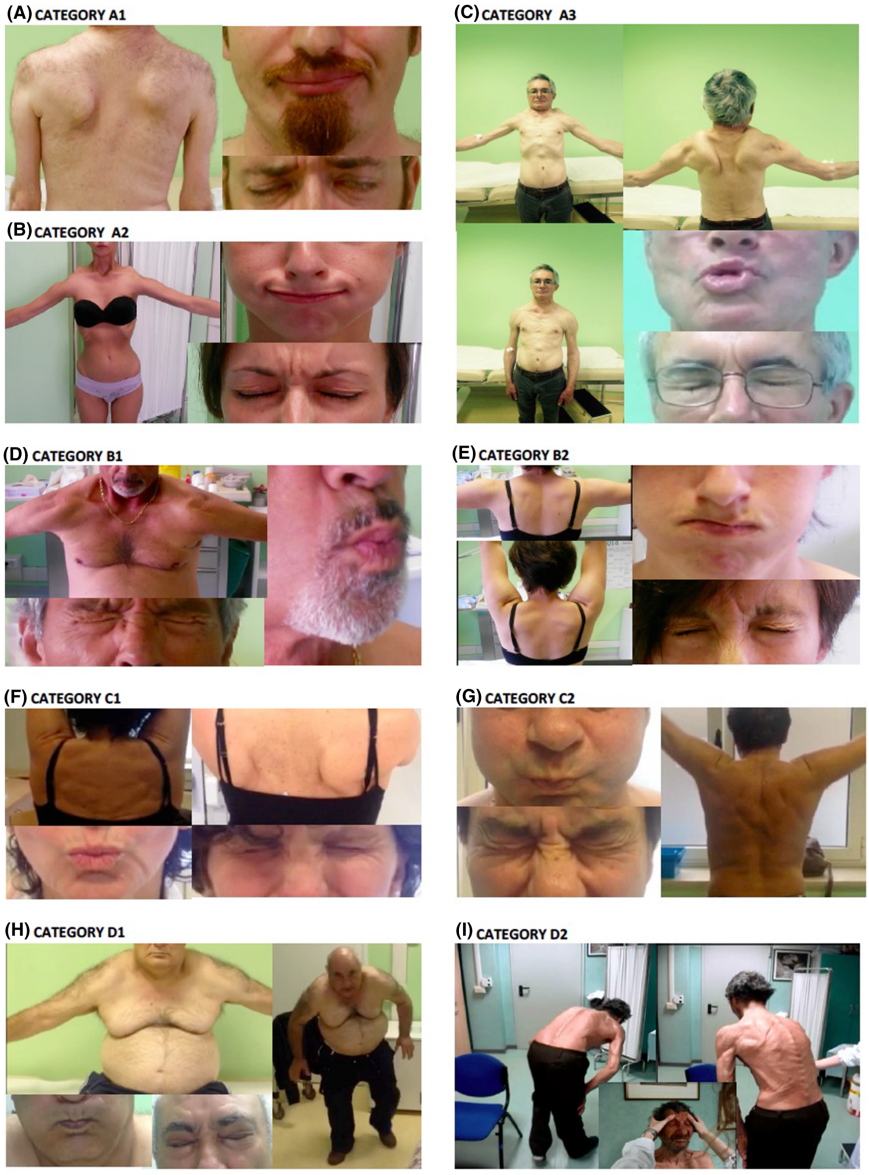
Category D comprises myopathic subjects presenting some FSHD features in association with other uncommon characteristics suggestive of a possible comorbidity (D1) or patients that do not fulfill the diagnostic criteria for FSHD and can be affected by an alternative disease (D2) (Figure 4H,I). Atypical features were chosen based on evidences from literature [Ricci et al., 2014]. This category may facilitate the discovery of factors that contribute to the disease expression or identify those subjects who are wrongly considered FSHD because of a diagnostic bias due to the random finding of DRA.

Finally, we decided to further differentiate non penetrant carriers: the asymptomatic subjects without motor impairment that present minor signs suggestive of FSHD ("Typical features-Other signs" Figure 2) are described as category C1, whereas category C2 includes subjects with a neurological examination completely normal (Figure 4F,G). This distinction might be of particular importance for studying the natural history of disease (i.e. subjects described as C1 might develop clinical FSHD later or remain asymptomatic).

Overall, the categories we generated aim at describing different phenotypes thus capturing clinical diversity, regardless the severity of motor impairment, otherwise reported as FSHD score.

Figure 4: *Examples of clinical categories: case reports. a Category A1: male, 38-year old, showing severe upper and lower facial weakness (unable to close both eyelids completely, puff cheeks and protrude lips), and impairment of upper limb abduction with winged scapula. b Category A2: female, 31-year old, with moderate upper (partial ability to close eyes, without the presence of widened palpebral fissures) and lower facial weakness (partial*

ability to puff out cheeks), impairment of upper limb abduction with winged scapula. c Category A3: male, 60-year old, with moderate lower facial weakness (partial ability to protrude lips), impairment of upper limb abduction with winged scapula. d Category B1: male, 66-year old, with impairment of upper limb abduction with winged scapula, no facial weakness. e Category B2: female, 34-year old, with moderate lower facial weakness (partial ability to puff out cheeks and to protrude lips), no scapular weakness. f Category C1: female, 55-year old, presenting asymmetric scapular winging on forward flexion without motor impairment (FSHD score 0). g Category C2: male, 56-year old, without motor impairment or other FSHD typical signs of muscle atrophy/weakness (FSHD score 0). h Category D1: male, 66-year old: onset after 50 age at shoulder girdle, without facial motor impairment and ''bent spine''. i Category D2: male, 75-year old, with isolated bent spine syndrome, without signs suggestive of FSHD



The characteristics of the 56 FSHD patients enrolled in the inter-rater reliability study are shown in Supplementary table 1. The sample is almost balanced by sex, 34% aged less than 40 years, 12.5% had an FSHD score higher than 10, all but three carried a DRA with 8 or fewer repeats (p13E-11 EcoRI fragments ≤ 35 kb).

The concordance between the clinical assessments performed by the two neurologists was evaluated for the nine CCEF categories described in Figure 3. As shown in Table 2, a good/excellent agreement [Kappa = 0.75; 95% CI (0.57; 0.87)] was observed using the nine CCEF classifications. The overall kappa statistic combine the reliability of the nine categories with a perfect agreement observed for categories B2, C2, D1, D2; a good/excellent agreement for categories A1, A2, B1 and C2, and a good agreement observed for the category A3. The results of the concordance of the final four CCEF categories are presented in Table 3. As expected, the reliability increased with a kappa equal to 0.90; 95% CI (0.71; 0.97). A perfect agreement was observed for categories C and D, an excellent agreement for categories A [Kappa = 0.88; 95% CI (0.75; 1.00)], and a good agreement for categories B [Kappa= 0.79; 95% CI (0.57; 1.00)]. A lower level of kappa, when compared with values obtained for each subcategory, is due to the increased number of categories taken into account in the final score and reflects the sensitivity of the test.

Table 2: *Agreement between Observer 1 and Observer 2 with respect to the nine CCEF categories classification.*

		Observer 2									
	CCEF categories	***A1***	***A2***	***A3***	***B1***	***B2***	***C1***	***C2***	***D1***	***D2***	***Total***
Observer 1	***A1***	6	2	0	0	0	0	0	0	0	8
	A2	1	18	2	0	0	0	0	0	0	21
	A3	0	2	4	2	0	0	0	0	0	8
	B1	0	0	1	5	0	0	0	0	0	6
	B2	0	0	0	0	2	0	0	0	0	2
	C1	0	0	0	0	0	2	0	0	0	2
	C2	0	0	0	0	0	1	4	0	0	5
	D1	0	0	0	0	0	0	0	2	0	2
	D2	0	0	0	0	0	0	0	0	2	2
	Total	7	22	7	7	2	3	4	2	2	56

Kappa=0.75; 95% CI (0.57; 0.87)

Table 3: *Agreement between Observer 1 and Observer 2 with respect to the fourth CCEF categories classification.*

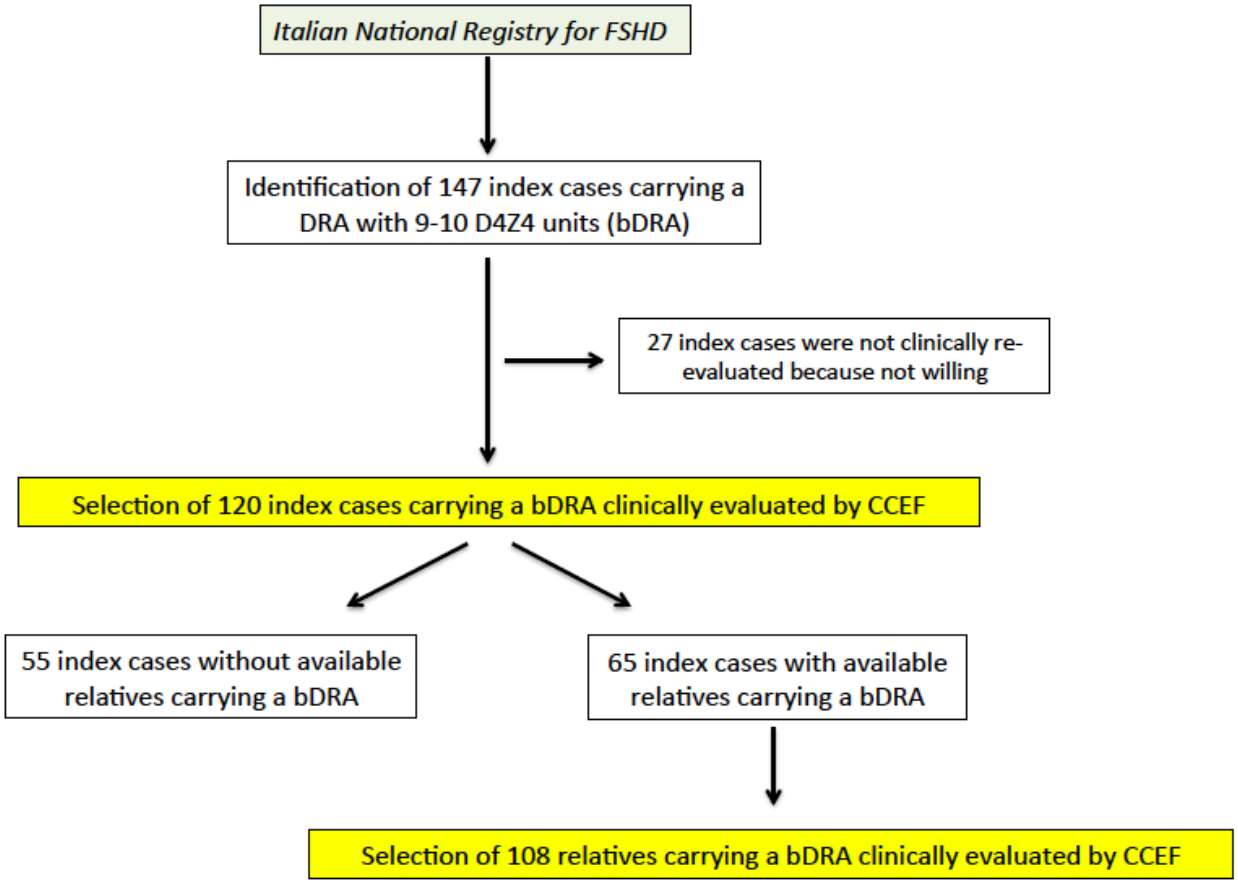
		Observer 2				
	CCEF categories	***A***	***B***	***C***	***D***	***Total***
Observer 1	***A***	35	2	0	0	37
	B	1	7	0	0	8
	C	0	0	7	0	7
	D	0	0	0	4	4
	Total	36	9	7	4	56

Kappa=0.90; 95%CI (0.71; 0.97)

Genotype-phenotype correlations analysis in carriers of 9-10 DRA

A large and comprehensive genotype-phenotype correlation study was performed in families in wich a 9-10 DRA segregates, recruited from the Italian National Registry for FSHD, in order to carry out a detailed clinical characterization/subclassification of probands and relatives, analyse disease penetrance and expression and study the mode of inheritance (familial/sporadic cases). Amnog 147 index cases carrying DRA with 9-10 repeats (bDRA) from Italian National Registry, 120 index cases were clinically evaluated by CCEF; it was not possible to re-evaluate 27 probands. Fifty five index cases were sporadic, without available relatives carrying a bDRA. One hundred and eight relatives carrying a bDRA from were identified from the other 65 unrelated families and clinically assessed by CCEF (Figure 5).

Figure 5: *Selection of the cohort of index cases and their relatives carrying a bDRA for genotype–phenotype correlation analysis.*



Age at onset and severity of motor impairment in index cases carrying 9-10 DRA

We examined 120 unrelated index cases (76 males, 42 females, mean age 55.0 ± 15.4) carriers of a borderline DRA from the Italian National Registry for FSHD, by using the new clinical tool CCEF. We firstly evaluated the disease severity in term of motor impairment and age at onset in this cohort of patients. The severity of motor impairment was assessed by the FSHD score, that translates disability into a number (FSHD score 0-15). The age at onset was collected retrospectively, on the basis of patient records. When subjects did not complain of motor impairment, but a mild muscle weakness was observed, the age at examination was set as the age at onset, according to previous reports [Ricci et al., Brain 2013].

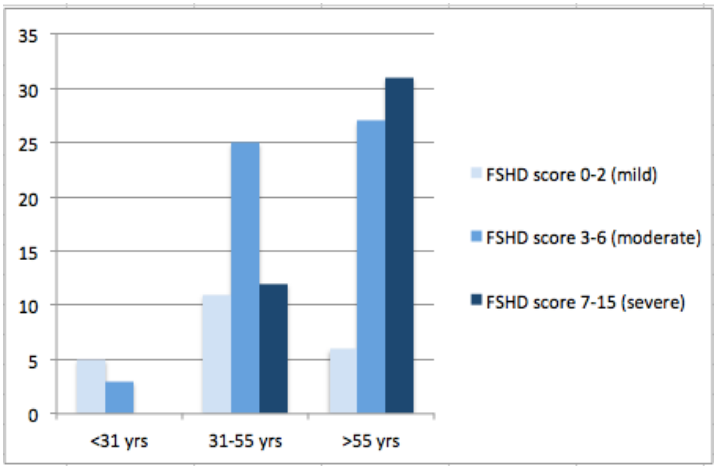
The mean FSHD score was 5.5 ±3.3 (mediana 5), corresponding to a moderate degree of motor impairment [Ricci et al., 2013] and the mean age at onset resulted 33.5 ±16.5 years. We did not detect any difference in term of FSHD score among males and females (in males mean FSHD score 5.3 ±3.1, in females FSHD score 5.8 ±3.6; Student's T test p-value >0.05), regardless the age at examination. Also when we considered the age at onset, we did not detected a significant difference

between males and females (in males age at onset 33.7 ±16.4, in females age at onset 32.9 ±16.8; Student's T test p-value >0.05).

Accordingly to previous analysis on FSHD patients [Ricci et al., 2013], the degree of motor impairment among index cases was also evaluated in relationship to age at examination. Index cases were subdivided in three subgroups by age: (1) < 31 years, (2) 31–55 years, (3) > 55 years. As it is expected for a progressive disease, the mean FSHD score increased with age: mean FSHD score was 1.8 ±1.9 (mediana 1.5) in index cases with age <31 years, and it increased respectively to 4.6 ±2.9 (mediana 4) and 6.6 ±3.2 (mediana 6) in the subgroups 2 and 3.

We also evaluated the distribution of different degrees of motor disability (mild: FSHD score 0-2, moderate: FSHD score 3-6, severe FSHD score: 7-15) according to age, as reported in Figure 6. We confirmed the variabily of clinical expression, since we described subjects minimally affected in all three subgroups, including subjects with age >55 years. However, in adulthood a moderate-severe motor impairment was more frequently observed among the index cases carrying a borderline DRA.

Figure 6: *Distribution of clinical severity among 120 index cases carrying a borderline DRA. Subjects were subdivided by age: <31 years, 31-55 years, >55 years. In each subgroup, number of subjects who received FSHD score equal to 0-2, 3-6 and 7-15 are reported.*



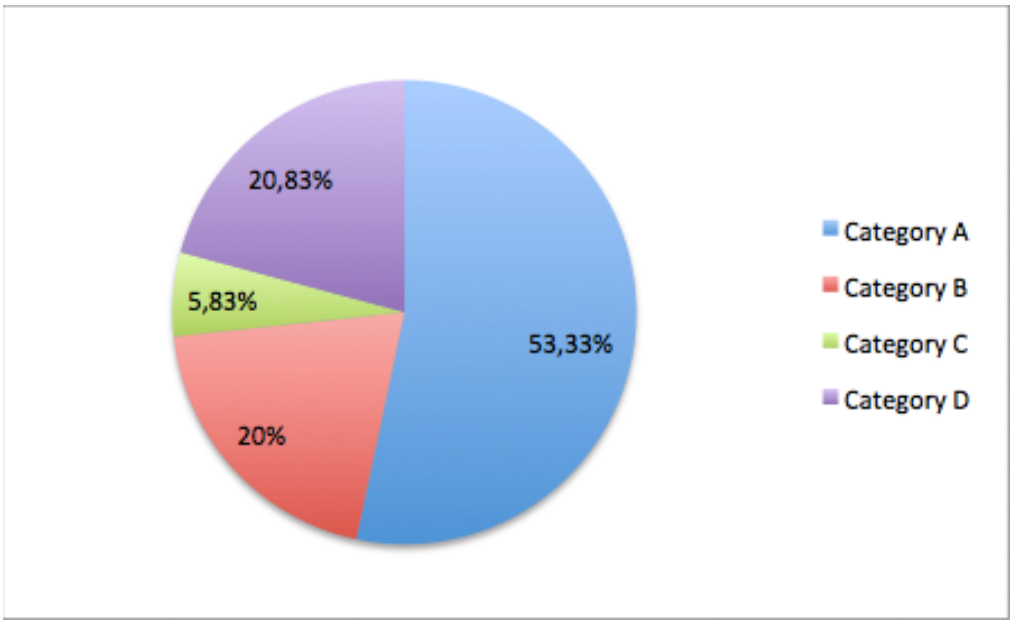
Phenotypic characterizaction of index cases carrying 9-10 DRA: distribution of clinical categories

The FSHD clinical form [Lamperti et al., 2019] assesses the severity of motor impairment, by translating disability into a number (FSHD score), but it does not capture clinical features that may describe various phenotypes. The CCEF has been designed to overcome this limitation. We therefore describe the clinical phenotypes observed in carriers of borderline DRA.

We evaluated by CCEF and classified 120 index cases. Figure 7 illustrates the distribution of the clinical categories. Overall, we observed that only 53.3% of patients showed a FSHD phenotype, classified as category A. Twenty percent of subjects presented muscle weakness limited to scapular

girdle (n°24) or facial muscles (n°2), therefore identifed as category B. Seven index cases that did not showed motor impairment (FSHD score 0), but at neurological examination presented minor signs of shoulder girdle involvement with winged scapula, were classified as category C1 (5.7%). Notably, 20.8% of index cases presented myopathic phenotypes with other anomalous clinical features not consistent with FSHD and they were listed as category D.

Figure 7: *Distribution of the clinical categories among 120 index cases.*



Moreover, by considering the clinical subcategories (Figure 8), our analysis showed that the most common FSHD phenotype was the category A3 (34.17%), characterized by a mild facial involvement. Notably, only 2 patients (1.67%) of patients were classified as category A1, with a more severe phenotypes characterized by an early and prominent weakness of orbicularis oculi and oris with facial diplegia. Among index cases with an incomplete FSHD phenotype, the shoulder involvement without facial weakness was quite frequent (18.33%), while an isolated facial weakness was rarely seen (1.67%). In our cohort some index cases (5.83%) carried out the molecular test for FSHD because they presented a mild hyperckemia and winged scapula, without motor impairment.

Among subjects with atypical features, according to the new clinical classification, 7.5% of them showed a phenotype non consistent with FSHD, suggestive of an alternative diagnosis (category D2); 13.33% of index cases presented additional clinical features other uncommon characteristics suggestive of a possible comorbidity (category D1).

Figure 8: *Distribution of the nine clinical subcategories among 120 index cases carrying bDRA.*

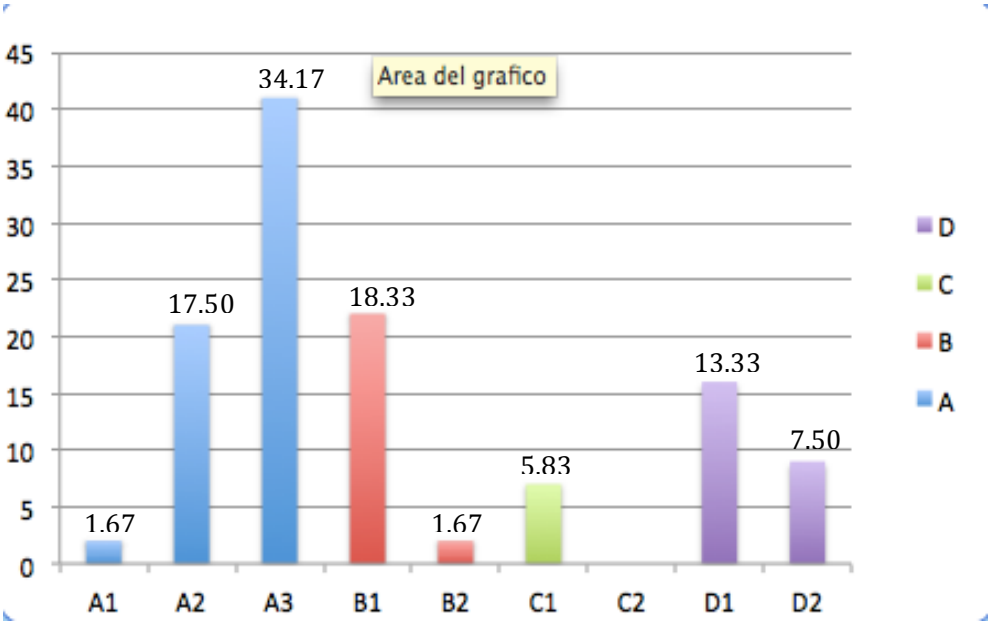


Table 4 shows mean age at onset and FSHD score of index cases for each category. FSHD index cases (category A) reported a mean age at onset of 33.5±15.8, similarly to patients carriers of alleles of 7-8 D4Z4 repeats [Ricci et al., 2013]. A comparable mean age at onset was also referred in probands with incomplete phenotype (category B), although the average FSHD score resulted significantly lower, regardless the age at examination (3.2±1.9 in subjects of category B versus 6.6±2.9 in subjects of category A), suggesting a milder phenotype in term of disease progression in this subgroup of patients.

Table 4: *Distribution of mean age at onset and FSHD score among index cases with bDRA according to clinical categories.*

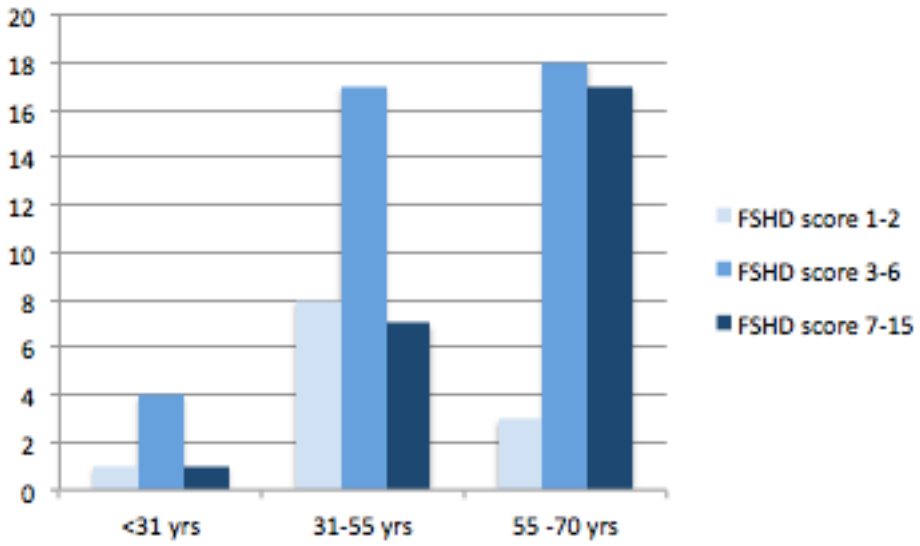
Index cases Category A (42 M, 22 F)		
Mean age at evaluation	*Mean age at onset*	*Mean FSHD score*
58.3±14.6	33.5±15.8	6.6±2.9
Index cases Category B (19 M, 5 F)		
Mean age at evaluation	*Mean age at onset*	*Mean FSHD score*
50.4±14.3	32.8±17	3.2±1.9
Index cases Category C (3 M, 4 F)		
Mean age at evaluation	*Mean age at onset*	*Mean FSHD score*
36.4±23.2	---	0
Index cases Category D (14 M, 11 F)		
Mean age at evaluation	*Mean age at onset*	*Mean FSHD score*
53.9±10.2	28±18.3	6.2±3.6

When we evaluated the percentage of index cases with classical (category A) or incomplete (category B) FSHD phenotype with different degree of motor disability (mild, moderate and severe), subdivided by age (<31years, 31-55 years and 55-70 years), we confirmed a prevalent moderate-severe form of disease over 31 years (Table 5, Figure 9).

Table 5: *Distribution of clinical severity among index cases carrying a borderline DRA classifed as clinical category A and B and subdivided by age: <31 years, 31-55 years, >55 years.*

Age at evaluation	***FSHD score 1-2***	***FSHD score 3-6***	***FSHD score 7-15***
<31 yrs	1	4	1
31-55 yrs	8	17	7
55 -70 yrs	3	18	17
Total	12	39	25

Figure 9: *Distribution of index cases carrying bDRA with classical (category A) or incomplete (category B) FSHD phenotype with different degree of motor disability (mild, moderate and severe), subdivided by age (<31years, 31-55 years and 55-70 years).*



As expected for a slowly progressive disease as FSHD, we observed that there is a mild correlation (Pearson coefficient <0.3) between the FSHD score and the age at examination (Figure 10) and between the FSHD score and the duration of disease (Figure11), in all category groups, although the above correlations were statically significant only in patients of category A (respectively p= 0.009 and 0.003).

Figure 10: *Correlation between FSHD score and age at evaluation according to the clinical category in the index cases (Pearson coefficient <0.3).*

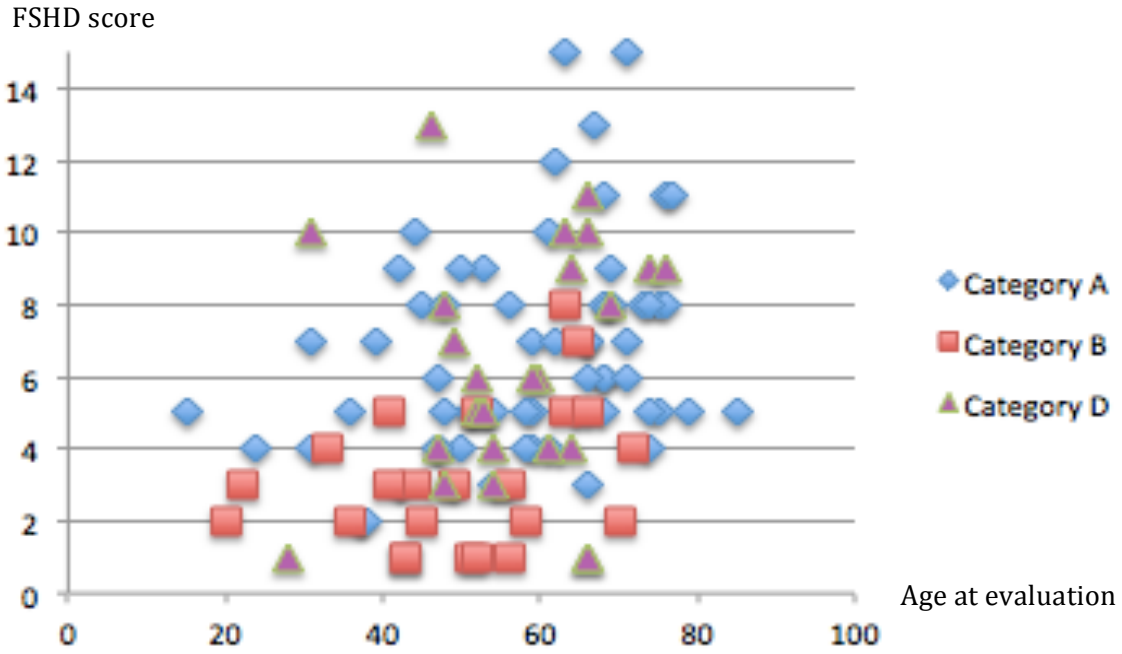
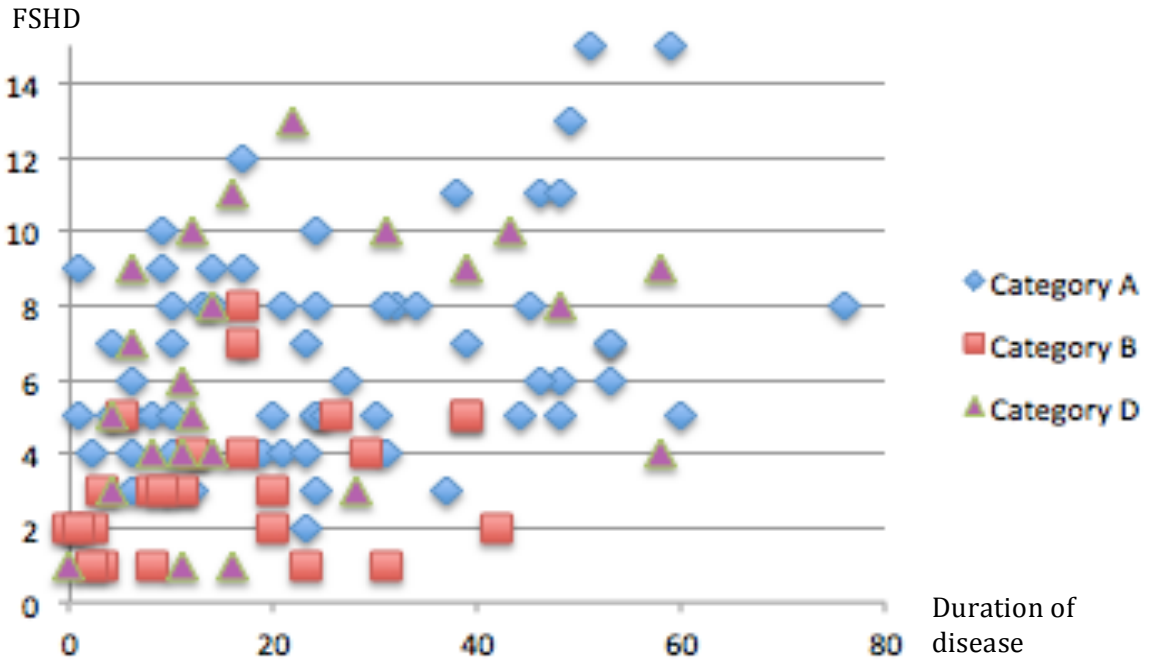


Figure 11: *Correlation between FSHD score and duration of disease according to the clinical category in the index cases (Pearson coefficient <0.3).*



Finally, we investigated the site of muscle weakness at onset reported by patients. Overall, we found that 66.6% of index cases experienced scapular girdle onset, 7.5% facial muscle onset (all of them classified as category A), and 8.3% distal lower limb onset. Notably, some patients, with a no

FSHD phenotype (as described below), reported an atypical muscle weakness to onset, such as axial involvement with bent syndrome (7.5%) and pelvic girdle weakness (5%); a sporadic case, female, 31 years old, presented a congenital onset with hemiparesis; in 6 subjects it was not possible to receive information about the onset of muscle symptoms.

Index cases with atypical phenotypes

As for the index cases of category D, it should consider subjects individually, since the presence of atypical clinical features can be indicative of an alternative diagnosis. We believe that it is not correct to consider in the group of FSHD patients subjects with atypical phenotype, because we risk to draw misleading conclusions.

More than 20% of index cases was classified as category D, for the presence of uncommon features (D1) or atypical phenotypes not consistent with FSHD (D2). These subjects performed the molecular test for FSHD during the medical investigations in suspicion of myopathy. Table 6 summarizes the atypical clinical features of patients. The available relatives of 9 index cases, carrying the same DRA, was not affected (clinical category C). Only 3 cases (248/12, 286/12 and 119/14) referred a positive family history for myopathy. Patients have conducted several investigations, but, to date, they do not receive an alternative diagnosis, except the case 286/12, classified as category D1, that resulted also affected by myasthenia gravis.

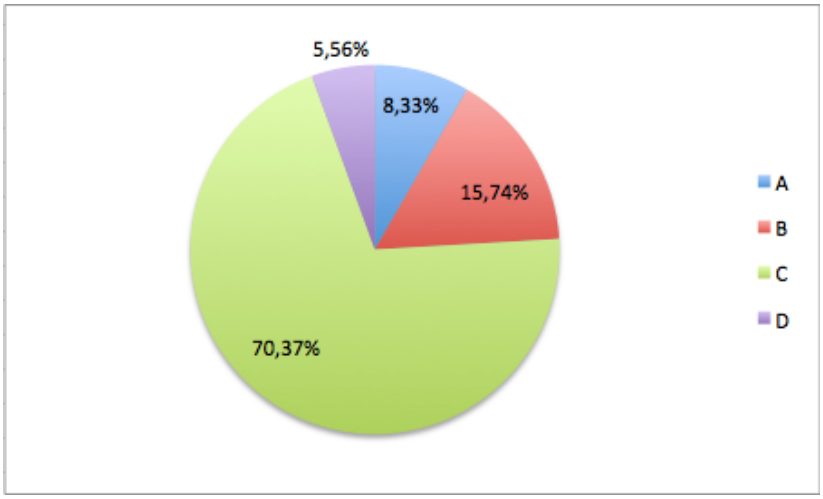
Table 6: *Index cases with atypical clinical features (clinical category D).*

ID	Sex	Age	Age at onset	Atypical phenotypic features	Family history	Clinical category	Other relatives with bDRA (category)
22/01	F	60	40	Axial involvement (bent syndrome), cardiac involvement	Negative	D2	
104/01	F	59	48	Pelvic limb girdle onset	Negative	D1	Daughter (C)
104/02	M	48	n.a.	LGMD-like, with contractures	Negative	D2	
1028/01	M	28	17	Recurrent myoglobinuria	Negative	D2	
464/02	F	64	25	Pelvic limb girdle onset, LGMD-like	Negative	D1	
494/03	F	66	50	Isolated pelvic girdle involvement	Negative	D2	Two sons (both C)
510/04	M	74	16	Dropped head	Negative	D1	
27/07	M	54	43	Pelvic limb girdle onset	Negative	D1	Three relatives (all C)
58/07	F	31	0	Congenital facio-brachio-crural hemiparesis	Negative	D2	Mother and aunt (both C)
174/07	F	64	6	Prevaling pelvic girdle involvement	Negative	D1	
198/07	M	66	54	Axial involvement (bent syndrome)	Negative	D1	Son (C)
169/08	F	63	20	LGMD-like	Negative	D2	Brother and sister (both C)
347/08	M	69	55	Axial involvement	Negative	D2	
35/09	M	47	39	Early gastrocnemius atrophy and weakness	Negative	D1	
309/09	M	66	50	LGMD-like	Negative	D2	
361/09	M	66	66	Isolated pelvic girdle involvement	Negative	D2	Two son (both C)
125/11	M	61	47	Prevailing axial involvement	Negative	D1	
352/11	F	49	43	LGMD-like	Negative	D1	Three relatives (C)
421/11	M	52	48	Dropped head	Negative	D1	
44/12	F	54	50	Bent syndrome	Negative	D2	
140/12	M	76	70	Axial involvement	Negative	D1	
248/12	F	53	41	LGMD-like	Positive	D1	
286/12	F	48	20	Diagnosis of myasthenia	Positive	D1	Two son (both C) and sister (A)
494/12	F	46	24	Prevailing pelvic girdle involvement	Negative	D1	
119/14	M			Pelvic girdle onset	Positive	D1	

Penetrance and disease expression among relatives carrying 9-10 DRA

For each proband evaluated by the new CCEF (120 unrelated patients), the clinical and molecular examinations were extended to the available relatives at various degrees of kinship. Fifty-five index cases were sporadic, without any available relatives carrying a bordeline DRA. It was possible to clinically evaluate 108 relatives (54 males, 54 females, mean age at evaluation 44.59±18.38) from 65 unrelated families, carriers of a borderline allele as the family index proband. Notably, 70.37% of relatives (mean age at evaluation 41.31±16.22) did not show motor impairment (non penetrant) and was classified as category C (Figure 12). Only 8.33% was affected by FSHD, identified as category A, while 15.74% showed an incomplete FSHD phenotype (category B). A small percentage of relatives (5.56%) presented an atypical phenotype (category D).

Figure 12: *Distribution of the clinical categories among 108 relatives from 65 unrelated families.*



The further analysis of the nine clinical subcategories (Figure 13) confirmed that in the majority of relatives the neurological examination was completely normal (category C2, 57.4%), while in 13% minor signs (winged scapula and/or horizontal clavicles), without motor impairment, was detected (category C1). Notably, none of relatives showed a phenotype A1 (Figure 12). Only in 9 families (13.8%) we identified one affected relative with classical FSHD phenotype (clinical category A2 and A3); all index cases of these families presented a FSHD phenotype, except in a family in which the index case was classified as category D1 (subject 286/12, see table 6). In these families, the mode of inheritance was likely autosomal dominant with incomplete penetrance in four families (example in Figure 14A) ; in the other we observed affected subjects only in one generation, resembling an autosomal recessive pattern (example Figure 14B).

Figure 13: *Distribution of the nine clinical subcategories among 108 relatives from 65 unrelated families*.

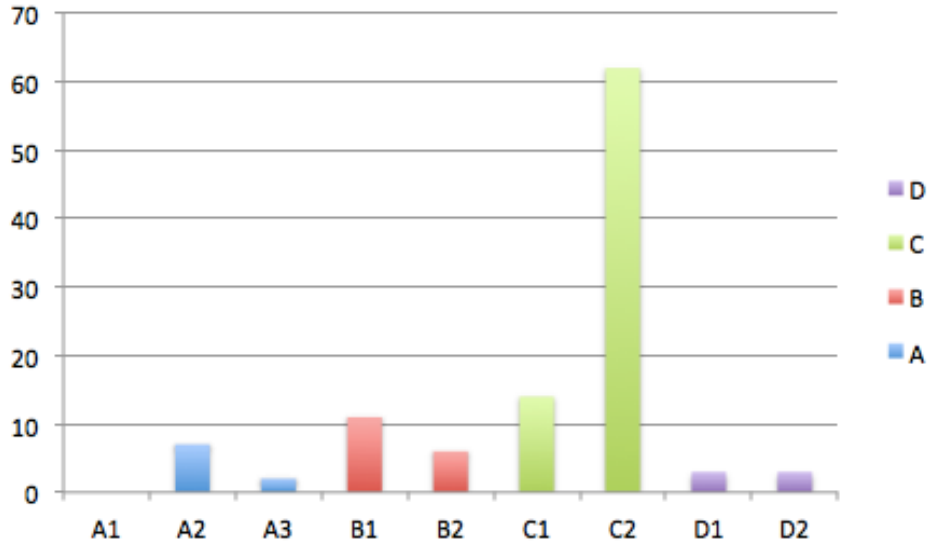


Figure 14A: *Family with a pattern of likely autosomal dominant inheritance and incomplete penetrance. SMCHD1 analysis in the index case II2 was negative*.

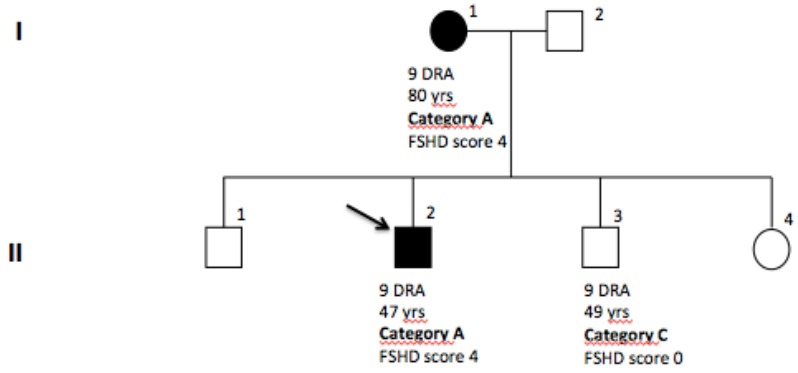
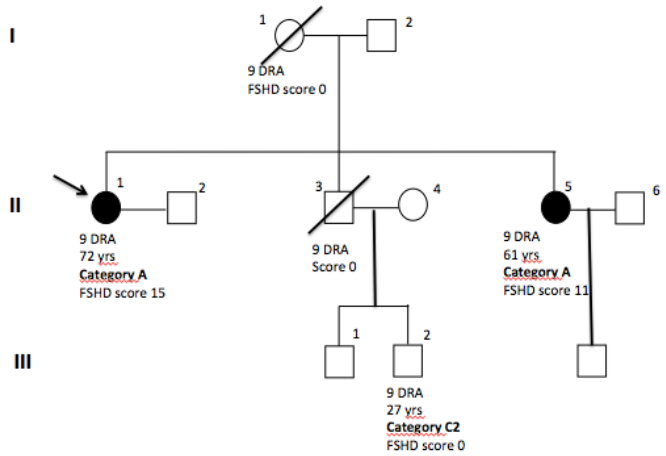


Figure 14B: *Family with a pattern of likely recessive inheritance. SMCHD1 analysis in the index case II1 was negative.*



Overall, table 7 summarizes the clinical data of index cases and relatives.

Table 7: *Clinical data of index cases and relatives carrying a borderline DRA.*

	Category A			**Category B**			**Category C**			**Category D**		
	N°	*Age*	*FSHD score*	*N°*	*Age*	*FSHD score*	*N°*	*Age*	*FSHD score*	*N°*	*Age*	*FSHD score*
Index cases	64	58.30 ±14.64	6.60* (6^)	24	50.42 ±14.29	3.21* (3^)	7	36.43 ±23.27	0	25	56.64 ±11.77	6.28* (6^)
Relatives	9	52.33 ±19.7	5.7* (4)	17	45.82 ±21.2	2* (2^)	76	41.5 ±16.11	0	6	68.67 ±18.11	5.83* (5)

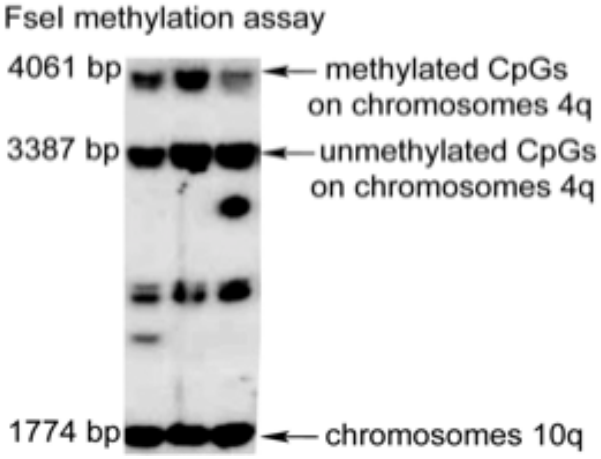
*media; ^mediana

Study of D4Z4 methylation in index cases and relatives carrying 9-10 DRA

We investigated the relevance of D4Z4 methylation in a well-clinically and molecularly-characterized cohort of index cases and relatives carrying bDRA.

Firstly, we analyzed the D4Z4 methylation at alleles of 4q35 chromosomes (MSRE1 method, Figure 15) in 50 consecutive index cases with a clear pattern of 4q-10q alleles. In fact, MSRE1 allows the assessment of the level of DNA methylation from one CpG positioned in the most proximal D4Z4 repeat on both 4q chromosomes. In individuals carrying standard allele constitution of 4-type repeat units on both chromosomes 4 and 10-type on both chromosomes 10 (disomic)[van Overveld et al, 2003] it is possible to access the methylation only at chromosomes 4q excluding chromosome 10q.

Figure 15: *Autoradiography of blotted DNA, digested with methylation-sensitive restriction enzyme FseI and hybridized by with probe p13E-11.*



In this group, we assessed that the average percentage of D4Z4 4q35 methylation was 28% (±12.99). In order to test the possibility that a differential clinical expression might lay in a different degree of D4Z4 methylation, we subdivided the index cases in two subgroups using a methylation threshold of 25%: a) index cases with low level of D4Z4 methylation ≤25% (subgroup 1); b) index cases with normal level of D4Z4 methylation >25% (subgroup 2). We chose the methylation threshold of 25% on the basis of published data [Lemmers et al., 2012; Sacconi et al., 2013]. In fact, levels of methylation <25% has been reported in FSHD2 patients and in FSHD1 subjects carrying upper-sized D4Z4 repeats arrays of 8-10 units with a severe phenotype. In particular, it was supposed that among patients with bDRA (that generally are more likely to have a less severe form of disease or to be asymptomatic) the hypomethylation might be a modifier of disease severity contributing in some subjects to an unusual high clinical severity [Sacconi et al., 2013].

Our analysis showed that, in the group of 50 index cases with bDRA, 25 subjects displayed a level of methylation ≤25%. Table 8 summarizes the clinical features of the two subgroup a) and b).

Table 8-A: *Clinical features of index cases with methylation level ≤25% (MSRE1), subgroup a.*

Index cases with methylation level ≤25% (MSRE1)				
	Number	*Mean FSHD score*	*Mean age at onset (yrs)*	*Mean age at evaluation (yrs)*
Category A	18	6.67±3.02	30.05±12.29	52.67±14.11
Category B	5	4±1.41	21.4±9.81	47.8±11.71
Category C	0	--	--	--
*Category D**	2	6.5	58.5	68.5
Total	25	6.12±2.92	30.6±14.72	52.96±13.94

**The two subjects classified as category D are 125/11 and 140/12: see table 5.*

Table 8-B: *Clinical features of index cases with methylation level >25% (MSRE1), subgroup b.*

Index cases with methylation level >25% (MSRE1)				
	Number	*Mean FSHD score*	*Mean age at onset (yrs)*	*Mean age at evaluation (yrs)*
Category A	17	6.65±3.37	35.94±19.99	63.29±12.51
*Category B^*	2	2.5	38.5	48.5
Category C	1	0	--	71
*Category D^^*	5	7.2±2.49	47.4±5.59	57.8±9.04
Total	25	6.16±3.42	38.54±18.22	61.32±12.06

^ The two subjects classified as category B were respectively 41 and 56 years old, reported an age at onset of 21 and 56 years and received FSHD score 3 and 2.

^^ The subjects classified as category D are 421/11, 248/12, 352/11, 347/08 and 309/09: see table 5.

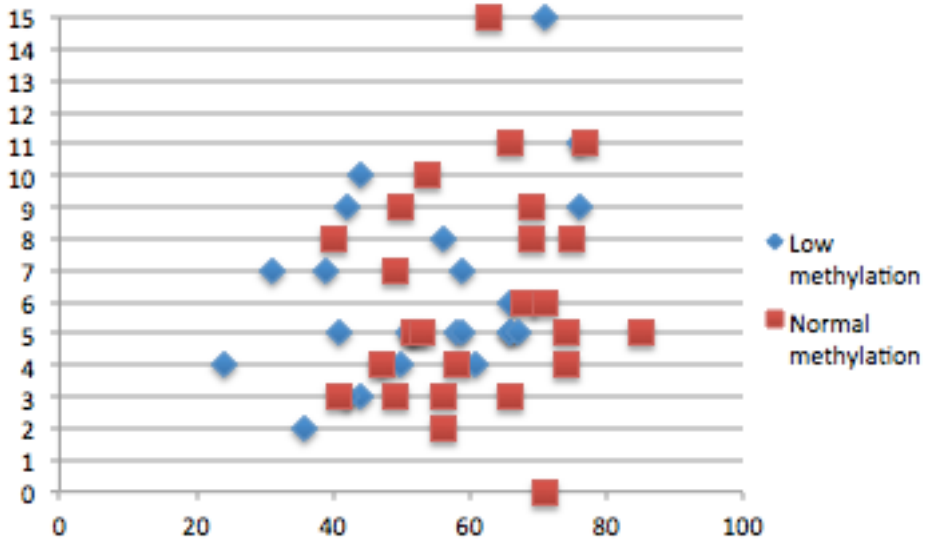
Notably, in our cohort patients classified as category A were identified in both subgroups (18 and 17 subjects respectively in subgroup a and b), with a mean FSHD score which does not differ statistically (Student's T test p-value >0.05). A general linear model (GLM) was also computed to test the relationship between degree of motor impairment and the presence of hypomethylation, considering age at examination as covariate; this model failed to reach statistical significance (p=0.593).

Moreover, the level of methylation does not seem to discriminate subjects with atypical phenotype, since both a low level and a normal level of methylation has been found in subjects classified as category D, respectively in 2 and 5 patients.

We also evaluated if subjects with low level of methylation differed on basis of age at onset and severe of motor impairment according to age at examination. Figures 16 shows and graphically

points out the wide variability of clinical expression in both subgroup a and b.

Figure 16: *FSHD score according to age at examination in index cases with MSRE1 hypomethylation (≤25%, subgroup a, blue dots) and in index cases with level of methylation >25% (subgroup b, red dots).*



Thus we focus our attention on the FSHD families where bDRA segregates and where the reduced penetrance in family is present, and we tested possibility that differential clinical expression might lay in a different degree of D4Z4 methylation between FSHD patients and their non-penetrant relatives carrying the same bDRA. We chose this approach for familial analysis because MSRE2 does not discriminate between the repeat array at 4q and 10q, therefore it is also applicable in subjects without suitable chromosomal profile for MSRE1 analysis. Overall, we analyzed the methylation level of the proximal D4Z4 repeat at 4q35 10q26 chromosomes (MSRE2 method) in 17 consecutive unrelated families in which the index case was classified as category A and there was non penetrant relative. In particular, for each family we study the level of D4Z4 methylation by MSRE2 in two family members, the index case and an available relative who received an FSHD score equal to 0 at the time of examination.

Comparing the D4Z4 methylation status in the 17 non-penetrant bDRA carriers (mean MSER2 methylation 47±14.45) with the level of D4Z4 methylation assessed in the 17 index cases (40±16.82), we did not observe a significant difference of methylation status between the two groups (Student's T test p-value = 0.097).

We also tested the possible link between the MSRE2 methylation status and total number of D4Z4 repeats at 4q35 10q26 chromosomes in non-penetrant bDRA relatives and FSHD patients

separately, but we did not detect any significant correlation between the two variables (Spearman's test p-value >0.05). This observation is in agreement with the assumption that the methylation status detected by using methylation-sensitive restriction enzymes depend on the total number of D4Z4 units carried by each individual in healthy subjects [Lemmers et al., 2015], but in subjects with DRA there is lack of correlation that might indicate the complexity of the molecular mechanisms underlying FSHD pathogenesis.

Analysis of SMCHD1 gene in subgroup of FSHD index cases with 9-10 DRA

Out of 50 index cases in which we studied the level of 4q35 D4Z4 methylation by MSRE1, we then analyzed *SMCHD1* gene by NGS approach in a subgroups of 21 patients, including 11 hypomethylated cases (≤25%). Table 9 shows the distribution of degree of motor impairment in hypomethylated and normal methylated patients.

Table 9: *Distribution of degree of motor impairment in hypomethylated and normal methylated patients in which we analyzed SMCHD1 gene.*

	Methylation level ≤25%	**Methylation level >25%**
FSHD score 3-6 (moderate)	6	8
FSHD score 7-15 (severe)	5	2

None of MSRE1 hypomethylated index cases was carrier of *SMCHD1* mutations.

Discussion

Clinical expression of facioscapulohumeral muscular dystrophy in carriers of 1-3 DRA: experience of the FSHD Italian National Registry

Since molecular analysis of the D4Z4 region was introduced to study FSHD, it has been suggested that very severe forms of disease are associated with a very short DRA. This notion has supported the idea that a rough inverse correlation exists between the size of D4Z4 allele and disease severity. Moreover, it has been long debated whether "infantile FSHD" might exist as a distinct nosological entity, characterised by specific peculiarities that distinguish it from classical FSHD with onset in the second decade of life [Brouwer et al., 1994]. "Infantile FSHD" has been defined by childhood onset and severe muscle impairment, associated with high-frequency hearing loss, retinal vascular

abnormalities, mental retardation and epilepsy. Our analysis, conducted on the largest cohort of 1–3 DRA carriers to date, shows that 1–3 DRAs are not always predictive of infantile onset or severe disease outcome. Importantly, the finding that only 27.9% of 1–3 DRA carriers present extra-muscular clinical conditions supports the notion that additional defects contribute to a more complex clinical phenotype [Annex 4, Nikolic et al., BMJ Open 2016].

Overall, our detailed analysis highlights a clinical variability also in carriers of 1-3 DRA allele, in spite of the assumption that this subgroup could be considered phenotypically homogenous with almost complete penetrance and more severe disease expression, thus indicating the presence of complex pathogenetic mechanisms with additional factors beside the short DRA.

A novel clinical tool to classify phenotypes associated with a DRA

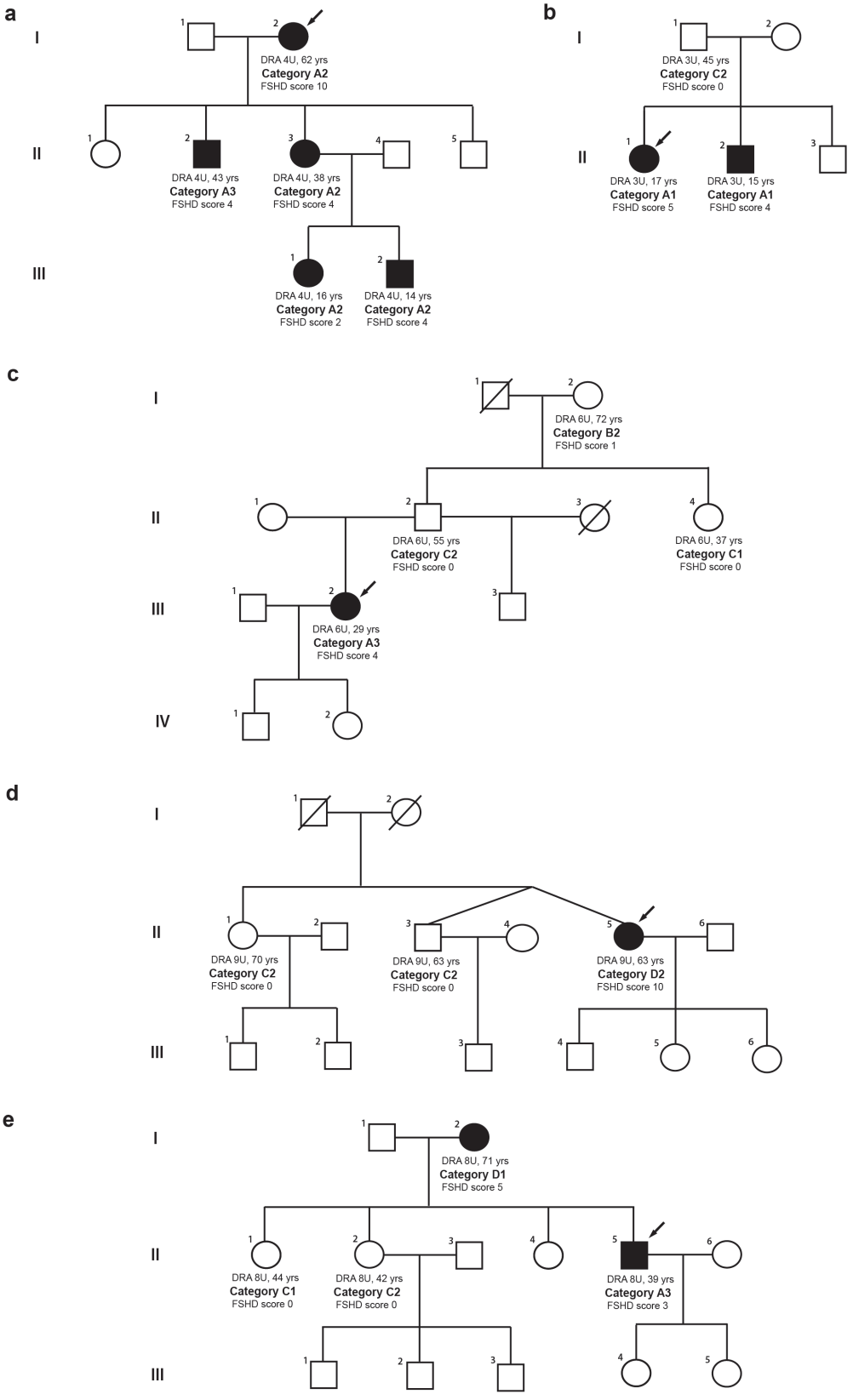
The recently published Guidelines on FSHD of the American Academy of Neurology [Tawil et al., 2015] represent an attempt towards the formulation of optimal standards of diagnosis and care for patients. In these recent Guidelines on FSHD a relevant diagnostic significance is attributed to the detection of D4Z4 alleles associated with the 4qA polymorphism regardless the phenotypic features. However large-scale genotype-phenotype studies have revealed incomplete penetrance and wide variable expressivity in FSHD [Ricci et al., 2013; Ricci et al., 2014] supporting the role of modifying loci or epigenetic mechanisms influencing the clinical expression of disease [Lemmers et al., 2012]. Moreover the FSHD molecular signature has a frequency of 1.3% [Scionti et al., 2012b], which decreases the specificity of the molecular testing for FSHD. So, in our opinion, diagnosis of FSHD must be supported by the harmonized description of the observed clinical phenotypes and the family history.

Nowadays studies suggest the role of epigenetic modifiers in FSHD onset and expression, including the level of 4q35 methylation and/or mutations in *SMCHD1* gene [Lemmers et al., 2012; Sacconi et al., 2013]. Besides, a vast number of reports describe subjects with peculiar/atypical phenotypes carrying a DRA and suggest that mutations in other genes, i.e. gene associated with other neuromuscular diseases, might contribute to disease phenotype [Ricci et al., 2014, Annex 1]. This genetic heterogeneity requires the harmonized classification of clinical phenotypes among patients and within families to serve clinical practice. In FSHD intra-familial clinical variability is one of the most relevant challenges affecting clinical practice and genetic counseling. Our work shows that the CCEF is an easy clinical tool useful to capture various phenotypes from classic FSHD to individuals with incomplete phenotype, or asymptomatic carriers as well as subjects with atypical signs for which alternative diagnoses may be supposed. The choice of the 9 categories responds to the necessity of describing the wide clinical spectrum of FSHD patients and their relatives with a

simple and direct approach. Notably, the CCEF collects several items regarding anamnestic data, including onset, disease progression, distribution and degree of motor impairment (measured as the FSHD Evaluation Scale).

By applying the CCEF it will be possible to quickly classify families on the basis of the harmonized description of genotypes and phenotypes. This classification will support genetic counseling taking into account disease penetrance and expression within a single family. Figure 17 shows some examples. Figure 17A displays a family with the canonical autosomal dominant pattern of inheritance. The disease is present in all three generations and all subjects, carrying a DRA, display facial and scapular girdle weakness typical of FSHD, categories A2 and A3. Figure 17B shows a family in which two sibs are severely affected (A1) whereas the father carrying the same 3U DRA (no somatic mosaicism of the DRA was detected) is healthy (C2). Figure 17C presents a four-generation pedigree in which a single 29 yrs old subject, III.2, developed mild weakness of orbicularis oris and weakness of scapular girdle muscle (category A3). She carries a 6U DRA inherited by her healthy 55 yrs old father, II.2, (category C2). The paternal 37 old yrs aunt, carrying the 6U DRA, is asymptomatic with non-specific signs as horizontal clavicles and axillary creases (category C1) and the paternal 72 yrs old grandmother, I.2, carrying the 6U DRA, presents only incomplete and mild weakness of facial muscle (category B2). Figure 17D describes a family with a single patient presenting severe myopathy with atypical phenotype (D2). The 63 yrs old proband carries a DRA with 9 units as do the twin brother and the 70 yrs old sister, both healthy (C2). Finally, Figure 17E displays a family that may mimic an autosomal dominant inheritance. The proband (II.5), carrying a DRA, presents a typical FSHD phenotype (A3). His mother (I.2) carries the same DRA, but she displays an atypical phenotype (D1) without the facial muscle involvement and with an early and predominant involvement of the pelvic girdle probably related to old age. Instead his two older sisters (II.1 and II.2) are asymptomatic carriers. In our opinion, all these unexpected distribution of clinical phenotypes require particular attention in evaluating the risk of disease onset and expression and the possible contribution of genetic modifiers. Indeed the systematic application of the CCEF might support physicians in the identification of these critical families that might be suitable for further investigations and promote the understanding of disease pathophysiology.

Figure 17: *Clinical characterization of families in which a DRA segregates. Five families are presented. For each subject carrying a 4qA-type DRA, age at evaluation, size of the DRA, clinical category and FSHD score are reported.*



Moreover by using the CCEF it is possible to obtain the longitudinal trajectory of disease progression for each patients and describe the disease's natural history, including the follow-up of non-manifesting carriers.

Overall, the CCEF is a flexible tool that can assist novel strategies to study the etiology of rare diseases. It can support a catalogue of the phenotypes observed among and within families facilitating the phenotypic stratification of FSHD patients, the search of genetic modifiers, and studies on the natural history of disease. Finally, the harmonized clinical classification of subjects is fundamental for the stratification of patients eligible for clinical trials. In this perspective the CCEF can be an instrument for observational studies or randomized clinical trials [Ricci et al., 2016, Annex 6].

Disease penetrance and clinical variability in subjects carrying a borderline DRA

Before the discovery of rearranged D4Z4 alleles, the diagnosis and counselling of FSHD families was entirely based on clinical evidence [Lunt et al., 1989]. Over the years, DNA testing of the D4Z4 locus and flanking polymorphisms has been considered highly sensitive and specific and extensively used to diagnose FSHD [Tawil et al., 2010]. Initially, FSHD patients were shown to exhibit on one allele a repeat number smaller than seven units, whereas their non-FSHD allele shows, like control alleles, higher D4Z4 numbers (8–100) [Wijmenga et al., 1992]. Later, the critical number of remaining D4Z4 repeats was raised for diagnostic purposes (n=9-10). But it became also evident that 3% healthy individuals carry D4Z4 reduced alleles (DRA) <11 repeats [Orrell et al., 1999; Scionti et al., 2012]. Thus, a remarkable overlap seems to exist between D4Z4 alleles in controls and in FSHD individuals, making a definition of a clear cut-off point difficult.

Notably, our previous study [Ricci et al., 2013], according to other reports in literature [Ricci et al., Curr Mol Gen 2014, Annex 1], confirmed that prognosis for subjects carrying or at risk of carrying D4Z4 reduced alleles has become more complicated, since FSHD penetrance in DRA carriers is not complete by age 20, as previously proposed [Tawil et al., 2010], as asymptomatic carriers in all the classes of ages up to 70 years were found. In addition, a wide variability of clinical expression, both in term of age at onset and severity of motor impairment, has been well documented among index cases and affected relatives, specially in carriers of DRA with >4 units. We observed that almost 30% of carriers of DRA with 4-8 units older than 55 years display no muscle weakness and only 24% of them develop disease with high degree of severity by the same age [Ricci et al., 2014]. For all of these reasons, to date the predictive value of DRA is not clear in clinical practice.

The above considerations seem to be particularly true for cases carrying alleles with 9-10 D4Z4 repeats, considered borderline alleles. A better knowledge of the significance of the 9-10 D4Z4 alleles is needed to allow their use in clinical practice and for genetic counseling. Today, only a few reports on penetrance and spectrum of clinical features in subjects carrying 9-10 D4Z4 reduced alleles (DRA) are available; however, often these studies considered not only individuals with

borderline alleles, but included cohorts of patients with a wider range of alleles size [Butz et al., 2003; Statland et al., 2015].

The present study is the largest comprehensive genotype-phenotype correlation analysis in families in which a 9-10 DRA segregates, recruited from the Italian National Registry for FSHD. The application of new clinical tool CCEF has allowed the phenotypes classification of index cases and their relatives.

We firstly evaluated severity of motor impairment in index cases carrying a bDRA, assessed by the FSHD score, that translates disability into a number (FSHD score 0-15). A rough and inverse relationship exists between the residual repeat size and the age at onset and severity of disease, although there is extreme variability in disease severity even within single families [Ricci et al., 2013; Nikolic et al., 2016]. Therefore, one might expect predominantly mild affections in patients carrying a bDRA with repeat numbers close to normal [Statland et al., 2015].

Our analysis shows that the mean age at onset reported by index cases carrying bDRA (33.5 years) resulted comparable to that has been seen in subjects with DRA with 4-6 and 7-8 D4Z4 repeats (29.2 and 34.6 years, respectively) [Ricci et al., 2013]. Similarly, the observed mean FSHD score (5.5) correspondes to a moderate degree of motor impairment, as also observed in carrying 4-8 D4Z4 repeats [Ricci et al., 2013]. When the distribution of different degrees of motor disability was analysed according to age, we confirmed the variabily of clinical expression, since we described subjects minimally affected also in subjects with age >55 years, but we also observed that the majority of index cases showed a moderate-severe motor impairment with age >30 years. We can therefore deduce that affected subjects with a bDRA show a disease expression very simil to that observed in probands carriers with a DRA with 4-8 D4Z4 repeats. Besides, also in this cohort of patients mild phenotypes up to very severe forms with loss of ambulation after IV dedade of life can coexist. Our data are consistent with that was observed previously by Butz and coworkers in 2003 in a series of 39 patients with DRA of 32-45 kb size. Also the authors did not find a specifically mild phenotype in patients with borderline repeat numbers and even observed severe phenotypes in the grey zone of 10-11 repeats.

The above observations strongly suggest that other factors than D4Z4 repeat number contribute to the phenotypic severity. Notably, this concept is further strengthened by the fact that in 75% of families in which a bDRA segregates the only affected subject is the index case, while the other family members carrying the same DRA are asymptomatic. Moreover, the systematic analysis of available and willing relatives carrying bDRA revealed that 70% of them did not show motor impairment and was non penetrant. This percentage is considerably higher than the percentage of non-penentrant subjects detected among carriers of DRA with 4-6 and 7-8 repeats (28.6% and

39.6%, respectively).

Thus, through the systematic collection of families in the Italian National Registry for FSHD has been possible to reach these informations that, to date, become crucial for genetic counseling of subjects carrying or at risk of carrying a DRA in the borderline region.

Phenotypic spectrum in carriers of a borderline DRA

A wide range of myopathic phenotypes have been well documented in subjects carrying DRA. Notably, in 2003 Butz and corworkers, by evaluating 39 unrelated FSHD patients carrying a bDRA, firstly highlighted the broad myopathic spectrum with four phenotypes (typical FSHD, facialsparing FSHD, FSHD with atypical features, non-FSHD muscle disease) in the borderline region.

The clinical and genetic heterogeneity requires a harmonized clinical classification of phenotypes among patients and within families to serve clinical practice, support genetic counseling, study the natural history of disease and search of genetic modifiers. The definition of molecular basis of FSHD and the understanding of the predictive significance of new molecular markers can not be disregarded from a precise selection of patients on the basis of the phenotype, in order to not draw misleading conclusions. CCEF has been designed for these purpose. Our clinical analysis allows the further classification of carriers of bDRA on the basis of the phenotypic features, regardless the degree of motor impairment. It is significant that only 53% of index cases showed a classical FSHD phenotype, as much as 20.8% of them presented myopathic phenotypes with other anomalous clinical features not consistent with FSHD.

In the last years, the assessment of the D4Z4 array size as diagnostic test for FSHD has led to the identification of phenotypes that differs at various degrees from the original description of disease. To date, it has become clear that the finding of an allele in a patient with atypical phenotype should prompt the clinician to search for other possible diagnoses. This is even more mandatory if there is the absence of a clear segregation of the disease with DRA within the family. It is important to highlight that an accurate estimate of the frequency of atypical forms between carriers of DRA is not still available [Ricci et al., 2016]. Preliminary data on a cohort of 228 probands carriers of 4q alleles of ≤ 8 D4Z4 units recruited from the Italian National Registry suggested that almost 6% of subjects did not fullfilled the diagnostic criteria for FSHD and resulted affected by other myopathic conditions (unpublished data). Likewise, during the assessement of the inter-rater reproducibility of the CCEF, we observed that 7% of subjects (4/56) was classified as category D. The systematic clinical re-evaluation by using CCEF of all available families recruited in the Italian Registry is currently ongoing and it will allow to establish the frequency of atypical phenotypes within families, also in relation to DRA size. To date, the present work is the first study that investigates

the presence of atypical forms in a large cohort of patients with bDRA. The number of patients classified as category D is higher than expected, based on previous observations, and significant for clinical practice. Notably, all available relatives of index cases D (with the exception of the family members of patient 286/12, who was classified as category D1 for the concomitant diagnosis of myasthenia gravis) resulted asymptomatic. Starting from the hypothesis that in these subjects with atypical phenotype the DRA is not causative, or may act as a susceptibility factor, it would be interesting to study the frequency of the DRA in other groups of myopathic patients with alternative diagnoses (for instance limb girdle muscular dystrophies).

The mode of inheritance is not autosomal dominant in the majority of families in wich a borderline DRA segregates

In FSHD1 the inheritance pattern has been considered autosomal dominant, according to the genetic diagnostic criteria defined by the European Expert Group on FSHD in 1991 [Padberg et al., 1991]. However, to date, it is well-known that sporadic FSHD1 cases can occur. Thus, in your recent genotype-phenotype correlation study [Ricci et al., 2013, Annex 2] we observed that 13% of FSHD families in which a DRA with 4-8 units segregates did not show an autosomal dominant inheritance because affected subjects were present only in one generation. Notably, the mode of inheritance appears difference between FSHD1 and FSHD2, since the majority (67%) of FSHD2 patients without DRA resulted sporadic, suggesting that the familial to sporadic ratio in FSHD2 seem to be inverse to the ratio in FSHD1. The present analysis revealed that, among 120 index cases evaluated by CCEF, 55 were sporadic, without available relatives carrying a bDRA; however, among them, only 10 subjects referred a positive family history for muscle disease, while in the other the family history was inconsistent. Importantly, by evaluating 65 unrelated families with at least two subjects carrying a bDRA, we observed that in 75% of families the only affected subjects was the index case. Moreover, in the familial cases, the mode of inheritance was often uncertain, due to incomplete penetrance or lack of extended family investigation. Overall, we can conclude that our observations are not consistent with a clear autosomal dominant pattern in the group of subjects carrying a bDRA, as observed in FSHD2 patients. The lack of autosomal dominant inheritance prompts to consider the presence of different undelying pathogenic mechanisms and of additional genetic defects, beside the DRA.

The level of 4q35 D4Z4 methylation is not predictive of disease outcome in subjects carrying a borderline DRA

The partial loss of D4Z4 methylation in FSHD1 and FSHD2 has been demonstrated by Southern blot analysis using several methylation-sensitive restriction enzymes and, more recently, by bisulfite sequencing and methylated DNA immunoprecipitation (MeDIP) analysis at D4Z4. These studies have shown that the different approaches revealed similar patterns of D4Z4 methylation. In FSHD2, the D4Z4 arrays are hypomethylated on 4q and 10q, whereas in FSHD1, hypomethylation is restricted to the contracted alleles. One of the most commonly used methylation-sensitive restriction site to measure D4Z4 methylation is the FseI site as it is highly predictive to FSHD; D4Z4 methylation analysis by FseI digestion can be done on large cohorts of individuals, in contrast to the more elaborate alternatives.

Since D4Z4 methylation is also repeat size dependent, it could be expected that in FSHD1 individuals with similarly sized pathogenic repeat arrays, those with longer arrays on the remaining three chromosomes have higher FseI methylation levels than those with shorter alleles on the other chromosomes. Insteed, Lemmers and coworkers [Lemmers et al., 2015] observed that in FSHD1 individuals, carrying 1-10 DRA, the FseI methylation is dependent from the short DRA, but it is independent from the other repeats on normal-size allele of chromosome 4 and on the alleles of chromosome 10. For the above considerations, in order to quantify D4Z4 chromatin relaxation, it is possible to determine the percentage of CpG methylation on the basis of measurements following cleavage with the methylation-sensitive FseI endonuclease in an assay that averaged the percentage of D4Z4 methylation on alleles of chromosomes 4 or on both alleles of chromosomes 4 and 10.

Moreover, a rough correlation has been reported between residual pathogenic array repeat size, methylation and disease severity in FSHD1 individuals. In particular, it has been suggested that in FSHD1, for individuals with D4Z4 repeat arrays of 1-6 units, the clinical severity mainly depends on the size of the D4Z4 repeat. Insteed, in individuals with arrays of 7-10 units the clinical severity could depend on other factors that regulate D4Z4 methylation because affected individuals, but not non-penetrant mutation carriers, have a greater reduction of D4Z4 CpG methylation than can be expected based on the size of the pathogenic D4Z4 repeat array [Lemmers et al., 2015]. The recent study of Lemmers and coworkers in 2015 reached the above observations by studying 85 subjects from 62 unrelated families carried a DRA with 7-10 unit, including 25 non-penetrant carriers. Therefore, it has been proposed that the striking variability in onset and disease progression, that is a clinical hallmark of FSHD, coud have epigenetic basis.

Starting from the above considerations, we have tested in our cohort of index cases and relatives carrying a bDRA if the level D4Z4 CpG methylation might clarify some aspects influencing FSHD

manifestation and it might be predictive of disease penetrance and outcome, also with diagnostic purpose. Firstly, we analyzed the D4Z4 methylation at alleles of 4q35 chromosomes (MSRE1 method, Figure 13) in 50 consecutive index cases with a clear pattern of 4q-10q alleles.

We subdived the index cases in two subgroups on basis of the level of methylation (≤25% and >25%) and we tested the hypothesis that the hypomethylation (≤25%) might be a modifier of disease severity among patients with bDRA, according to previous reports [Sacconi et al., 2013].

We chose the methylation threshold of 25% on basis of published data [Lemmers et al., 2012; Sacconi et al., 2013]. In fact, levels of methylation ≤25% has been reported in FSHD1 subjects carrying upper-sized D4Z4 repeats arrays of 8-10 units with a more severe phenotype, thus it was supposed that among patients with bDRA the hypomethylation might be a modifier of disease severity [Sacconi et al., 2013].

Our analysis showed a low level of 4q D4Z4 methylation only in 50% of index cases, and confirmed a wide variability of clinical expression in term of motor disability in both subgroups, regardless the level of methylation. Notably, a methylation level ≤25% was detected also in index cases that did not fulfilled the diagnostic criteria of FSHD.

The observed phenotypic variability in the FSHD families and the presence of asymptomatic subjects carrying the same DRA as their FSHD relatives proned us also to study the intra-familial methylation status. Comparing the D4Z4 methylation status in the non-penetrant bDRA carriers with the level of D4Z4 methylation assessed in the index cases, we did not observe ant significant difference of methylation status between the two groups.

Therefore, these results highlight how a low level of methylation should not be considered predictive of disease outcome in clinical practice and nor even can be used as diagnostic marker.

As regards the hypothetical role of *SMCHD1* gene in FSHD pathogenesis, studies on a larger number of FSHD patients and families of INRF are currently ongoing. In fact, *SMCHD1*, a chromatin modifier necessary for the establishment and maintenance of CpG methylation of the inactive X chromosome, has been proposed as a modifier for disease severity in families with FSHD1 [Sacconi et al., 2013]. However, our preliminary data on 21 FSHD index cases carrying bDRA failed to identify *SMCHD1* mutations, also in these patients with low level of D4Z4 4q35 methylation and/or severe motor impairment.

Conclusions

In the last years, there has been a tendency to simplify the phenotypic complexity and to give greater importance to molecular tests in the diagnostic flow chart of human genetic diseases. However, the extensive use over the past 20 years of DNA analysis for studying Mendelian disorders has revealed many disease mechanisms that are more complex than single mutations. For example, identical phenotypes may be produced by mutations in different genes, the same mutation can cause different phenotypes, and distinct mutations in the same gene may result in different disorders that segregate with diverse Mendelian or even multifactorial patterns. In addition, the incomplete penetrance of some mutations argues for the role of modifying loci or epigenetic mechanisms influencing the clinical expression in many Mendelian disorders. Moreover the possibility of conducting extensive studies in different human populations has revealed the large variability of human DNA variations blurring the distinction between a polymorphism and a detrimental mutation more subtle. Thus, establishing the value of mutational events underlying genetic diseases may be complex even in diseases with simple patterns of inheritance and well-characterized pathologic course. FSHD seems to fall in this complex pattern and the Italian National Registry represents an unique opportunity to study and dissect the clinical and genetic complexity for the muscle disease.

In conclusion, the systematic and standardized collection of clinical and molecular data of FSHD families in the Italian National Registry has allowed to highlight how, to date, the molecular mechanisms are still not entirely clear, there is an extreme clinical variability of disease expression both in terms of severity and phenotypic features, the pattern of inheritance is not always autosomal dominant and there is not a definite knowledge of natural history of disease. Notably, there are several pitfalls in the genetic diagnosis of FSHD. The molecular markers proposed for FSHD are not predictive of disease outcome and could be confounding for the clinician wanting to differentiate between the diagnosis of FSHD and a myopathy presenting with FSHD-like features. The future goal of FSHD clinical research will be the selection of patients and families with homogeneous clinical and genetic features, in term of mode of inheritance, regardless of the size of D4Z4 alleles, to provide the appropriate background for molecular studies aimed at dissecting the complex pathogenesis of this disease.

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References

- Awerbuch GI, Nigro MA, Wishnow R. Beevor's sign and facioscapulohumeral dystrophy. Arch Neurol 1990; 47:1208-9.
- Bailey RO, Marzulo DC, Hans MB. Infantile facioscapulohumeral muscular dystrophy: new observations. Acta Neurol Scand 1986;74:51-8.
- Becker PE. Dystrophia musculorum progressiva. Eine genetische und klinische untersuchung der muskeldystrophien. Stuttgart: Georg Thieme Verlag, 1953.
- Blewitt, M.E. et al. An N-ethyl-N-nitrosourea screen for genes involved in variegation in the mouse. Proc. Natl. Acad. Sci. USA 2005;102, 7629–7634.
- Brouwer OF, Padberg GW, Wijmenga C, Frants RR. Facioscapulohumeral muscular dystrophy in early childhood. Arch Neurol 1994;51:387–94.
- Brouwer OF, Wijmenga C, Frants RR, Padberg GW. Facioscapulohumeral muscular dystrophy: the impact of genetic research. Clin Neurol Neurosurg 1993; 95: 9-21.
- Buj-Bello A, Furling D, Tronchère H, Laporte J, Lerouge T, Butler-Browne GS, Mandel JL. Muscle-specific alternative splicing of myotubularin-related 1 gene is impaired in DM1 muscle cells. Hum Mol Genet. 2002 Sep 15;11(19):2297-307.

- Butz M, Koch MC, Müller-Felber W, Lemmers RJ, van der Maarel SM, Schreiber H. Facioscapulohumeral muscular dystrophy. Phenotype-genotype correlation in patients with borderline D4Z4 repeat numbers. J Neurol. 2003 Aug;250(8):932-7.

- Butz M, Koch MC, Müller-Felber W, Lemmers RJ, van der Maarel SM, Schreiber H. Facioscapulohumeral muscular dystrophy. Phenotype-genotype correlation in patients with borderline D4Z4 repeat numbers. J Neurol. 2003 Aug;250(8):932-7.

- Celegato B, Capitanio D, Pescatori M, Romualdi C, Pacchioni B, Cagnin S, Viganò A, Colantoni L, Begum S, Ricci E, Wait R, Lanfranchi G, Gelfi C. Parallel protein and transcript profiles of FSHD patient muscles correlate to the D4Z4 arrangement and reveal a common impairment of slow to fast fibre differentiation and a general deregulation of MyoD-dependent genes. Proteomics. 2006 Oct;6(19):5303-21.
- Chuenkongkaew WL, Suphavilai R, Vaeusorn L, Phasukkijwatana N, Lertrit P, Suktitipat B. Proportion of 11778 mutant mitochondrial DNA and clinical expression in a thai population with Leber hereditary optic neuropathy. J Neuroophthalmol 2005 Sep; 25(3): 173-175.

- Chuenkongkaew WL, Suphavilai R, Vaeusorn L, Phasukkijwatana N, Lertrit P, Suktitipat B. Proportion of 11778 mutant mitochondrial DNA and clinical expression in a thai population with Leber hereditary optic neuropathy. J Neuroophthalmol 2005 Sep; 25(3): 173-175.

- Chung CS, Morton NE. Discrimination of genetic entities in muscular dystrophy. Am J Hum Genet 1959;11:339-59.
- Clapp J, Mitchell LM, Bolland DJ, Fantes J, Corcoran AE, Scotting PJ, Armour JA, Hewitt JE. Evolutionary conservation of a coding function for D4Z4, the tandem DNA repeat mutated in facioscapulohumeral muscular dystrophy. Am J Hum Genet. 2007 Aug;81(2):264-79.
- Clark LN, Koehler U, Ward DC, Wienberg J, Hewitt JE. Analysis of the organisation and localisation of the FSHD-associated tandem array in primates: implications for the origin and evolution of the 3.3 kb repeat family. Chromosoma. 1996 Sep;105(3):180-9.

- de Greef JC, Frants RR, van der Maarel SM. Epigenetic mechanisms of facioscapulohumeral muscular dystrophy. Mutat Res. 2008 Dec 1;647(1-2):94-102.
- de Greef JC, Lemmers RJ, Camaño P, Day JW, Sacconi S, Dunand M, van Engelen BG, Kiuru-Enari S, Padberg GW, Rosa AL, Desnuelle C, Spuler S, Tarnopolsky M, Venance SL, Frants RR, van der Maarel SM, Tawil R. Clinical features of facioscapulohumeral muscular dystrophy 2. Neurology. 2010 Oct 26;75(17):1548-54.
- de Greef JC, Lemmers RJ, van Engelen BG, Sacconi S, Venance SL, Frants RR, Tawil R, van der Maarel SM. Common epigenetic changes of D4Z4 in contraction-dependent and contraction-independent FSHD. Hum Mutat. 2009 Oct;30(10):1449-59.
- Deidda G, Cacurri S, Grisanti P, Vigneti E, Piazzo N, Felicetti L. Physical mapping evidence for a duplicated region on chromosome 10qter showing high homology with the facioscapulohumeral muscular dystrophy locus on chromosome 4qter. Eur J Hum Genet. 1995;3(3):155-67.
- Deidda G, Cacurri S, Piazzo N, Felicetti L. Direct detection of 4q35 rearrangements implicated in facioscapulohumeral muscular dystrophy (FSHD). J Med Genet 1996; 33: 361-5.
- Dixit M, Ansseau E, Tassin A, Winokur S, Shi R, Qian H, Sauvage S, Mattéotti C, van Acker AM, Leo O, Figlewicz D, Barro M, Laoudj-Chenivesse D, Belayew A, Coppée F, Chen YW. DUX4, a candidate gene of facioscapulohumeral muscular dystrophy, encodes a transcriptional activator of PITX1. Proc Natl Acad Sci U S A. 2007 Nov 13;104(46):18157-62.
- Doerner A, Pauschinger M, Badorff A, Noutsias M, Giessen S, Schulze K, Bilger J, Rauch U, Schultheiss HP. Tissue-specific transcription pattern of the adenine nucleotide translocase isoforms in humans. FEBS Lett. 1997 Sep 8;414(2):258-62.

- Dorobek M, Szmidt-Sałkowska E, Rowińska-Marcińska K, Gaweł M, Hausmanowa-Petrusewicz I. Relationships between clinical data and quantitative EMG findings in facioscapulohumeral muscular dystrophy. Neurol Neurochir Pol 2013; 47: 8-17.
- Duchenne G. Album de photographies pathologiques. 1862
- Duchenne G. Revue photographique des hopitaux de Paris.1869
- Emmrich P, Ogunlade V, Gradistanac T, Daneschnejad S, Koch MC, Schober R. Facioscapulohumeral muscle dystrophy and heart disease. Z Kardiol 2005; 94: 348-54.
- Engel AG, Franzini-Armstrong C. Miology. McGraw-Hill 2004 (Third Edition).
- Filosto M, Tonin P, Scarpelli M, et al. Novel mitochondrial tRNA Leu(CUN) transition and D4Z4 partial deletion in a patient with a facioscapulohumeral phenotype. Neuromuscul Disord 2008 Mar; 18(3): 204-209.
- Fleiss JL. Statistical Methods for Rates and proportions. 1981 2nd edn. Wiley, New York.
- Gabellini D, D'Antona G, Moggio M, Prelle A, Zecca C, Adami R, Angeletti B, Ciscato P, Pellegrino MA, Bottinelli R, Green MR, Tupler R. Facioscapulohumeral muscular dystrophy in mice overexpressing FRG1. Nature. 2006 Feb 23;439(7079):973-7.
- Gabellini D, Green MR, Tupler R. Inappropriate gene activation in FSHD: a repressor complex binds a chromosomal repeat deleted in dystrophic muscle. Cell. 2002 Aug 9;110(3):339-48.
- Gaillard MC, Roche S, Dion C, Tasmadjian A, Bouget G, Salort-Campana E, Vovan C, Chaix C, Broucqsault N, Morere J, Puppo F, Bartoli M, Levy N, Bernard R, Attarian S, Nguyen K, Magdinier F. Differential DNA methylation of the D4Z4 repeat in patients with FSHD and asymptomatic carriers. Neurology. 2014 Aug 19;83(8):733-42.
- Galluzzi G, Deidda G, Cacurri S, Colantoni L, Piazzo N, Vigneti E, Ricci E, Servidei S, Merico B, Pachì A, Brambati B, Mangiola F, Tonali P, Felicetti L. Molecular analysis of 4q35 rearrangements in fascioscapulohumeral muscular dystrophy (FSHD): application to

family studies for a correct genetic advice and a reliable prenatal diagnosis of the disease. Neuromuscul Disord. 1999 May;9(3):190-8.

- Gehring WJ, Müller M, Affolter M, Percival-Smith A, Billeter M, Qian YQ, Otting G, Wüthrich K. The structure of the homeodomain and its functional implications. Trends Genet. 1990 Oct;6(10):323-9.
- Ginisty H, Sicard H, Roger B, Bouvet P. Structure and functions of nucleolin. J Cell Sci. 1999 Mar;112 (Pt 6):761-72.
- Goto K, Nishino I, Hayashi YK. Very low penetrance in 85 Japanese families with facioscapulohumeral muscular dystrophy 1A. J Med Genet. 2004; 41: e12.
- Hartweck LM, Anderson LJ, Lemmers RJ, Dandapat A, Toso EA, Dalton JC, Tawil R, Day JW, van der Maarel SM, Kyba M. A focal domain of extreme demethylation within D4Z4 in FSHD2. Neurology. 2013 Jan 22;80(4):392-9.
- Hewitt JE, Lyle R, Clark LN, Valleley EM, Wright TJ, Wijmenga C, van Deutekom JC, Francis F, Sharpe PT, Hofker M, et al. Analysis of the tandem repeat locus D4Z4 associated with facioscapulohumeral muscular dystrophy. Hum Mol Genet. 1994 Aug;3(8):1287-95.
- Jacobsen SJ, Diala ES, Dorsey BV, Rising MB, Graveline R, Falls K, Schultz P, Hogan C, Rediker K, D'Amico C, et al. A clinically homogeneous group of families with facioscapulohumeral (Landouzy-Déjérine) muscular dystrophy: linkage analysis of six autosomes. Am J Hum Genet. 1990 Sep;47(3):376-88.
- Jiang G, Yang F, van Overveld PG, Vedanarayanan V, van der Maarel S, Ehrlich M. Testing the position-effect variegation hypothesis for facioscapulohumeral muscular dystrophy by analysis of histone modification and gene expression in subtelomeric 4q. Hum Mol Genet. 2003 Nov 15;12(22):2909-21.
- Jones TI, King OD, Himeda CL, Homma S, Chen JC, Beermann ML, Yan C, Emerson CP Jr, Miller JB, Wagner KR, Jones PL. Individual epigenetic status of the pathogenic D4Z4

macrosatellite correlates with disease in facioscapulohumeral muscular dystrophy. Clin Epigenetics. 2015 Mar 29;7(1):37.

- Jordan B, Eger K, Koesling S, Zierz S. Camptocormia phenotype of FSHD: a clinical and MRI study on six patients. J Neurol 2011; 258: 866-73.
- Kanadia RN, Johnstone KA, Mankodi A, Lungu C, Thornton CA, Esson D, Timmers AM, Hauswirth WW, Swanson MS. A muscleblind knockout model for myotonic dystrophy. Science. 2003 Dec 12;302(5652):1978-80.
- Korngut L, Siu VM, Venance SL, et al. Phenotype of combined Duchenne and facioscapulohumeral muscular dystrophy. Neuromuscul Disord 2008 Jul; 18(7): 579-582.
- Korngut L, Siu VM, Venance SL, et al. Phenotype of combined Duchenne and facioscapulohumeral muscular dystrophy. Neuromuscul Disord 2008 Jul; 18(7): 579-582.
- Lamperti C, Fabbri G, Vercelli L, D'Amico R, Frusciante R, Bonifazi E, Fiorillo C, Borsato C, Cao M, Servida M, Greco F, Di Leo R, Volpi L, Manzoli C, Cudia P, Pastorello E, Ricciardi L, Siciliano G, Galluzzi G, Rodolico C, Santoro L, Tomelleri G, Angelini C, Ricci E, Palmucci L, Moggio M, Tupler R. A standardized clinical evaluation of patients affected by facioscapulohumeral muscular dystrophy: The FSHD clinical score. Muscle Nerve. 2010 Aug;42(2):213-7.
- Landouzy L, Dejerine J. De la myopathie atrophique progressive. Rev Med Francaise 1885;5:81.
- Laoudj-Chenivesse D, Carnac G, Bisbal C, Hugon G, Bouillot S, Desnuelle C, Vassetzky Y, Fernandez A. Increased levels of adenine nucleotide translocator 1 protein and response to oxidative stress are early events in facioscapulohumeral muscular dystrophy muscle. J Mol Med (Berl). 2005 Mar;83(3):216-24.
- Lecky BR, MacKenzie JM, Read AP, Wilcox DE. X-linked and FSH dystrophies in one family. Neuromuscul Disord 1991; 1: 275-278.

- Lee J, Fung KP. Confidence interval of the kappa coefficient by bootstrap resampling [letter]. Psychiatry Res 1993; 49:97–98.
- Leidenroth A, Hewitt JE. A family history of DUX4: phylogenetic analysis of DUXA, B, C and Duxbl reveals the ancestral DUX gene. BMC Evol Biol. 2010 Nov 26;10:364.
- Lemmers RJ, Goeman JJ, van der Vliet PJ, van Nieuwenhuizen MP, Balog J, Vos-Versteeg M, Camano P, Ramos Arroyo MA, Jerico I, Rogers MT, Miller DG, Upadhyaya M, Verschuuren JJ, Lopez de Munain Arregui A, van Engelen BG, Padberg GW, Sacconi S, Tawil R, Tapscott SJ, Bakker B, van der Maarel SM. Inter-individual differences in CpG methylation at D4Z4 correlate with clinical variability in FSHD1 and FSHD2. Hum Mol Genet. 2015 Feb 1;24(3):659-69.
- Lemmers RJ, de Kievit P, Sandkuijl L, Padberg GW, van Ommen GJ, Frants RR, van der Maarel SM. Facioscapulohumeral muscular dystrophy is uniquely associated with one of the two variants of the 4q subtelomere. Nat Genet. 2002 Oct;32(2):235-6.
- Lemmers RJ, van der Vliet PJ, Klooster R, Sacconi S, Camaño P, Dauwerse JG, Snider L, Straasheijm KR, van Ommen GJ, Padberg GW, Miller DG, Tapscott SJ, Tawil R, Frants RR, van der Maarel SM. A unifying genetic model for facioscapulohumeral muscular dystrophy. Science. 2010 Sep 24;329(5999):1650-3.
- Lemmers RJ, van der Wielen MJ, Bakker E, Padberg GW, Frants RR, van der Maarel SM. Somatic mosaicism in FSHD often goes undetected. Ann Neurol 2004; 55, 845-850.
- Lemmers RJ, Van Overveld PG, Sandkuijl LA, Vrieling H, Padberg GW, Frants RR, van der Maarel SM. Mechanism and timing of mitotic rearrangements in the subtelomeric D4Z4 repeat involved in facioscapulohumeral muscular dystrophy.Am J Hum Genet. 2004 Jul;75(1):44-53 (a).

  Lemmers RJ, Wohlgemuth M, Frants RR, Padberg GW, Morava E, van der Maarel SM. Contractions of D4Z4 on 4qB subtelomeres do not cause facioscapulohumeral muscular dystrophy. Am J Hum Genet. 2004 Dec;75(6):1124-30 (b).

- Lemmers RJ, Wohlgemuth M, van der Gaag KJ, van der Vliet PJ, van Teijlingen CM, de Knijff P, Padberg GW, Frants RR, van der Maarel SM. Specific sequence variations within the 4q35 region are associated with facioscapulohumeral muscular dystrophy. Am J Hum Genet. 2007 Nov;81(5):884-94.
- Lin MY, Nonaka I. Facioscapulohumeral muscular dystrophy: muscle fiber type analysis with particular reference to small angular fibers. Brain Dev 1991; 13: 331-8.
- Lunt PW, Compston DA, Harper PS. Estimation of age dependent penetrance in facioscapulohumeral muscular dystrophy by minimising ascertainment bias. J Med Genet. 1989 Dec;26(12):755-60.
- Lunt PW, Jardine PE, Koch MC, Maynard J, Osborn M, Williams M, Harper PS, Upadhyaya M. Correlation between fragment size at D4F104S1 and age at onset or at wheelchair use, with a possible generational effect, accounts for much phenotypic variation in 4q35-facioscapulohumeral muscular dystrophy (FSHD) Hum Mol Genet. 1995 May;4(5):951-8 (a).
- Lunt PW, Jardine PE, Koch M, Maynard J, Osborn M, Williams M, Harper PS, Upadhyaya M. Phenotypic-genotypic correlation will assist genetic counseling in 4q35-facioscapulohumeral muscular dystrophy. Muscle Nerve. 1995;2:S103-9 (b).
- Lyle R, Wright TJ, Clark LN, Hewitt JE. The FSHD-associated repeat, D4Z4, is a member of a dispersed family of homeobox-containing repeats, subsets of which are clustered on the short arms of the acrocentric chromosomes. Genomics. 1995 Aug 10;28(3):389-97.
- Masciullo M, Iannaccone E, Bianchi ML, Santoro M, Conte G, Modoni A, Monforte M, Tasca G, Laschena F, Ricci E, Silvestri G. Myotonic dystrophy type 1 and de novo FSHD mutation double trouble: a clinical and muscle MRI study. Neuromuscul Disord 2013; 23: 427-31.

- Matsumura T, Goto K, Yamanaka G, Lee JH, Zhang C, Hayashi YK, Arahata K. Chromosome 4q;10q translocations; comparison with different ethnic populations and FSHD patients. BMC Neurol. 2002 Aug 20;2:7.
- McGonigal A, Thomas AM, Petty RK. Facioscapulohumeral muscular dystrophy and myasthenia gravis coexisting in the same patient: a case report. J Neurol 2002; 249: 219-20.
- Mostacciuolo ML, Pastorello E, Vazza G, Miorin M, Angelini C, Tomelleri G, Galluzzi G, Trevisan CP. Facioscapulohumeral muscular dystrophy: epidemiological and molecular study in a north-east Italian population sample. Clin Genet. 2009 Jun;75(6):550-5.
- Nikolic A, Ricci G, Sera F, Bucci E, Govi M, Mele F, Rossi M, Ruggiero L, Vercelli L, Ravaglia S, Brisca G, Fiorillo C, Villa L, Maggi L, Cao M, D'Amico MC, Siciliano G, Antonini G, Santoro L, Mongini T, Moggio M, Morandi L, Pegoraro E, Angelini C, Di Muzio A, Rodolico C, Tomelleri G, Grazia D'Angelo M, Bruno C, Berardinelli A, Tupler R. Clinical expression of facioscapulohumeral muscular dystrophy in carriers of 1-3 D4Z4 reduced alleles: experience of the FSHD Italian National Registry. BMJ Open. 2016 Jan 5;6(1):e007798.
- Olsen DB, Gideon P, Jeppesen TD, Vissing J. Leg muscle involvement in facioscapulohumeral muscular dystrophy assessed by MRI. J Neurol. 2006 Nov;253(11):1437-41.
- Osborne RJ, Welle S, Venance SL, Thornton CA, Tawil R. Expression profile of FSHD supports a link between retinal vasculopathy and muscular dystrophy. Neurology. 2007 Feb 20;68(8):569-77.
- Ottaviani A, Schluth-Bolard C, Rival-Gervier S, Boussouar A, Rondier D, Foerster AM, Morere J, Bauwens S, Gazzo S, Callet-Bauchu E, Gilson E, Magdinier F. Identification of a perinuclear positioning element in human subtelomeres that requires A-type lamins and CTCF. EMBO J. 2009 Aug 19;28(16):2428-36.
- Padberg G. Facioscapulohumeral disease. MD thesis, University of Leiden, 1982.

- Padberg GW, Lunt PW, Koch M, Fardeau M. Diagnostic criteria for facioscapulohumeral muscular dystrophy. Neuromuscul Disord. 1991;1(4):231-4.
- Padberg GW Frants RR, Brouwer OF, Wijmenga C, Bakker E, Sandkuijl LA. Facioscapulohumeral muscular dystrophy in the Dutch population. Muscle Nerve 1995; 2, S81-84.Palmucci L, Mongini T, Doriguzzi C, et al. Association of FSHD and CMT1A in two Italian families. Neuromuscular Disorders 2001;11(6-7):602–613.
- Palmucci L, Mongini T, Doriguzzi C, et al. Association of FSHD and CMT1A in two Italian families. Neuromuscular Disorders 2001;11(6-7):602–613.
- Pearson. Two new pedigrees of muscular dystrophy. Ann Eugen 1933 5:179-191.
- Reilich P, Schramm N, Schoser B, Schneiderat P, Strigl-Pill N, Müller-Höcker J, Kress W, Ferbert A, Rudnik-Schöneborn S, Noth J, Lochmüller H, Weis J, Walter MC. Facioscapulohumeral muscular dystrophy presenting with unusual phenotypes and atypical morphological features of vacuolar myopathy. J Neurol 2010; 257: 1108-18.
- Ricci E, Galluzzi G, Deidda G, Cacurri S, Colantoni L, Merico B, Piazzo N, Servidei S, Vigneti E, Pasceri V, Silvestri G, Mirabella M, Mangiola F, Tonali P, Felicetti L. Progress in the molecular diagnosis of facioscapulohumeral muscular dystrophy and correlation between the number of KpnI repeats at the 4q35 locus and clinical phenotype. Ann Neurol 1999; 45: 751-57.
- Ricci G, Ruggiero L, Vercelli L, Sera F, Nikolic A, Govi M, Mele F, Daolio J, Angelini C, Antonini G, Berardinelli A, Bucci E, Cao M, D'Amico MC, D'Angelo G, Di Muzio A, Filosto M, Maggi L, Moggio M, Mongini T, Morandi L, Pegoraro E, Rodolico C, Santoro L, Siciliano G, Tomelleri G, Villa L, Tupler R. A novel clinical tool to classify facioscapulohumeral muscular dystrophy phenotypes. J Neurol. 2016 Jun;263(6):1204-14.
- Ricci G, Scionti I, Alì G, Volpi L, Zampa V, Fanin M, Angelini C, Politano L, Tupler R, Siciliano G. Rippling muscle disease and facioscapulohumeral dystrophy-like phenotype in a patient carrying a heterozygous CAV3 T78M mutation and a D4Z4 partial deletion:

Further evidence for "double trouble" overlapping syndromes. Neuromuscul Disord. 2012 Jun;22(6):534-40.

- Ricci G, Scionti I, Sera F, Govi M, D'Amico R, Frambolli I, Mele F, Filosto M, Vercelli L, Ruggiero L, Berardinelli A, Angelini C, Antonini G, Bucci E, Cao M, Daolio J, Di Muzio A, Di Leo R, Galluzzi G, Iannaccone E, Maggi L, Maruotti V, Moggio M, Mongini T, Morandi L, Nikolic A, Pastorello E, Ricci E, Rodolico C, Santoro L, Servida M, Siciliano G, Tomelleri G, Tupler R. Large scale genotype-phenotype analyses indicate that novel prognostic tools are required for families with facioscapulohumeral muscular dystrophy. Brain. 2013 Nov;136(Pt 11):3408-17.
- Ricci G, Zatz M, Tupler R. Facioscapulohumeral muscular dystrophy: more complex than it appears. Curr Mol Med. 2014 Oct 10.
- Rudnik-Schöneborn S, Weis J, Kress W, Häusler M, Zerres K. Becker's muscular dystrophy aggravating facioscapulohumeral muscular dystrophy--double trouble as an explanation for an atypical phenotype. Neuromuscul Disord 2008; 18: 881-885.
- Rudnik-Schöneborn S, Weis J, Kress W, Häusler M, Zerres K. Becker's muscular dystrophy aggravating facioscapulohumeral muscular dystrophy--double trouble as an explanation for an atypical phenotype. Neuromuscul Disord 2008; 18: 881-885.
- Sacconi S, Lemmers RJ, Balog J, van der Vliet PJ, Lahaut P, van Nieuwenhuizen MP, Straasheijm KR, Debipersad RD, Vos-Versteeg M, Salviati L, Casarin A, Pegoraro E, Tawil R, Bakker E, Tapscott SJ, Desnuelle C, van der Maarel SM. The FSHD2 gene SMCHD1 is a modifier of disease severity in families affected by FSHD1. Am J Hum Genet. 2013 Oct 3;93(4):744-51.
- Sakellariou P, Kekou K, Fryssira H, Sofocleous C, Manta P, Panousopoulou A, Gounaris K, Kanavakis E. Mutation spectrum and phenotypic manifestation in FSHD Greek patients. Neuromuscul Disord. 2012 Apr;22(4):339-49.

- Sarfarazi M, Upadhyaya M, Padberg G, Pericak-Vance M, Siddique T, Lucotte G, Lunt P. An exclusion map for facioscapulohumeral (Landouzy-Déjérine) disease. J Med Genet. 1989 Aug;26(8):481-4.
- Sarfarazi M, Wijmenga C, Upadhyaya M, Weiffenbach B, Hyser C, Mathews K, Murray J, Gilbert J, Pericak-Vance M, Lunt P, Frants RR, Jacobsen S, Harper PS, Padberg GW. Regional mapping of facioscapulohumeral muscular dystrophy gene on 4q35: combined analysis of an international consortium. Am J Hum Genet 1992; 51: 396-403.
- M, Savarese, Di Fruscio G, Mutarelli M, et al. MotorPlex provides accurate variant detection across large muscle genes both in single myopathic patients and in pools of DNA samples. Acta Neuropathol Commun 2014;2:100.
- Savarese M, Di Fruscio G, Torella A, Fiorillo C, Magri F, Fanin M, Ruggiero L, Ricci G, Astrea G, Passamano L, Ruggieri A, Ronchi D, Tasca G, D'Amico A, Janssens S, Farina O, Mutarelli M, Marwah VS, Garofalo A, Giugliano T, Sanpaolo S, Del Vecchio Blanco F, Esposito G, Piluso G, D'Ambrosio P, Petillo R, Musumeci O, Rodolico C, Messina S, Evilä A, Hackman P, Filosto M, Di Iorio G, Siciliano G, Mora M, Maggi L, Minetti C, Sacconi S, Santoro L, Claes K, Vercelli L, Mongini T, Ricci E, Gualandi F, Tupler R, De Bleecker J, Udd B, Toscano A, Moggio M, Pegoraro E, Bertini E, Mercuri E, Angelini C, Santorelli FM, Politano L, Bruno C, Comi GP, Nigro V. The genetic basis of undiagnosed muscular dystrophies and myopathies: Results from 504 patients. Neurology. 2016 Jul 5;87(1):71-6.
- Scionti I, Fabbri G, Fiorillo C, Ricci G, Greco F, D'Amico R, Termanini A, Vercelli L, Tomelleri G, Cao M, Santoro L, Percesepe A, Tupler R. Facioscapulohumeral muscular dystrophy: new insights from compound heterozygotes and implication for prenatal genetic counselling. J Med Genet. 2012 Mar;49(3):171-8 (a).
- Scionti I, Greco F, Ricci G, Govi M, Arashiro P, Vercelli L, Berardinelli A, Angelini C, Antonini G, Cao M, Di Muzio A, Moggio M, Morandi L, Ricci E, Rodolico C, Ruggiero L, Santoro L, Siciliano G, Tomelleri G, Trevisan CP, Galluzzi G, Wright W, Zatz M, Tupler R.

Large-scale population analysis challenges the current criteria for the molecular diagnosis of fascioscapulohumeral muscular dystrophy. Am J Hum Genet. 2012 Apr 6;90(4):628-35 (b).

- Snider L, Geng LN, Lemmers RJ, Kyba M, Ware CB, Nelson AM, Tawil R, Filippova GN, van der Maarel SM, Tapscott SJ, Miller DG. Facioscapulohumeral dystrophy: incomplete suppression of a retrotransposed gene. PLoS Genet. 2010 Oct 28;6(10):e1001181.
- Statland JM, Donlin-Smith CM, Tapscott SJ, Lemmers RJ, van der Maarel SM, Tawil R. Milder phenotype in facioscapulohumeral dystrophy with 7-10 residual D4Z4 repeats. Neurology. 2015 Dec 15;85(24):2147-50.

  Tasca G, Pescatori M, Monforte M, Mirabella M, Iannaccone E, Frusciante R, Cubeddu T, Laschena F, Ottaviani P, Ricci E. Different molecular signatures in magnetic resonance imaging-staged facioscapulohumeral muscular dystrophy muscles. PLoS One 2012; 7: e38779.
- Tawil R, Forrester J, Griggs RC, Mendell J, Kissel J, McDermott M, King W, Weiffenbach B, Figlewicz D. Evidence for anticipation and association of deletion size with severity in facioscapulohumeral muscular dystrophy. The FSH-DY Group. Ann Neurol. 1996 Jun;39(6):744-8.
- Tawil R, Kissel JT, Heatwole C, Pandya S, Gronseth G, Benatar M; Guideline Development, Dissemination, and Implementation Subcommittee of the American Academy of Neurology; Practice Issues Review Panel of the American Association of Neuromuscular & Electrodiagnostic Medicine. Evidence-based guideline summary: Evaluation, diagnosis, and management of facioscapulohumeral muscular dystrophy: Report of the Guideline Development, Dissemination, and Implementation Subcommittee of the American Academy of Neurology and the Practice Issues Review Panel of the American Association of Neuromuscular & Electrodiagnostic Medicine. Neurology. 2015 Jul 28;85(4):357-64.
-

- Tawil R, van der Maarel S, Padberg GW, van Engelen BG. 171st ENMC international workshop: Standards of care and management of facioscapulohumeral muscular dystrophy. Neuromuscul Disord. 2010 Jul;20(7):471-5.
- Thomas MJ, Seto E. Unlocking the mechanisms of transcription factor YY1: are chromatin modifying enzymes the key? Gene. 1999 Aug 20;236(2):197-208.
- Tonini MM, Passos-Bueno MR, Cerqueira A, Matioli SR, Pavanello R, Zatz M. Asymptomatic carriers and gender differences in facioscapulohumeral muscular dystrophy (FSHD). Neuromuscul Disord. 2004 Jan;14(1):33-8.
- Tonini MM, Passos-Bueno MR, Cerqueira A, Pavanello R, Vainzof M, Dubowitz V, Zatz M. Facioscapulohumeral (FSHD1) and other forms of muscular dystrophy in the same family: is there more in muscular dystrophy than meets the eye? Neuromuscul Disord 2002; 6: 554-57.
- Trevisan CP, Pastorello E, Ermani M, Angelini C, Tomelleri G, Tonin P, Mongini T, Palmucci L, Galluzzi G, Tupler RG, Marioni G, Rimini A. Facioscapulohumeral muscular dystrophy: a multicenter study on hearing function. Audiol Neurootol 2008; 13: 1-6. (a)
- Trevisan CP, Pastorello E, Tomelleri G, Vercelli L, Bruno C, Scapolan S, Siciliano G, Comacchio F. Facioscapulohumeral muscular dystrophy: hearing loss and other atypical features of patients with large 4q35 deletions. Eur J Neurol 2008; 15: 1353-8. (b)
- Tupler R, Barbierato L, Memmi M, Sewry CA, De Grandis D, Maraschio P, Tiepolo L, Ferlini A. Identical de novo mutation at the D4F104S1 locus in monozygotic male twins affected by facioscapulohumeral muscular dystrophy (FSHD) with different clinical expression. J Med Genet. 1998 Sep;35(9):778-83.
- Tupler R, Berardinelli A, Barbierato L, Frants R, Hewitt JE, Lanzi G, Maraschio P, Tiepolo L. Monosomy of distal 4q does not cause facioscapulohumeral muscular dystrophy. J Med Genet. 1996 May;33(5):366-70.

- Tsien F, Sun B, Hopkins NE, Vedanarayanan V, Figlewicz D, Winokur S, Ehrlich M Methylation of the FSHD syndrome-linked subtelomeric repeat in normal and FSHD cell cultures and tissues. Mol Genet Metab. 2001;74:322-331.
- Tsuji M, Kinoshita M, Imai Y, Kawamoto M, Kohara N. Facioscapulohumeral muscular dystrophy presenting with hypertrophic cardiomyopathy: a case study. Neuromuscul Disord 2009; 19: 140-2.
- Tyler FH, Stephens FE: Studies in disorders of muscle: II. Clinical manifestations and inheritance of facioscapulohumeral dystrophy in a large family. Ann Intern Med 1950; 32:640-660.
- Umapathi T, Chaudhry V, Cornblath D, Drachman D, Griffin J, Kuncl R. Head drop and camptocormia. J Neurol Neurosurg Psychiatry 2002; 73: 1-7.
- Upadhyaya M, Jardine P, Maynard J, Farnham J, Sarfarazi M, Wijmenga C, Hewitt JE, Frants R, Harper PS, Lunt PW. Molecular analysis of British facioscapulohumeral dystrophy families for 4q DNA rearrangements. Hum Mol Genet. 1993 Jul;2(7):981-7.
- Upadhyaya M, Lunt PW, Sarfarazi M, Broadhead W, Daniels J, Owen M, Harper PS. A closely linked DNA marker for facioscapulohumeral disease on chromosome 4q. J Med Genet. 1991 Oct;28(10):665-71.
- Upadhyaya M, Lunt PW, Sarfarazi M, Broadhead W, Daniels J, Owen M, Harper PS. DNA marker applicable to presymptomatic and prenatal diagnosis of facioscapulohumeral disease. Lancet. 1990 Nov 24;336(8726):1320-1.
- Upadhyaya M, Maynard J, Rogers MT, Lunt PW, Jardine P, Ravine D, Harper PS. Improved molecular diagnosis of facioscapulohumeral muscular dystrophy (FSHD): validation of the differential double digestion for FSHD. J Med Genet. 1997 Jun;34(6):476-9.
- van den Boogaard ML, Lemmers RJ, Camaño P, van der Vliet PJ, Voermans N, van Engelen BG, Lopez de Munain A, Tapscott SJ, van der Stoep N, Tawil R, van der Maarel

SM. Double SMCHD1 variants in FSHD2: the synergistic effect of two SMCHD1 variants on D4Z4 hypomethylation and disease penetrance in FSHD2. Eur J Hum Genet. 2016 Jan;24(1):78-85.

- van der Maarel SM, Deidda G, Lemmers RJ, van Overveld PG, van der Wielen M, Hewitt JE, Sandkuijl L, Bakker B, van Ommen GJ, Padberg GW, Frants, RR. De novo facioscapulohumeral muscular dystrophy: frequent somatic mosaicism, sex-dependent phenotype, and the role of mitotic transchromosomal repeat interaction between chromosomes 4 and 10. Am J Hum Genet 2000; 66, 26-35.
- van Deutekom JC, Bakker E, Lemmers RJ, van der Wielen MJ, Bik E, Hofker MH, Padberg GW, Frants RR. Evidence for subtelomeric exchange of 3.3 kb tandemly repeated units between chromosomes 4q35 and 10q26: implications for genetic counselling and etiology of FSHD1. Hum Mol Genet. 1996 Dec;5(12):1997-2003 (a).
- van Deutekom JC, Lemmers RJ, Grewal PK, van Geel M, Romberg S, Dauwerse HG, Wright TJ, Padberg GW, Hofker MH, Hewitt JE, Frants RR. Identification of the first gene (FRG1) from the FSHD region on human chromosome 4q35. Hum Mol Genet. 1996 May;5(5):581-90 (b).
- van Deutekom JC, Wijmenga C, van Tienhoven EA, Gruter AM, Hewitt JE, Padberg GW, van Ommen GJ, Hofker MH, Frants RR. FSHD associated DNA rearrangements are due to deletions of integral copies of a 3.2 kb tandemly repeated unit. Hum Mol Genet. 1993 Dec;2(12):2037-42.
- van Geel M, Dickson MC, Beck AF, Bolland DJ, Frants RR, van der Maarel SM, de Jong PJ, Hewitt JE. Genomic analysis of human chromosome 10q and 4q telomeres suggests a common origin. Genomics. 2002 Feb;79(2):210-7.
- van Koningsbruggen S, Dirks RW, Mommaas AM, Onderwater JJ, Deidda G, Padberg GW, Frants RR, van der Maarel SM. FRG1P is localised in the nucleolus, Cajal bodies, and speckles. J Med Genet. 2004 Apr;41(4):e46.

- van Koningsbruggen S, Straasheijm KR, Sterrenburg E, de Graaf N, Dauwerse HG, Frants RR, van der Maarel SM. FRG1P-mediated aggregation of proteins involved in pre-mRNA processing. Chromosoma. 2007 Feb;116(1):53-64.
- van Overveld PG, Enthoven L, Ricci E, Rossi M, Felicetti L, Jeanpierre M, Winokur ST, Frants RR, Padberg GW, van der Maarel SM. Variable hypomethylation of D4Z4 in facioscapulohumeral muscular dystrophy. AnnNeurol. 2005;58:569-576.
- van Overveld PG, Lemmers RJ, Deidda G, Sandkuijl L, Padberg GW, Frants RR, van der Maarel SM. Interchromosomal repeat array interactions between chromosomes 4 and 10: a model for subtelomeric plasticity. Hum Mol Genet 2000 22; 9: 2879-84.
- van Overveld PG, Lemmers RJ, Sandkuijl LA, Enthoven L, Winokur ST, Bakels F, Padberg GW, van Ommen GJ, Frants RR, van der Maarel SM. Hypomethylation of D4Z4 in 4q-linked and non-4q-linked facioscapulohumeral muscular dystrophy. Nat Genet. 2003 Dec;35(4):315-7.
- Wijmenga C, Frants RR, Brouwer OF, Moerer P, Weber JL, Padberg GW. Location of facioscapulohumeral muscular dystrophy gene on chromosome 4. Lancet. 1990 Sep 15;336(8716):651-3.
- Wijmenga C, Hewitt JE, Sandkuijl LA, Clark LN, Wright TJ, Dauwerse HG, Gruter AM, Hofker MH, Moerer P, Williamson R, et al. Chromosome 4q DNA rearrangements associated with facioscapulohumeral muscular dystrophy. Nat Genet. 1992 Sep;2(1):26-30.
- Wijmenga C, Padberg GW, Moerer P, Wiegant J, Liem L, Brouwer OF, Milner EC, Weber JL, van Ommen GB, Sandkuyl LA, et al. Mapping of facioscapulohumeral muscular dystrophy gene to chromosome 4q35-qter by multipoint linkage analysis and in situ hybridization. Genomics. 1991 Apr;9(4):570-5.
- Wijmenga C, van Deutekom JC, Hewitt JE, Padberg GW, van Ommen GJ, Hofker MH, Frants RR. Pulsed-field gel electrophoresis of the D4F104S1 locus reveals the size and the

parental origin of the facioscapulohumeral muscular dystrophy (FSHD)-associated deletions. Genomics. 1994 Jan 1;19(1):21-6.

- Winokur ST, Bengtsson U, Feddersen J, Mathews KD, Weiffenbach B, Bailey H, Markovich RP, Murray JC, Wasmuth JJ, Altherr MR, Schutte BC. The DNA rearrangement associated with facioscapulohumeral muscular dystrophy involves a heterochromatin-associated repetitive element: implications for a role of chromatin structure in the pathogenesis of the disease. Chromosome Res 1994; 2: 225-34.
- Winokur ST, Bengtsson U, Vargas JC, Wasmuth JJ, Altherr MR, Weiffenbach B, Jacobsen SJ. The evolutionary distribution and structural organization of the homeobox-containing repeat D4Z4 indicates a functional role for the ancestral copy in the FSHD region. Hum Mol Genet. 1996 Oct;5(10):1567-75.
- Winokur ST, Chen YW, Masny PS, Martin JH, Ehmsen JT, Tapscott SJ, van der Maarel SM, Hayashi Y, Flanigan KM. Expression profiling of FSHD muscle supports a defect in specific stages of myogenic differentiation. Hum Mol Genet. 2003 Nov 15;12(22):2895-907.
- Zatz M, Marie SK, Cerqueira A, Vainzof M, Pavanello RC, Passos-Bueno MR. The facioscapulohumeral muscular dystrophy (FSHD1) gene affects males more severely and more frequently than females. Am J Med Genet. 1998 May 1;77(2):155-61.
- Zatz M, Marie SK, Passos-Bueno MR, Vainzof M, Campiotto S, Cerqueira A, Wijmenga C, Padberg G, Frants R. High proportion of new mutations and possible anticipation in Brazilian facioscapulohumeral muscular dystrophy families. Am J Hum Genet. 1995 Jan;56(1):99-105.
- Zeng W, de Greef JC, Chen YY, Chien R, Kong X, Gregson HC, Winokur ST, Pyle A, Robertson KD, Schmiesing JA, Kimonis VE, Balog J, Frants RR, Ball AR Jr, Lock LF, Donovan PJ, van der Maarel SM, Yokomori K. Specific loss of histone H3 lysine 9 trimethylation and HP1gamma/cohesin binding at D4Z4 repeats is associated with facioscapulohumeral dystrophy (FSHD). PLoS Genet. 2009 Jul;5(7):e1000559.

Annexes

Annex 1

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Facioscapulohumeral Muscular Dystrophy: More Complex than it Appears

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Abstract: Facioscapulohumeral muscular dystrophy (FSHD) has been classified as an autosomal dominant myopathy, linked to rearrangements in an array of 3.3 kb tandemly repeated DNA elements (D4Z4) located at the 4q subtelomere (4q35). For the last 20 years, the diagnosis of FSHD has been confirmed in clinical practice by the detection of one D4Z4 allele with a reduced number (≤8) of repeats at 4q35. Although wide inter- and intra-familial clinical variability was found in subjects carrying D4Z4 alleles of reduced size, this DNA testing has been considered highly sensitive and specific. However, several exceptions to this general rule have been reported. Specifically, FSHD families with asymptomatic relatives carrying D4Z4 reduced alleles, FSHD genealogies with subjects affected with other neuromuscular disorders and FSHD affected patients carrying D4Z4 alleles of normal size have been described. In order to explain these findings, it has been proposed that the reduction of D4Z4 repeats at 4q35 could be pathogenic only in certain chromosomal backgrounds, defined as "permissive" specific haplotypes. However, our most recent studies show that the current DNA signature of FSHD is a common polymorphism and that in FSHD families the risk of developing FSHD for carriers of D4Z4 reduced alleles (DRA) depends on additional factors besides the 4q35 locus. These findings highlight the necessity to re-evaluate the significance and the predictive value of DRA, not only for research but also in clinical practice. Further clinical and genetic analysis of FSHD families will be extremely important for studies aiming at dissecting the complexity of FSHD.

Keywords: D4Z4 reduced allele, diagnostic criteria, facioscapulohumeral muscular dystrophy, genetic counseling, genetic heterogeneity, genotype-phenotype correlation, molecular test, muscle disease.

INTRODUCTION

Facioscapulohumeral muscular dystrophy (FSHD, OMIM #158900) is the third most common form of hereditary myopathy with a prevalence of 1 in 20.000 [1]. The disease is characterized by progressive atrophy and weakness of a highly selective set of muscle groups. Before the finding of D4Z4 reduced alleles (DRA) at 4q35, which have been considered pathognomonic for disease, the diagnosis and counseling of FSHD families were entirely based on clinical evidence and family history. Over the years, DNA testing for FSHD has been considered highly sensitive and specific [2, 3]. Thus there has been a tendency to associate clinical findings and molecular data, even when the phenotype did not completely fulfill the clinical criteria of FSHD. However, the specificity and the sensitivity of DNA testing in FSHD have come into question since it was observed that 1) 3% of healthy subjects from the general population carry DRA [4-7] and 2) 20% of FSHD probands do not carry DRA [7-10]. These clinical and molecular studies show that FSHD is a more complex disorder than previously thought. Therefore the current molecular signature is insufficient to diagnose FSHD and has to be carefully re-evaluated as predictor of disease outcome. Moreover, a wide range of myopathic phenotypes have been observed in subjects carrying DRA, indicating that a careful clinical diagnosis and molecular characterization of each family should be performed to determine the significance of DRA. It is indeed possible that the lack of specificity of DRA might have led to biased interpretations of clinical observations. Additionally, genetic heterogeneity must be considered in FSHD families in which no DRA segregates. An unbiased analysis of such genealogies as well as in families with no clear autosomal dominant inheritance might allow identifying additional genes involved in FSHD pathogenesis, as it has happened in other complex genetic diseases such as Alzheimer's disease or amyotrophic lateral sclerosis [11-17]. These critical aspects must be considered both in research and in clinical practice.

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THE CLASSICAL PHENOTYPE OF FSHD

The disease was firstly reported in 1862 by Duchenne de Boulogne, who published a picture of an affected patient in his *Album de photographies pathologiques* [18]. Duchenne described the disease in his famous series of papers in Archives of General Medicine in 1869 [19], which is often cited as the earliest reference of FSHD [20]. In 1885, Landouzy and Dejerine [21] described in detail the clinical features of FSHD, thus also called "Landouzy-Dejerine form of muscular dystrophy", characterized by progressive facial, shoulder girdle and pectoral muscle weakness and atrophy, subsequent involvement of abdominal muscles with lumbar hyperlordosis and anterior leg muscles with steppage gait. Subsequently, in 1982, the thesis of Padberg provided the first modern clinical description of FSHD families. Padberg investigated a group of 107 subjects from 19 families, including 73 subjects displaying clinical signs of FSHD. These studies provided the first evidence for wide clinical variability in FSHD patients, even within the same family [22].

The clinical presentation in FSHD is characterized by initially restricted distribution of weakness starting with asymptomatic facial weakness followed by weakness of scapular fixator, humeral, truncal and lower extremity muscles. The onset at lower-extremity is often characterized by distal weakness, typically in the anterior leg compartment, presenting with footdrop. Extraocular and bulbar muscles are typically spared. Weak abdominal muscles result in a protuberant abdomen and contribute to the lumbar lordosis. Lower abdominal muscles are weaker than upper abdominal muscles, causing strikingly positive Beevor's s sign, a physical finding fairly specific for FSHD [23]. A notable distinctive feature of FSHD is that muscle weakness displays asymmetric distribution, which does not correlate with the handedness of the individual [24]. The creatine kinase (CK) level can be moderately increased or normal. Electromyography (EMG) and histological analysis reveal non-specific myopathic changes associated, in some cases, with neurogenic and/or inflammatory aspects [25, 26]. Muscle magnetic resonance imaging (MRI) can detect muscles showing normal MRI signal together with muscles showing abnormalities on T1-weighted MRI sequences, corresponding to areas of fatty fibrous replacement, or areas characterized by increased signal on T2- short tau inversion recovery (T2-STIR) sequences also in muscles not yet replaced by fat tissue, reflecting an increase in tissue water content due to muscle oedema [27]. Ancillary features, such as sensorineural deafness or retinal vasculopathy have been also reported in infantile FSHD forms, but they are not to be considered decisive criteria for FSHD diagnosis [28, 29]. FSHD has been considered a fully penetrant autosomal dominant disease with age-dependent penetrance estimated to be >95% by age 20 [30]. However, in contrast with the expected course for a classical autosomal dominant Mendelian disorder, the chronology of disease progression is unpredictable, and disease expressivity ranges from subjects with very mild muscles weakness, almost unaware of being affected, to wheelchair-dependent patients (Fig. **1**).

DEFINITION OF DIAGNOSTIC CRITERIA FOR FSHD IN PRE-MOLECULAR ERA

In 1991 an International Consortium established the clinical, laboratory and genetic criteria for FSHD diagnosis, in absence of a diagnostic DNA test. This work responded for the need of selecting families that could be included in the linkage analysis [31] towards the identification of the FSHD gene. Four main criteria were identified: (1) onset of the disease in facial or shoulder girdle muscles; sparing of the extra-ocular, pharyngeal and lingual muscles and the myocardium; (2) facial weakness in more than 50% of the affected family members; (3) autosomal dominant inheritance in familial cases; and (4) evidence of myopathic disease in EMG and muscle biopsy in at least one affected member. By contrast, (1) involvement of extra-ocular, masticatory, pharyngeal and lingual muscles; (2) regression of symptoms and signs; (3) presence of severe and diffuse contractures; (4) involvement of myocardium with presence of cardiomyopathy; (5) persistently high CK values above five times the upper limit, were considered suggestive of alternative diagnosis [31].

THE DISCOVERY OF DNA ALTERATIONS ASSOCIATED WITH FSHD

The need for an accurate pre-symptomatic test led to an active search for the identification of the FSHD gene [30]. In 1990 the FSHD locus was assigned to chromosome 4 by positional mapping performed in 10 Dutch families with autosomal dominance inheritance [32]. This chromosomal position was confirmed using additional polymorphic markers in other families [33, 34]. Subsequently, Wijmenga and coworkers [35] reported that D4S139, a Variable Number Tandem Repeat structure (VNTR) locus, was the most closely linked to FSHD. Because D4S139 represents the most telomeric 4q-specific marker, it established the location of the FSHD gene in the subtelomeric region of chromosome 4q. The assignment of the FSHD locus to region 4q35 was definitively established in 1992 by six laboratories, based on the genotyping of 504 affected patients and 559 unaffected subjects from 65 families [36]. Later, Wijmenga and coworkers [37] identified a 3.3 kb tandemly repeated sequence (D4Z4) located at the 4q subtelomeric region that could be detected by hybridization of *Eco*RI digested DNA using the p13E-11 DNA sequence as probe. This study included 11 Dutch families, 6 *de novo* cases, 29 healthy individuals. One family presenting compound heterozygosity for two D4Z4 alleles smaller than 28 kb was excluded from the study. The authors showed that in healthy individuals the majority (72%) of *Eco*RI fragments detected by the p13E-11 probe were larger than 28 kb, while in FSHD patients there was an overrepresentation of fragments smaller than 28 kb [37]. It was also shown that 5 out of

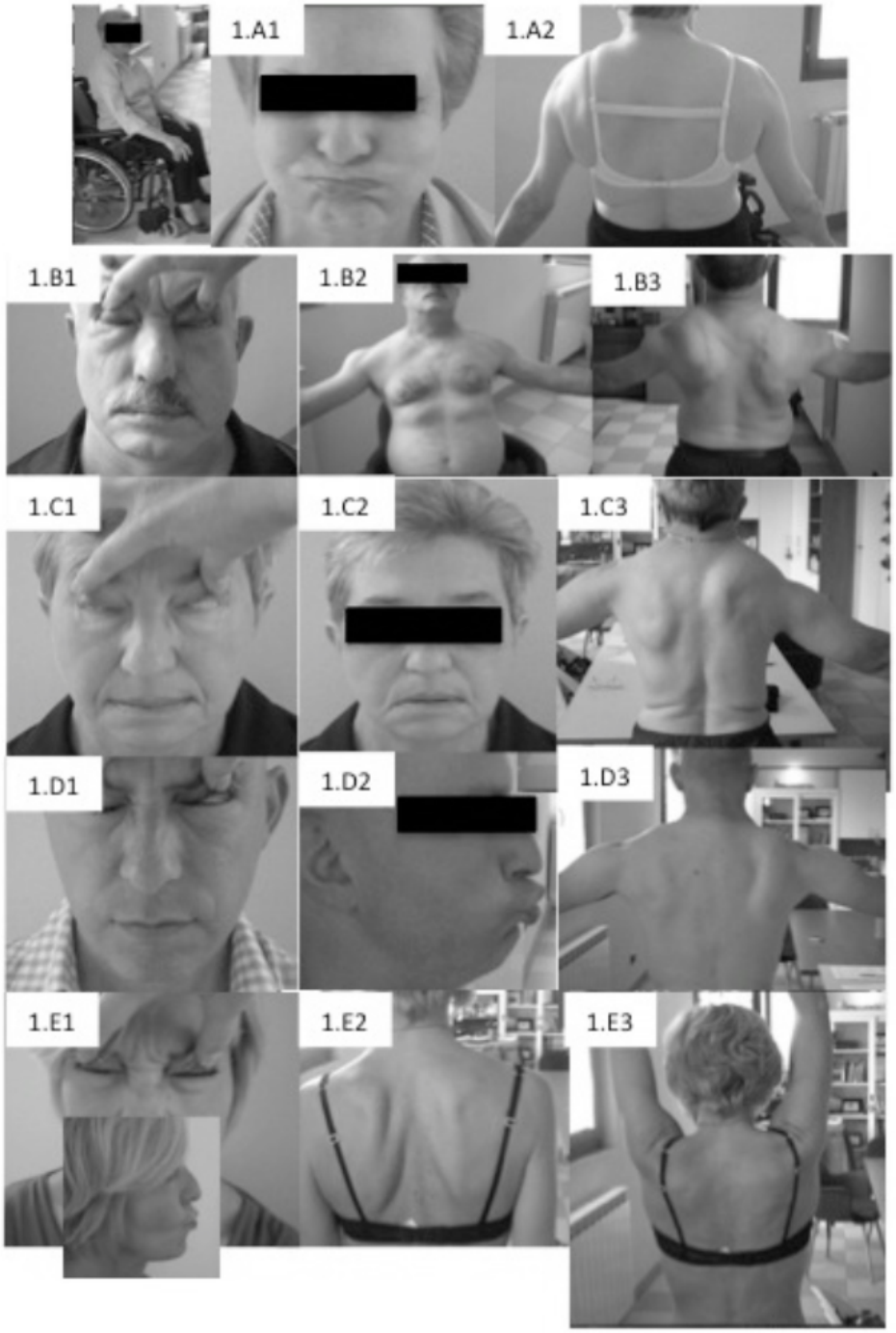

Fig. (1). Wide variability of clinical expression in a FSHD family. The proband (aged 66 years, Fig. **1A**), her brother (aged 60 yrs, Fig. **1.B1**, **1.B2**, **1.B3**), her sisters (respectively aged 65 and 52 yrs; Fig. **1.C1**, **1.C2**, **1.C3** and Fig. **1.E1**, **1.E2**, **1.E3**) and her son (aged 42 yrs, Fig. **1.D1**, **1.D2**, **1.D3**), all carrying a 23 kb 161qA DRA. The family members **1.A**, **1.B**, **1.C**, **1.D** display facial weakness (Fig. **1.A1**, **1.B1**, **1.C1**, **1.C2**, **1.D1**), limitation in raising the arms (the Fig. **1.A2**, **1.B3**, **1.C3**, **1.D3** show the maximum capacity of arms abduction), scapula winging (Fig. **1.B3**, **1.C3**, **1.D3**), fulfilling the clinical diagnostic criteria of FSHD The sister, **1.E**, aged 52 yrs, is asymptomatic. Mild scapula winging is detected at clinical examination (Fig. **1.E2**) without any motor impairment (Fig. **1.E1**, **1.E3**).

6 affected individuals with unaffected parents carried a *de novo* p13E-11 allele smaller than 28 kb. On this basis it was proposed that FSHD is caused by DNA rearrangements of p13E-11 *Eco*RI alleles. However, 8 healthy individuals presented in the study carried p13E-11 alleles smaller than 28 kb, providing an early clue that D4Z4 allele size alone would be unlikely to explain all cases of FSHD pathogenesis.

THE D4Z4 LOCUS

The probe p13E-11 detects a highly polymorphic locus with a VNTR structure constituted by a tandemly arrayed sequence of DNA repetitive elements named D4Z4 [38]. The variation in size of *Eco*RI fragments is due to variability in the number of D4Z4 repeats [39]. In normal subjects the p13E-11 *Eco*RI alleles usually range from 40 kb to approximately 300 kb (>10 D4Z4 units), whereas alleles of 35 kb or shorter (≤ 8 D4Z4 units) are present in the majority of either de novo or familial FSHD patients [40-42].

The D4Z4 repeats belong to a family of 3.3 repeats scattered within the human genome including chromosome 1 secondary constriction, and the heterochromatin of the acrocentric chromosomes [38, 43, 44]. Importantly, an almost identical D4Z4 array was located at chromosome 10q, with 98% homology between 4q35 and 10q26 regions [45]. The homology between 4q35 and 10q26 is not confined to the 3.3-kb repeats but extends both proximally (42 kb) and distally to include the telomere [46]. Notably, the size of D4Z4 alleles on chromosome 10 overlaps with those on chromosome 4.

The presence of a polymorphism on the D4Z4 copy on chromosome 10, creating a *BlnI* restriction site, has facilitated the distinction between 4q and 10q D4Z4 alleles by using *Eco*RI/*Bln*I double digestion followed by p13E-11 Southern hybridization [47] (Fig. **2**). This approach has led to the discovery that in 20-30% of the population translocated 4-type repeats reside on chromosome 10q and, viceversa, translocated 10-type repeats on chromosome 4q [48-51]. *De novo* reduced allele account for a surprisingly high percentage of FSHD patients (10%–33%) [52, 53]. This high incidence can be partly explained by the presence of parental mosaicism for 4q short alleles that has been reported in 19% of *de novo* cases [54-56]. The presence of somatic mosaicism for a rearrangement of

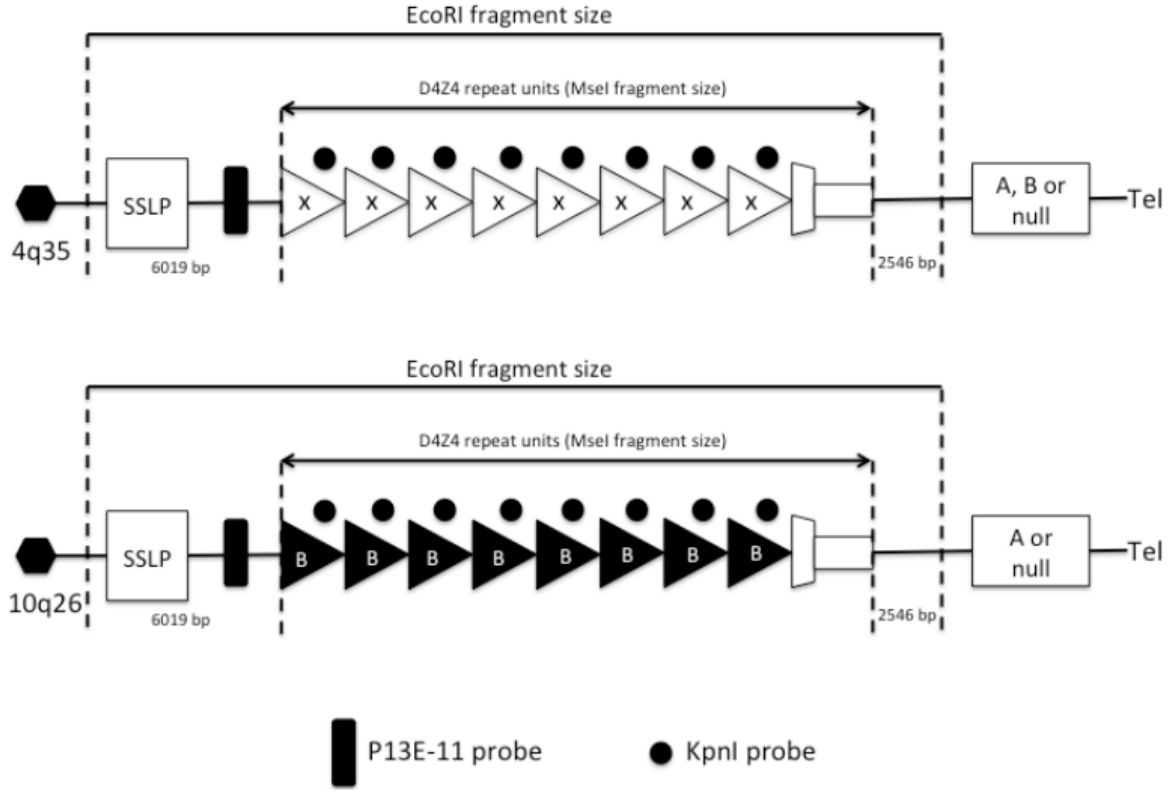

Fig. (2). Schematic representation of Polymorphisms at the 4q and 10q subtelomeres. Schematic representation of the method used to calculate D4Z4 repeat numbers from *Eco*RI-fragment sizes. Seven and eight D4Z4 repeats (31-36 kb *Eco*RI fragment size) were defined to be the upper diagnostic range for FSHD. D4Z4 repeat units on chromosomes 4 and 10 can be distinguished because all repeats on 10q contain *BlnI* restriction sites (B), while all D4Z4 repeats on 4q contain *XapI* restriction sites (X).

D4Z4 was found in as much as 3% of the general population [57]. These observations, demonstrate that the D4Z4 array is highly recombinogenic. Notwithstanding, only DRAs located at 4q have been associated with FSHD regardless of the repeat type composition.

Despite the identification of the molecular defect associated with FSHD, its pathologic effects remain largely unknown. Each D4Z4 repeat unit harbors GC-rich sequences, which are predominantly found in heterochromatic regions of the genome [38, 43] This led to the hypothesis that deletion of D4Z4 repeats might modify the chromatin organization of the 4q subtelomeric region and alter gene expression [43]. Consistently, an element within D4Z4 has been shown to behave as a silencer that provides a binding site for a transcriptional repressing complex [58]. More recent findings raise the possibility that epigenetic markings like DNA methylation [59], histone modifications [60] or chromosomal architectures [61] can be altered at the disease locus, suggesting that the chromosomal context in which the D4Z4 deletions arise can be considered crucial for clinical development of FSHD. A recent study indicates that the Polycomb group of epigenetic repressors targets D4Z4 in healthy subjects and that D4Z4 deletion is associated with reduced Polycomb silencing in FSHD patients. Cells from FSHD patients produce a chromatin-associated noncoding RNA, DBE-T, which recruits the Trithorax group protein Ash1L to the FSHD locus, driving histone H3 lysine 36 dimethylation, chromatin remodeling, and 4q35 gene transcription [62]. Collectively, these results suggest a model in which reduction of D4Z4 leads to the inappropriate transcriptional derepression of proximal chromosome 4-specific genes. Indeed, 4q35 proximal genes such as FSHD Region Gene 2 (*FRG2*), FSHD Region Gene 1 (*FRG1*), and Adenine Nucleotide Translocator 1 (*ANT1*), with high myopathic potential, were observed to be transcriptionally upregulated in FSHD muscle [58] and mice over-expressing FRG1 develop a muscular dystrophy with features of human disease [63]. Involvement of the proximal 4q35 genes added a different level of complexity on FSHD molecular mechanism other than repeats reduction, which indeed does not account for all FSHD cases. However, different studies testing this model showed controversial results. Some were in accordance [58, 64-66], while others not [67-69] preventing a consensus regarding whether protein-coding genes within 4q35 are upregulated and contribute to FSHD pathogenesis.

Another potential mechanism was suggested when detailed sequence analysis revealed that the D4Z4 repeat contains an open reading frame (ORF) encoding a double-homeobox transcription factor, *DUX4* [38, 70]. It has thus been proposed that reduction of the D4Z4 array results in the transcription of the *DUX4* [71].

Although the abundance of the *DUX4* mRNA and protein results is extremely low (approximately 1 in 1000 FSHD muscle cell nuclei were detected with an abundant amount of *DUX4* mRNA) [72], it has been observed that the *DUX4*-expressing FSHD muscle nuclei show pathologic features consistent with DUX4 induced toxicity [73]. Thus, according to the current pathogenic model of FSHD, the inefficient chromatin-mediated repression, either related to the contraction of the array or to its hypomethylation, may result in the occasional escape from repression in muscle cells with a consequently inappropriate expression of DUX4 protein [71].

In addition gene expression analyses revealed alterations in FSHD muscle that could be linked to various pathologic processes such as altered angiogenesis, susceptibility to oxidative stress and abnormal muscle differentiation [67-69, 74]. Despite all these efforts the FSHD pathophysiology and the sequence of molecular events associating a potentially cytotoxic lesion at muscle level are still elusive.

THE IDENTIFICATION OF SPECIFIC HAPLO-TYPES ASSOCIATED WITH D4Z4 REDUCED ALLELES

Since there are individuals with DRA that do not have clinical signs of FSHD, it has been proposed that additional DNA sequences flanking the D4Z4 repeat array are necessary for disease development. In 2002, a polymorphic bi-allelic segment of 10 kb distal to D4Z4 was identified [46]. Of the two allelic forms, 4A and 4B, only 4A was found to be associated with FSHD [75]. Additional sequence variations, namely Simple Sequence Length Polymorphisms (SSLP), were found proximal to the D4Z4 repeat. Together with the 4A/4B polymorphisms, these SSLPs generate at least 17 and 8 genetically distinct variants, respectively, at the chromosome 4q and chromosome 10q subtelomeres [76]. Among these many haplotypes only the common variant 4A161 and the rare variants 4A159 and 4A168 were found associated with D4Z4 reduced alleles in FSHD patients. By contrast, D4Z4 reduced alleles associated with other haplotypes were not detected in the FSHD cases. Finally, a single nucleotide polymorphism (SNP) ATTAAA was found in the pLAM1 sequence of the 4qA alleles that provides a PolyAdenylation Signal (PAS) allowing the expression of the most distal copy of the DUX4 gene [77]. Thus, it has been proposed that the combination of (1) a reduction in the number of D4Z4 elements, (2) the presence of the 4qA allele, and (3) the PAS in the pLAM1 sequence together with the (159/161/168) SSLPs represents the molecular signature that defines alleles causally related to FSHD. On this basis it has been hypothesized that this particular chromosomal setting, named 4APAS, causes FSHD through a toxic gain of function attributable to the stabilized distal DUX4 transcript [77] (Fig. **3**).

MOLECULAR BASIS OF FSHD: PATHOGENIC HYPOTHESIS

However, as explained below, data coming from different genotype-phenotype studies and clinical reports on FSHD patients and families showed that

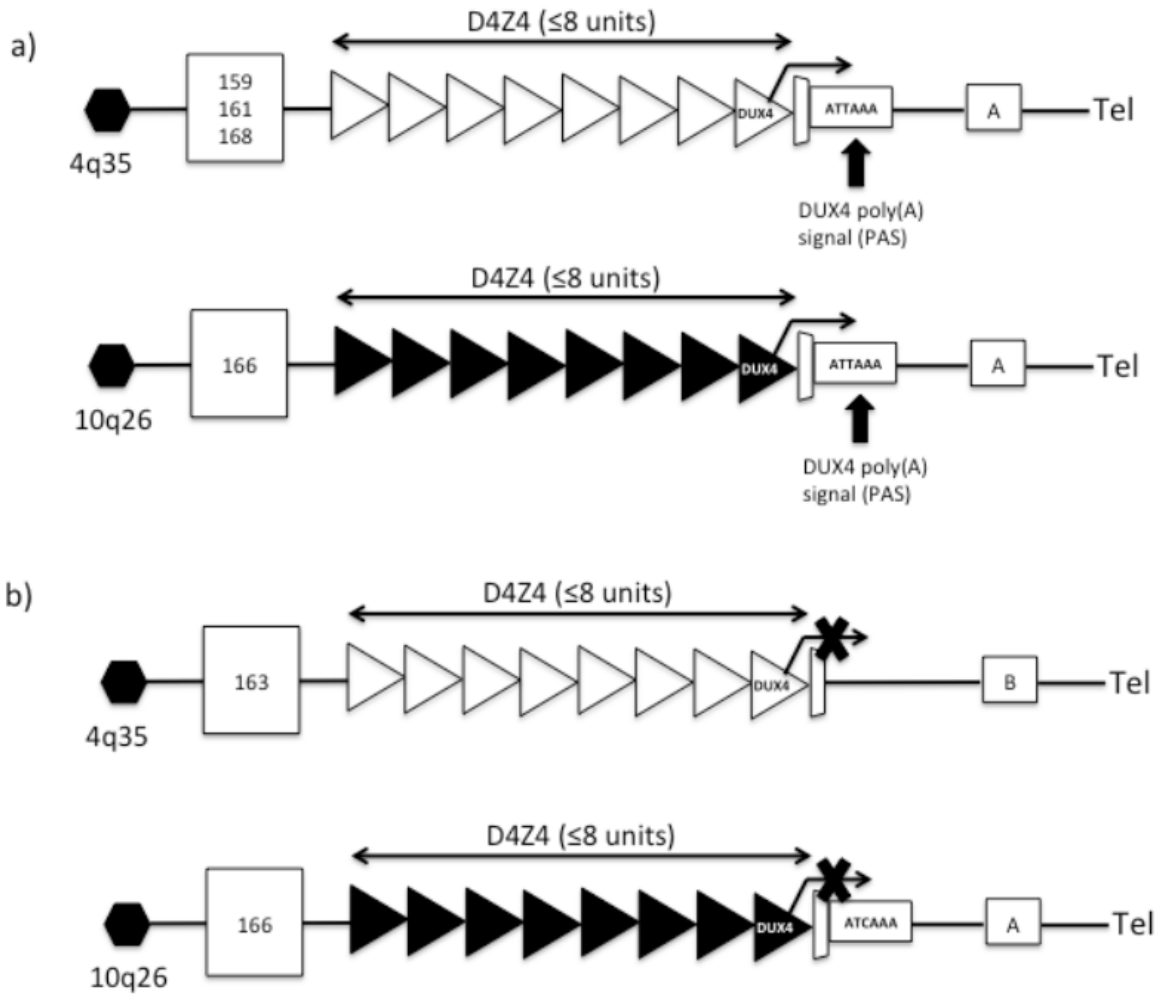

Fig. (3). Schematic representation of the current view of permissive and not-permissive haplotype. Fig. **3.A**: Permissive haplotypes. Fig. **3.B**: Non-permissive haplotype. The ATTAAA variant creates a polyadenylation signal (PAS) that stabilizes the *DUX4* transcript and has been postulated to be the critical factor causing FSHD.

exceptions that are not inconsistent with this hypothesis are frequent in human populations.

LARGE-SCALE POPULATION ANALYSIS CHALLENGES THE CURRENT CRITERIA FOR THE MOLECULAR DIAGNOSIS OF FSHD

The finding of FSHD families with compound heterozygosity [6, 78-80] suggests that in the general population DRA are more frequent than expected based on the prevalence of FSHD (1 in 20,000). This possibility was first predicted by van Overveld and coworkers [49], who found among 208 anonymous blood donors 6 subjects carrying DRA of size between 25-35 kb. This notion was definitely confirmed by the study of 801 normal control subjects from Italy and Brazil, which showed that 3% (25 of 801) of normal controls carried D4Z4 alleles of size ranging from 21 kb (4 D4Z4 units) and 35 kb (8 D4Z4 units) [7]. Remarkably 11 of them (~1.3%) carried the supposedly pathogenic 4A161PAS haplotype. The age of all these healthy carriers ranged between 40 to 78 years, an age in which FSHD is considered to be fully penetrant. Therefore it can be concluded that the current genetic signature of FSHD is a relatively common polymorphism and little predictive value can be attributed to the 4A161PAS haplotype in the absence of family history because 1.3% of healthy subjects carry this haplotype [7].

FACIOCAPULOHUMERAL MUSCULAR DYSTROPHY WITHOUT D4Z4 REPEATS CONTRACTION ON CHROMOSOME 4q35

Initially, FSHD patients were shown to exhibit one D4Z4 allele with a 8 or fewer repeats (35 kb), whereas

their non-FSHD allele shows, like control alleles, higher D4Z4 numbers (n≥ 9-100). Later, the critical number of remaining D4Z4 repeats was raised for diagnostic purposes (n=9-10) [81, 82]. At the same time it became evident that small D4Z4 alleles of 30-40 kb (6–10 D4Z4 repeats) were found also in normal controls [4-7, 83]. Thus, a remarkable overlap exists between D4Z4 alleles in controls and in FSHD patients and a definition of clear cut-off point is difficult. Furthermore it has been estimated that approximately 20% of FSHD patients carry DRA of 38 kb or larger [7-10], although these patients are clinically indistinguishable from those carrying a DRA. In 2003 Butz and coworkers conducted a systematic study including 37 unrelated myopathic patients carrying 32-41 kb D4Z4 alleles (7-10 units) and 102 healthy controls [83]. A broad myopathic spectrum with four phenotypes (typical FSHD, facial-sparing FSHD, FSHD with atypical features, non-FSHD muscle disease) was found among carriers of these alleles termed "borderline". Seven control subjects, out of 102 (6.8%), carried alleles of the same size-range. Therefore the study highlighted that in this group there is no definite D4Z4 diagnostic cut-off point separating FSHD, FSHD-like myopathies and controls, thus questioning the clinical significance of these "borderline" alleles [83].

More recently, De Greef and coworkers [84] performed a cross-sectional study on 33 patients from 27 families with D4Z4 allele with ≥11 repeats. These patients, termed the "FSHD2" cohort, displayed D4Z4 alleles of normal size on both chromosomes 4 alleles and appeared clinically undistinguishable from those carrying DRA. Of the 33 FSHD2 patients, 20 (61%) were males. The average age at onset was 26 years (range 0–60), which is almost 10 years later than in FSHD1. The initial symptom was scapular weakness in 61%, foot dorsiflexor weakness was reported in 27%, facial weakness in 10%, and hip girdle weakness in 3%. In contrast with the "FSHD1" cohort, in which there are significantly more males clinically and also more severely affected than females [10, 85-87] no gender difference in disease severity in FSHD2 was observed. Furthermore, there is a notable difference in the mode of inheritance between FSHD1 and FSHD2. In this study [84] pedigree analysis showed that the majority of cases (22/33) were sporadic, 11 belonged to 5 families. Two families showed autosomal dominant (parent-child pairs), two families displayed autosomal recessive inheritance (sibs pairs) and one family failed to present a clear Mendelian pattern of inheritance [84]. The major molecular feature of FSHD2 is loss of DNA methylation of the D4Z4 arrays at the 4q35 and the 10q26 [88]. Therefore it has been hypothesized that in FSHD2, in which D4Z4 alleles have normal size, there is a defect in establishing or maintaining the D4Z4 repeat chromatin structure. Consistent with this hypothesis, mutations in the *SMCHD1* (Structural maintenance of chromosomes flexible hinge domain-containing) gene have been found in FSHD2 patients [89]. This gene has been recently identified as an epigenetic modifier of chromatin structure. It has thus been proposed that lower levels of *SMCHD1* result in lower D4Z4 methylation contributing to disease onset. Therefore that lack of autosomal dominant inheritance in FSHD2 families would be explained by the presence of a mutated gene in association with D4Z4 hypomethylation.

GENOTYPE-PHENOTYPE CORRELATION STUDIES IN FSHD

Penetrance of Disease in Carriers of D4Z4 Reduced Allele

In pre-molecular era, the first observations performed on large families with clinical diagnosis of FSHD suggested an almost complete penetrance of the disease [90-92]. However, since the advent of molecular diagnosis for FSHD, subjects carrying DRA without signs of disease have been reported [4, 7, 10, 42, 80, 85-89, 93-95], challenging the notion that DRA alone can cause nearly full disease penetrance. Several such studies are summarized below.

In a previous study on 52 Brazilian families with DRA smaller than 35 kb [85], the estimated penetrance for FSHD allele was found to be 85% for patients until age 30. Furthermore, when the authors considered the sexes separately, the estimated penetrance of the FSHD allele was significantly greater for males (95%) than for females (69%). Interestingly, among 27 families with at least two clinically affected patients it was observed that in 21 families the pattern of inheritance was autosomal dominant (4 of them with incomplete penetrance). Surprisingly, in 3 pedigrees the pattern of inheritance was compatible with the presence of an autosomal recessive trait since there were at least two affected sibs born from asymptomatic parents. These observations suggested that FSHD phenotypes may result from distinct types of mutations in different families.

A study conducted on Italian families [94] reported 7 subjects, aged 20 to 69 years old, with DRA between 21 and 37 kb (4-8 units), without symptoms or signs of FSHD, who were classified as non-penetrant carriers. In this study, unaffected individuals were not observed in families with DRA smaller than 20 kb.

A retrospective analysis conducted on 85 Japanese patients with FSHD and both their parents documented parents with DRA who had no clinical symptoms, confirming an estimated low penetrance of 59% (excluding somatic mosaicism) [95].

Tonini *et al.* in 2004 [86], analyzing 238 subjects with DRA <35 kb from 106 unrelated families, observed that about 20% of individuals related to FSHD patients who carried a DRA remained asymptomatic or were minimally affected with a significantly higher proportion of females than males; asymptomatic carriers were found in about 30% of the families.

Recently, Sakellariou and coworkers [10] reported clinical and genetic analysis of 133 individuals carrying DRA (71 probands and 62 relatives) from 71 unrelated Greek families, revealing a high percentage (almost 50%) of asymptomatic relatives older than 30 years

and carrying DRA. The percentage of unaffected carriers was also lower in males than in females (29% *vs* 71%). It is also noteworthy that 16 among the 38 multiple-case families (42%) were found to have at least one symptom-free individual, with a greater proportion of asymptomatic or minimally affected gene carriers concentrating in some pedigrees, as previously observed by Tonini and coworkers [86]. A statistically significant association between the genders and the clinical manifestation of the disease was also observed: among the females the percentage of symptomatic patients was found to be 66.7% whereas among the males it was 86.6%.

The recent study performed by the Italian Clinical Network for FSHD [87] evaluated the degree of motor impairment in a large group of patients affected by facioscapulohumeral muscular dystrophy and their relatives who carry DRA. Clinical assessment was performed in 530 subjects, 163 probands and 367 relatives, from 176 unrelated families according to a standardized clinical score [96]. Overall, 32.2% of relatives did not display any muscle functional impairment. This phenotype was influenced by the degree of relation with proband, because 47.1% of second- through fifth-degree relatives were unaffected, while only 27.5% of first-degree family members did not show motor impairment. The estimated risk of developing motor impairment by age 50 for relatives carrying a DRA with 1-3 repeats or 4-8 repeats was 88.7% and 55% respectively. Male relatives had a mean score significantly higher than females (5.4 *vs* 4.0, p=0.003). No 4q haplotype was exclusively associated with the presence of disease. In 19 (13%) families in which DRA with 4-8 repeats segregate, the diagnosis of FSHD was reported only in one generation. In 5 pedigrees the pattern of inheritance was compatible with the presence of an autosomal recessive trait since there were at least two affected sibs born from asymptomatic parents.

Overall these studies on cohorts of FSHD families of different geographical origin showed that penetrance of disease is not complete, with females significantly less affected than males and in some families autosomal dominant mode of inheritance is not observed.

ATYPICAL PHENOTYPES ASSOCIATED WITH D4Z4 REDUCED ALLELES: CLINICAL SUB-TYPES OF FSHD OR MORE COMPLEX MYO-PATHIC CONDITIONS?

In the past 20 years, assessment of the D4Z4 array size as diagnostic test for FSHD has led to the identification of phenotypes that differs at various degrees from the original description of disease made by Landouzy-Dejerine [21]: two examples are shown in Figs. (**4**, **5**). This has provoked a trend towards the expansion of the clinical pattern associated with D4Z4 reduced allele. Several subtypes of FSHD with atypical clinical presentation have been described (Table **1**) [4, 83, 97-119].

For example, in 2000 van der Kooi and coworkers [102] described six sporadic cases that did not meet most of the diagnostic criteria defined in 1991 but were diagnosed as FSHD because they carried a DRA (range 26 to 38 kb) on 4q. The foot drop was the predominant clinical feature found in three patients; in three others, inability to walk on toes, shoulder pain, and pelvic limb weakness with difficulty in walking were reported, respectively. None of them had facial weakness and only one complained of shoulder weakness. Interestingly, none had a positive family history.

In the same year, Felice and coworkers [103] described 10 patients out of 14, with facial-sparing scapular myopathy associated with DRA (range 20 to 39 kb). Except for the absence of facial weakness, most patients had clinical and laboratory features otherwise consistent with FSHD. Five patients referred also a positive family history of similar weakness, although DNA analysis was not performed on other family members.

Felice and Moore in 2001 [104] also described four patients, each harboring DRA (range 25 to 34 kb), who presented with atypical phenotypes including facial-sparing scapular myopathy, limb-girdle muscular dystrophy (LGMD) distal myopathy and asymmetric brachial weakness. Only the first two patients had undergone muscle biopsies, which showed unspecific dystrophic features. None of these patients were subjected to other molecular investigations for differential diagnosis. Interestingly, the patient with LGMD phenotype and asymmetric brachial weakness did not report a positive family history for neuromuscular diseases. In this work, the authors concluded that the availability of the DNA test, considered as highly sensible and specific, allowed to establish definitively the diagnosis without the need for the more invasive and less specific muscle biopsy.

Krasnianski *et al.* [109] described three patients from a single family (father and two sons) in which a 23 kb DRA segregated. They showed signs consistent of typical FSHD associated with chronic progressive external ophthalmoplegia. The oculomotor impairment was reported as the initial manifestation of disease starting from infancy. The muscle biopsy of the father and one child demonstrated prominent myopathic changes without ragged red fibers or histopathological features of other neuromuscular diseases. The absence of singular or multiple deletions of mitochondrial DNA apparently excluded a coincidental diagnosis of Chronic Progressive External Ophthalmoplegia (CPEO) of mitochondrial origin. On the other hand, the classic FSHD distribution of the muscle weakness had been never described in patients with CPEO. The possibility of oculopharyngeal muscular dystrophy was not investigated. In the same paper [106], the authors further described other two familial cases and one sporadic case with facial-sparing FSHD syndrome associated with D4Z4 reduced allele (34 and 30 kb allele respectively).

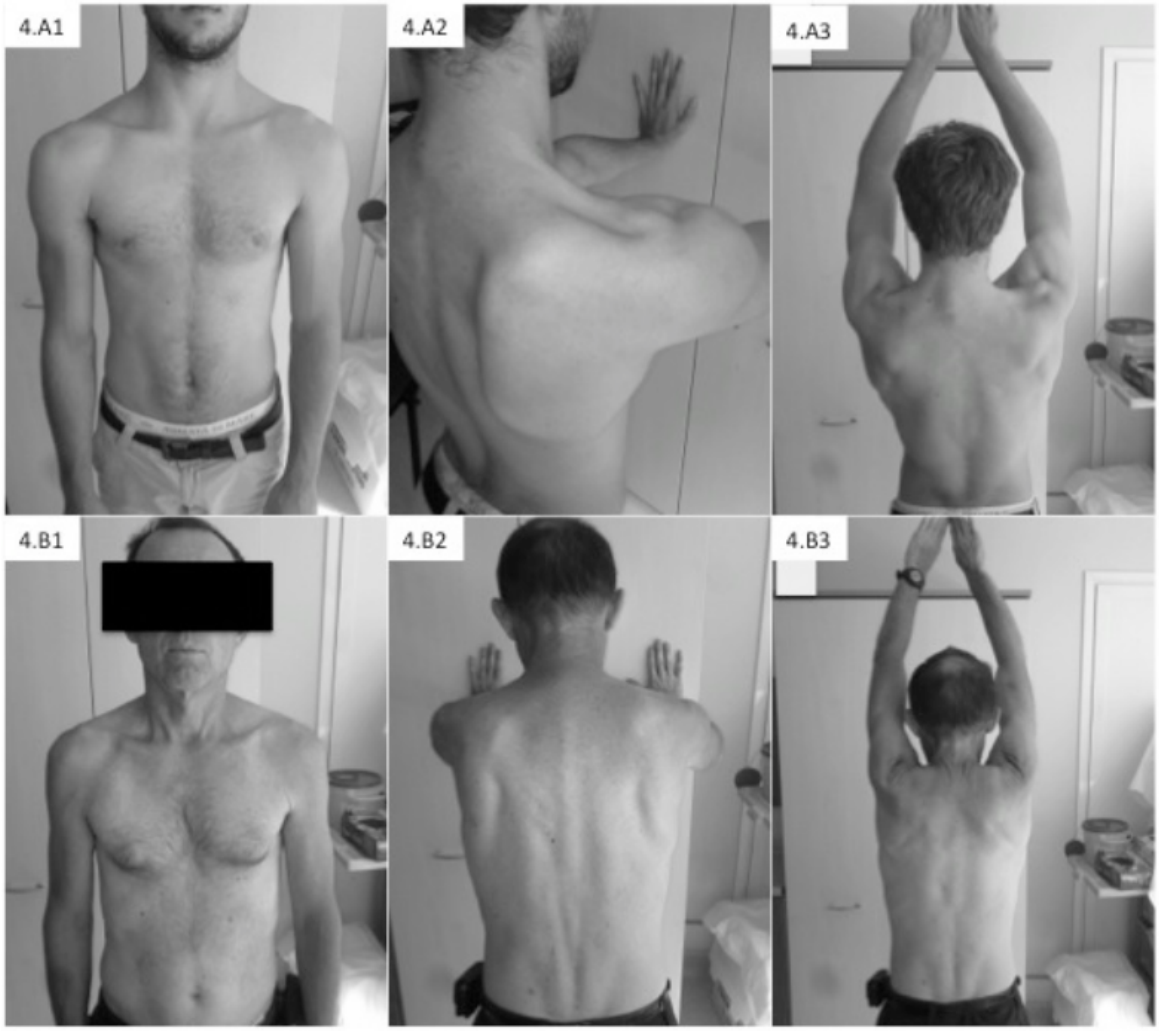

Fig. (4). A myopathic subject and his father, both carriers of a 25 kb 4qA161 DRA. The proband **4.A** (19 yrs old) complained a mild impairment of the right shoulder girdle. The neurological examination shows mild winged scapula in pushing against a wall with the hands at shoulder level and elbows straight (Fig. **4.A2**), hypotrophy of right *sovrapinatus* (Fig. **4.A2**) and *pectoralis* muscles (Fig. **4.A1**), with no limitation of arm abduction (Fig. **4.A3**). Facial weakness is not detected. The neurological examination of the father **4.B** (59 yrs old) is normal (Fig. **4.B1**, **4.B2** and **A.B3**).

Cardiac involvement, including hypertrophic cardiomyopathy, conduction defects and arrhythmia, has been reported in subjects carrying a DRA by several reports [98-101], although the European Expert Group on FSHD in 1991, that defined the Diagnostic Criteria for FSHD in pre-molecular era [31], defined that "cardiomyopathy is not part of the disease" and "when present it suggests an alternative diagnosis".

Reilich *et al.* [113] described five unrelated cases carrying DRA whose biopsies showed signs of vacuolar myopathy with rimmed vacuoles. The atypical clinical features included a form of LGMD phenotype with facial-sparing, a form of distal and proximal weakness, which was associated with dysphagia in one patient and a form of a prevalent asymmetric lower limb distal weakness. Scapular winging or facial weakness was also reported, suggesting the possibility of an overlapping FSHD syndrome. In these cases the family history was negative for neuromuscular disorders or motor impairment, although molecular analysis was not performed in other family members. Only in one family the DNA testing revealed the same DRA (size 35 kb) in the mother and two sisters of the proband affected by distal weakness; these relatives showed a mild facial involvement at clinical examination. The five muscle biopsies of the above unrelated cases showed a pattern of degenerative myopathy with rimmed vacuoles and inflammatory infiltrates. Immunohisto-

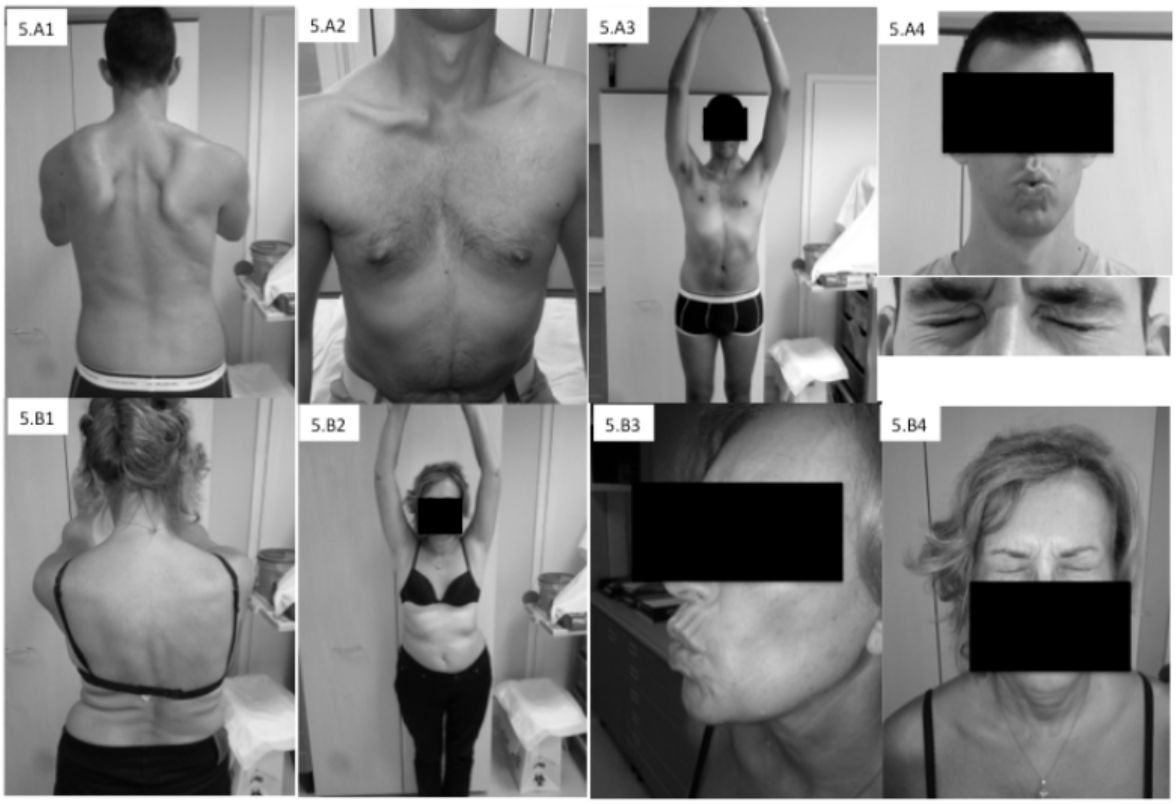

Fig. (5). A myopathic subject and his mother, both carriers of a 31 kb 4qA161 DRA. The proband **5.A** (29 yrs old) complained a mild impairment of the shoulder girdle. The neurological examination shows bilateral winged scapula (Fig. **5.A1**) and frank *pectus excavatum* (Fig. **5.A2**), with no limitation of arm abduction (Fig. **5.A3**); no evident facial weakness is detected (Fig. **5.A4**). The mother **5.B** (63 yrs old) reported congenital hip dysplasia and suffered of scoliosis since infancy (Fig. **5.B1**). The neurological examination does not show muscle impairment (Fig. **5.B1**, **5.B2**, **5.B3**).

chemistry did not detect abnormal desmin, myotilin or alphabeta-crystallin deposits, excluding the diagnosis of myofibrillar myopathies. Electron microscopy revealed autophagic vacuoles containing myelin-like material and filamentous nuclear inclusions. Interestingly, MRI imaging did not reveal the muscle lower limbs involvement typical of FSHD.

Table **1** summarizes other several atypical phenotypes associated with DRA, including the bent spine syndrome, a clinical condition characterized by a stooped posture in the standing position, which is exaggerated in walking or in exercise and disappears in the supine position, sometimes associated with a dropped head [107, 110, 115, 116]. The first reported case [107] was about a 59-years-old woman with a family history of FSHD presenting with an overlapping condition with camptocormia, scapular winging and mild facial and proximal weakness. Kottlors *et al.* [115] described the case of a 65-years-old man complaining of lower back pain and progressive bent spine syndrome, since the age of 60, carrying a 31 kb DRA. The patient recalled that his mother had a similar posture that began at age of 80. The genetic analysis performed on the available family members revealed the presence of DRA in the two daughters, who showed signs of myopathic facies. In one slight weakness of foot extensors was observed. Nonetheless, none in the family presented a typical FHSD phenotype. Jordan and coworkers [116] reported six sporadic cases carrying a DRA (range 21-34 kb) with prevalent axial weakness. All patients referred late disease onset in fourth-sixth decades. Muscle MRI imaging revealed that in all six patients the most severely affected muscles were the thoracic and lumbar spinal tract together with hamstrings.

The conclusion of some authors is that the extensive use of genetic analysis has expanded the clinical and morphological spectrum of FSHD, and many consider the detection of DRA in a patient sufficient to diagnose FSHD [3]. Interestingly, the atypical phenotypic cases are often sporadic. It may thus be supposed that in these cases the shorter D4Z4 fragment is not per se sufficient to trigger myopathy. Indeed the wide heterogeneity associated with alterations on chromosome 4q35 can suggest that other factors/pathologic conditions influence and

Table 1. Synopsis of atypical FSHD patiets and families.

Reference	Case Description	Family History
Jardine *et al.*, *Neuromuscul Disord* 1994 [97]	Dominantly inherited muscular dystrophy with onset in the shoulder girdle and later progression to the lower limbs, associated with DRA of 38 kb	Familial cases
Nakagawa *et al.*, *Internal Medicine* 1997 [4]	6 cases with severe limb and girdle muscular weakness (LGMD-like) with or without mild facial muscle involvement (DRA size range: 13 to 24 kb)	-5 familial cases -1 sporadic case
Laforêt *et al.*, *Neurology* 1998 [98]	5 FSHD subjects with conduction defects or arrhythmia without associated cardiovascular risk factors	-3 familial cases -2 sporadic cases
Finsterer *et al.*, *Cardiology* 2005 [99] Emmrich *et al.*, *Z Kardiol* 2005 [100] Tsuji *et al.*, *Neuromuscular Disorders* 2009 [101]	3 unrelated subjects with FSHD and cardiomyopathy	-2 familial cases -1 sporadic case
van der Kooi *et al.*, *J Neurol Neurosurg Psychiatry* 2000 [102]	FSHD cases with atypical presentation, characterized by foot drop (3 cases), shoulder pain and pelvic limb weakness (3 cases). Range of DRA size: 26 to 38 kb	All sporadic cases
Felice *et al.*, *Neurology* 2000 [103]	10 cases with facial sparing scapular myopathy (range of DRA size: 20 to 39 kb)	-5 familial cases -5 sporadic cases
Felice and Moore, *Muscle Nerve* 2001 [104]	1 subject with facial-sparing scapular myopathy (DRA size 25kb), 1 subject with limb-girdle muscular dystrophy (LGMD) (DRA size 34kb), 1 subject with distal myopathy (DRA size 30kb), and 1 subject with asymmetric brachial weakness (DRA size 34 kb)	-1 familial case -4 sporadic cases
Yamanaka *et al.*, *Neurology* 2001 [105]	7 subjects with a severe form of FSHD associated with atrophic tongue (DRA size range: 10 to 17 kb)	-2 familial cases -3 sporadic cases
Uncini *et al.*, *Neuromuscul Disord* 2002 [106]	A case with isolated monomelic atrophy of lower limb with calf muscle involvement (DRA size 26 kb)	Familial case
Umapathi *et al.*, *J Neurol Neurosurg Psychiatry* 2002 [107]	A case with camptocormia, scapular winging and mild facial and proximal weakness	Familial case
Krasnianski *et al.*, *Arch Neurol* 2003 [109]	3 subjects from a single family with FSHD and chronic progressive external ophthalmoplegia (DRA size 20 kb); 2 related subjects respectively with facial-sparing scapulohumeral and chronic pain/calf atrophy/mild minimal hip flexor paresis (DRA size 34 kb); a case with sporadic facial sparing scapulohumeral (DRA size 30 kb)	-5 familial cases -1 sporadic case
Butz *et al.*, *J Neurol* 2003 [83]	6 subjects with facial-sparing FSHD, 4 subjects with atypical FSHD (onset and predominance in left pelvi-femoral muscle, isolated atrophy M.pect., predominant weakness of axial and pelvic girdle, one-sided atrophy of Mm. Pect., trap., suprasp.), 4 subjects with no FSHD phenotype (bilateral atrophy of Mm. tib. ant., onset in lower limbs with dysarthria and dysphagia, discrete facial paresis with highly elevated CK, improvement under cortisol, onset and predominance in pelvic muscles). DRA size range: 32 to 45 kb	-3 possibly familial cases -12 sporadic cases
Wood-Allum *et al.*, *Neuropathol Appl Neurobiol* 2004 [110]	A case with clinical features of FSHD, but also kyphosis, weakness of neck flexion, and nemaline rods at muscle biopsy (DRA size 17 kb). A case with camptocormia due to weakness in the paraspinal muscles (DRA size 30 kb)	-1 familial case -1 sporadic case
Sugie *et al.*, *Neurology* 2009 [111]	A case with hemiatrophy (DRA size of 20 kb)	Sporadic case
Zouvelou *et al.*, *J Clin Neurosci* 2009 [112]	A case with persistent, asymptomatic hyperCKemia (DRA size 23 kb)	Sporadic case
Reilich *et al.*, *J Neurol* 2010 [113]	5 unrelated cases with an unusual phenotype (LGMD phenotype with facial sparing, distal and proximal weakness, dysphagia, prevalent asymmetric lower limb distal weakness) and vacuolar myopathy with rimmed vacuoles	-2 possibly familial cases -3 sporadic cases
Figueroa *et al.*, *J Neurol* 2010 [114]	2 siblings, one with isolated facial diplegia and the other with late onset facial and limb-girdle weakness (DRA size of 25 kb)	Familial cases
Kottlors *et al.*, *Muscle Nerve* 2010 [115]	A case with lower back pain and progressive bent spine syndrome	Familial case
Jordan *et al.*, *J Neurol* 2011 [116]	6 cases with bent spine syndrome (DRA size range 21 to 34 kb)	-2 familial cases -4 sporadic cases
Papadopoulos *et al.*, *Muscle Nerve* 2011 [117]	A case with bent spine syndrome (DRA size 28 kb)	Familial case
Hassan *et al.*, *Muscle Nerve* 2012 [118]	7 subjects with focal weakness (3 subjects with monomelic lower limb atrophy and weakness, 2 subjects with upper limb unilateral weakness or atrophy, 2 subjects with axial weakness)	Familial cases

modulate the disease expression, such as epigenetic or environmental factors, concomitant inflammatory disease. It may plausible that other genetic and/or environmental factors may participate in the onset of a myopathy that might present clinical features overlapping with FSHD. On the other hand, it must also be considered the possibility that other myopathies might have been misdiagnosed because of the random finding of a DRA in the affected subject.

SEVERAL REPORTS OF "DOUBLE TROUBLE" CONDITIONS IN FSHD FAMILIES

In 2002, Tonini and coworkers [120] reported two unrelated Brazilian families with members apparently affected by two different forms of muscular dystrophy. In the first one, the 35-years-old male proband showed LGMD with proximal weakness, elevated CK (16-fold above normal) and a myopathic muscle biopsy. Muscle protein immunohistochemical and immunoblotting analysis revealed a normal pattern for dystrophin, the four sarcoglycans, calpain, dysferlin and telethonin; DNA analysis for caveolin-3 gene was negative. Two of his sisters also complained of muscle weakness. The younger sister, aged 38 years, complained of proximal muscular weakness in upper and lower limbs, had calf hypertrophy, and a serum CK 5-fold above normal but she refused further investigations. The oldest sister, aged 51 years, showed mild clinical signs possibly consistent with FSHD, confirmed through the molecular analysis (30 kb DRA). The DRA was also found in another six relatives: four of them, aged 72, 45, 36 and 22 years, were asymptomatic and two (aged 19 and 16 years) showed only mild facial hyposthenia. Surprisingly the DRA was not detected in the affected proband. In the second family, a 57-years-old male with a typical FSHD phenotype was carrying a 17 kb DRA, which was also present in other affected relatives. However, in a 14-year-old severely affected male cousin, confined to a wheelchair since age 12, but without facial weakness, the small fragment was not found; the patient refused to undergo muscle biopsy. These families illustrate complicated situations that may occur for diagnosis and genetic counseling of neuromuscular disorders. Considering that the prevalence of hereditary neuromuscular disorders is very approximately 1/1000, we estimated that the finding of two families with an additional neuromuscular disorder was about three times higher than expected. Therefore, although the presence of different neuromuscular disorders in the same genealogy could be only a coincidence, we speculated that some epigenetic mechanisms present in particular families might turn individuals more prone to pathological mutations.

However in FSHD, more than in other neuromuscular disorders, several "double trouble" conditions patients are described. In these patients the D4Z4 reduced allele is associated with a well-known pathogenic mutation of other genes, causing complex and overlapping phenotypes as summarized in Table **2**. In particular, patients with mitochondrial myopathy/FSHD [121], Becker dystrophy/FSHD [122], Duchenne dystrophy/FSHD [123, 124], Leber's hereditary optic neuropathy/FSHD [125], LGMD1C with rippling disease/FSHD [126], myotonic dystrophy type 1/FSHD [127] were reported suggesting the possibility of a synergistic effect of those simultaneous mutations in reaching and in modulating the clinical expression.

Besides, the coexistence of facioscapulohumeral muscular dystrophy and myasthenia gravis in a same patient was also reported [128].

CONCLUSION

In the pre-molecular era, the diagnosis and counseling of FSHD families was entirely based on clinical evidence [30]. In 1992 the discovery of rearranged D4Z4 alleles generated a radical shift in the FSHD field [37]. Over the years, DNA testing of the D4Z4 locus and flanking polymorphisms has been considered highly sensitive and specific and extensively used to diagnose FSHD [3] and researchers have been trying to explain how rearrangements of repetitive elements at the 4q35 subtelomere might cause disease. Definitively, this region represents an important example of how repetitive elements can influence gene expression, and generates pathology [129]. However, several types of evidence challenge the current understanding the molecular basis for of FSHD: 1) 20% of FSHD cases do not carry alleles with reduced numbers (≤ 8) of D4Z4 repeats at 4q35 [7-10] and not all of them can be explained by DNA hypomethylation of the D4Z4 array; 2) alleles with reduced numbers (≤ 8) of D4Z4 repeats at 4q35 combined with 4A(159/161/168) PAS haplotype, have a frequency of 1.3% among healthy subjects from the general population. Thus there are millions of individuals carrying this molecular signature who do not have FSHD disease [7]; 3) the penetrance of the disease among relatives of FSHD patients is incomplete and several factors, including the genetic background [86, 87], play a role in disease outcome; 4) other genetic mechanisms should be considered to explain this large percentage of cases in which FSHD does not segregate in an autosomal dominant mode of inheritance [87] (Fig. **6**).

All these findings are not surprising if considered within the present context of human molecular genetics.

The extensive use over the past 20 years of DNA analysis for studying Mendelian disorders has revealed many disease mechanisms that are more complex than single mutations. For example, identical phenotypes may be produced by mutations in different genes [130], the same mutation can cause different phenotypes [131, 132], and distinct mutations in the same gene may result in different disorders that segregate with diverse Mendelian or even multifactorial patterns [133]. In addition, the incomplete penetrance of some mutations argues for the role of modifying loci or epigenetic mechanisms influencing the clinical expression in many Mendelian disorders. Moreover the

Table 2. Synopsis of documented genetic comorbities associated with the FSHD.

Reference	Molecular and Clinical Findings
Lecky *et al.*, *Neuromuscul Disord* 1991 [123]	Duchenne dystrophy FSHD
Chuenkongkaew *et al.*, *Eur J Neurol* 2005 [125]	Leber's hereditary optic neuropathy (G11778A mutation mutation in mitochondrial DNA) / FSHD (DRA size 17-27-kb)
Korngut *et al.*, *Neuromuscul Disord* 2008 [124]	Duchenne dystrophy (deletion c.367_368delGT in exon 6 of the dystrophin gene) FSHD (DRA size 31 kb)
Rudnik-Schöneborn *et al.*, *Neuromuscul Disord* 2008 [122]	Becker dystrophy (donor splice site mutation c.4071+1 G>T in exon 29 of the dystrophin gene) FSHD (DRA size 28 kb)
Filosto *et al.*, *Neuromuscul Disord* 2008 [121]	Mitochondrial myopathy (heteroplasmic transition T12313C of the $tRNA^{Leu(CUN)}$) FSHD (DRA size 25 kb)
Ricci *et al.*, *Neuromuscul Disord* 2012 [126]	LGMD1C with rippling disease (heterozygous *CAV3* T78M) FSHD (DRA size 35 kb)
Masciullo *et al.*, *Neuromuscul Disord* 2013 [127]	Myotonic dystrophy type 1 (CTG expansion at the DMPK locus, about 500 repeats) FSHD (DRA size 24 kb)

possibility of conducting extensive studies in different human populations has revealed the large variability of human DNA variations blurring the distinction between a polymorphism and a detrimental mutation more subtle. Thus, establishing the value of mutational events underlying genetic diseases may be complex even in diseases with simple patterns of inheritance and well-characterized pathologic course [7, 134, 135].

1992
discovery of D4Z4 deletion at 4q35 in FSHD patients [37].

1996
discovery of the BlnI restriction site in D4Z4 repeats on chromosome 10q26 [47].

1998
setting the standards of molecular diagnosis in FSHD [2].

2000
interchromosomal rearrangements between chromosome 4 and 10 [49].

2000
3% of healthy blood donors carry D4Z4 alleles with 8 or fewer repeats [49].

2002
discovery of bi-allelic (4A/4B) polymorphism distal to D4Z4 array.
Only 4A associated with FSHD [75].

2007
discovery of polymorphic SSLPs proximal to D4Z4.
Only 161 SSLP associated with 4A and D4Z4 reduced allele is "permissive" for FSHD [76].

2010
discovery of SNP (ATT/CAAA) in the pLAM sequence of the 4A allele.
Only (161/159/168) SSLP associated with 4A, ATTAAA SNP and D4Z4 reduced allele is "permissive" for FSHD [77].

2012
discovery that 161 SSLP associated with 4A, ATTAAA and D4Z4 reduced allele is present in 1.3% of healthy subjects [7].

2012
20% of FSHD patients do not carry D4Z4 reduced allele [7].

2013
13% of families in which D4Z4 reduced allele segregates do not show autosomal dominant inheritance [87].

2013
27.5% of 1st degree relatives with D4Z4 reduced allele do not have muscle weakness;
47.1% 2nd-5th degree relatives with D4Z4 reduced allele do not have muscle weakness [87].

Fig. (6). Synopsis of findings in 20 years of FSHD genetic studies. The extended use of D4Z4 analysis in FSHD has challenged the original notion that FSHD is a fully-penetrant autosomal dominant disease associated with reduction in size of D4Z4 alleles.

FSHD seems to fall in this complex pattern. A wide variability in clinical spectrum together with a growing number of atypical clinical presentations associated with the FHSD genetic marker has been extensively documented. Interestingly, several cases are sporadic with no clear autosomal dominant inheritance and in some families the occurrence of affected sibs with healthy parents suggests an autosomal recessive mode of inheritance. Different explanations should be considered for atypical cases previously considered as examples of the wide clinical spectrum of FSHD as well as for non-manifesting relatives usually believed examples of non-penetrance. First, clinical heterogeneity associated with DRA may indicate involvement of other mechanisms that influence and modulate the disease expression (such as genetic, epigenetic or environmental factors), thus emphasizing the concept that the current genetic signature of FSHD alone may not be sufficient to produce clinical symptoms. Second, in families that include asymptomatic members and/or atypical phenotypes, the significance of the DRA should be carefully evaluated, and whole Exome Sequencing or Whole Genome Sequencing should be conducted in an attempt to identify new susceptibility/causative factors contributing to FSHD. To reach this goal, the precise phenotypic classification of patients and families as well as the natural history of the disease and pattern of inheritance will be crucial to create parameters to subclassify FSHD patients. This approach will lay the basis for a more precise genetic counseling of at-risk families and a better understanding of FSHD pathogenesis leading to the identification of outcomes of interests for patients and clinicians to be used in clinical trials.

ABBREVIATIONS

FSHD = Facioscapulohumeral muscular dystrophy

DRA = D4Z4 reduced allele

ORF = Open reading frame

LGMD = Limb Girdle Muscular Dystrophy

MRI = Magnetic Resonance Imaging

CPEO = Chronic progressive external ophthalmoplegia

EMG = Electromyography

CONFLICT OF INTEREST

The authors have nothing to declare.

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REFERENCES

[1] Mostacciuolo ML, Pastorello E, Vazza G, *et al.* Facioscapulohumeral muscular dystrophy: epidemiological and molecular study in a northeast Italian population sample. Clin Genet 2009; 75: 550-55.

[2] Upadhyaya M, Maynard J, Rogers MT, *et al.* Improved molecular diagnosis of facioscapulohumeral muscular dystrophy (FSHD): validation of the differential double digestion for FSHD. J Med Genet 1997; 34: 476-79.

[3] Tawil R, van der Maarel S, Padberg GW, van Engelen BG. Standards of care and management of facioscapulohumeral muscular dystrophy. Neuromuscul Disord 2010; 20: 471-75.

[4] Nakagawa M, Matsuzaki T, Higuchi I, *et al.* Facioscapulohumeral muscular dystrophy: clinical diversity and genetic abnormalities in Japanese patients. Intern Med 1997; 36: 333-39.

[5] van Overveld PG, Lemmers RJ, Deidda G, *et al.* Interchromosomal repeat array interactions between chromosomes 4 and 10: a model for subtelomeric plasticity. Hum Mol Genet 2000; 9: 2879-84.

[6] Wohlgemuth M, Lemmers RJ, van der Kooi EL, *et al.* Possible phenotypic dosage effect in patients compound heterozygous for FSHD-sized 4q35 alleles. Neurology 2003; 61: 909-13.

[7] Scionti I, Greco F, Ricci G, *et al.* Large scale population analysis challenges the current criteria for the molecular diagnosis of fascioscapulohumeral muscular dystrophy (FSHD). Am J Hum Genet 2012; 90: 628-25.

[8] Funakoshi M, Goto K, Arahata K. Epilepsy and mental retardation in a subset of early onset 4q35-facioscapulohumeral muscular dystrophy. Neurology 1998; 50: 1791-94.

[9] Yamanaka G, Goto K, Hayashi YK, Miyajima T, Hoshika A, Arahata K. Clinical and genetical features of Japanese early-onset facioscapulohumeral muscular dystrophy. No To Hattatsu 2002; 34: 318-24.

[10] Sakellariou P, Kekou K, Fryssira H, *et al.* Mutation spectrum and phenotypic manifestation in FSHD Greek patients. Neuromuscul Disord 2012; 22: 339-49.

[11] Chiò A, Battistini S, Calvo A, *et al.* Genetic counselling in ALS: facts, uncertainties and clinical suggestions. J Neurol Neurosurg Psychiatry 2014; 85: 478-85.

[12] Ravits J, Appel S, Baloh RH, *et al.* Deciphering amyotrophic lateral sclerosis: what phenotype, neuropathology and genetics are telling us about pathogenesis. Amyotroph Lateral Scler Frontotemporal Degener 2013; 14 (Suppl 1): 5-18.

[13] Mok K, Laaksovirta H, Tienari PJ, *et al.* Homozygosity analysis in amyotrophic lateral sclerosis. Eur J Hum Genet 2013; 21: 1429-35.

[14] Turner MR, Hardiman O, Benatar M, *et al.* Controversies and priorities in amyotrophic lateral sclerosis. Lancet Neurol 2013; 12: 310-22.

[15] Bettens K, Sleegers K, Van Broeckhoven C. Genetic insights in Alzheimer's disease. Lancet Neurol 2013; 12: 92-104.

[16] Roberts JS, Uhlmann WR. Genetic susceptibility testing for neurodegenerative diseases: Ethical and practice issues. Prog Neurobiol 2013; 110: 89-101.

[17] Ertekin-Taner N, De Jager PL, Yu L, Bennett DA. Alternative Approaches in Gene Discovery and Characterization in Alzheimer's Disease. Curr Genet Med Rep 2013; 1: 39-51.

[18] Duchenne GB. Album de photographies pathologiques. Paris: J.B. Baillière et fils 1862.

[19] Duchenne G.B. Revue photographique des hopitaux de Paris. Paris: Adrien Delahaye 1869.

[20] Flanigan KM in Myology (eds Engel A & Franzini-Armstrong C) McGraw Hill Professional 2004; 1123-1133.

[21] Landouzy L, Dejerine J. De la myopathie atrophique progressive. Rev Med Francaise 1885; 5: 81.

[22] Padberg G. Facioscapulohumeral disease. MD Thesis, University of Leiden, 1982.
[23] Awerbuch GI, Nigro MA, Wishnow R. Beevor's sign and facioscapulohumeral dystrophy. Arch Neurol 1990; 47: 1208-9.
[24] Brouwer OF, Wijmenga C, Frants RR, Padberg GW. Facioscapulohumeral muscular dystrophy: the impact of genetic research. Clin Neurol Neurosurg 1993; 95: 9-21.
[25] Lin MY, Nonaka I. Facioscapulohumeral muscular dystrophy: muscle fiber type analysis with particular reference to small angular fibers. Brain Dev 1991; 13: 331-8.
[26] Dorobek M, Szmidt-Sałkowska E, Rowińska-Marcińska K, Gaweł M, Hausmanowa-Petrusewicz I. Relationships between clinical data and quantitative EMG findings in facioscapulohumeral muscular dystrophy. Neurol Neurochir Pol 2013; 47: 8-17.
[27] Tasca G, Pescatori M, Monforte M, *et al*. Different molecular signatures in magnetic resonance imaging-staged facioscapulohumeral muscular dystrophy muscles. PLoS One 2012; 7: e38779.
[28] Trevisan CP, Pastorello E, Ermani M, *et al*. Facioscapulohumeral muscular dystrophy: a multicenter study on hearing function. Audiol Neurootol 2008; 13: 1-6.
[29] Trevisan CP, Pastorello E, Tomelleri G, *et al*. Facioscapulohumeral muscular dystrophy: hearing loss and other atypical features of patients with large 4q35 deletions. Eur J Neurol 2008; 15: 1353-8.
[30] Lunt PW, Compston DA, Harper PS. Estimation of age dependent penetrance in facioscapulohumeral muscular dystrophy by minimising ascertainment bias. J Med Genet 1989; 26: 755-60.
[31] Padberg GW, Lunt PW, Koch M, Fardeau M. Diagnostic criteria for facioscapulohumeral muscular dystrophy. Neuromuscul Disord 1991; 1: 231-34.
[32] Wijmenga C, Frants RR, Brouwer OF, Moerer P, Weber JL, Padberg GW. Location of facioscapulohumeral muscular dystrophy gene on chromosome 4. Lancet 1990; 336: 651-3.
[33] Upadhyaya M, Lunt PW, Sarfarazi M, *et al*. DNA marker applicable to presymptomatic and prenatal diagnosis of facioscapulohumeral disease. Lancet 1990; 336: 1320-1.
[34] Upadhyaya M, Lunt PW, Sarfarazi M, *et al*. A closely linked DNA marker for facioscapulohumeral disease on chromosome 4q. J Med Genet 1991; 28: 665-71.
[35] Wijmenga C, Padberg GW, Moerer P, *et al*. Mapping of facioscapulohumeral muscular dystrophy gene to chromosome 4q35-qter by multipoint linkage analysis and in situ hybridization. Genomics 1991; 9: 570-5.
[36] Sarfarazi M, Wijmenga C, Upadhyaya M, *et al*. Regional mapping of facioscapulohumeral muscular dystrophy gene on 4q35: combined analysis of an international consortium. Am J Hum Genet 1992; 51: 396-403.
[37] Wijmenga C, Hewitt JE, Sandkuijl LA, *et al*. Chromosome 4q DNA rearrangements associated with facioscapulohumeral muscular dystrophy. Nat Genet 1992; 2: 26-30.
[38] Hewitt JE, Lyle R, Clark LN, *et al*. Analysis of the tandem repeat locus D4Z4 associated with facioscapulohumeral muscular dystrophy. Hum Mol Genet 1994; 3: 1287-95.
[39] van Deutekom JC, Wijmenga C, van Tienhoven EA, *et al*. FSHD associated DNA rearrangements are due to deletions of integral copies of a 3.2 kb tandemly repeated unit. Hum Mol Genet 1993; 2: 2037-42.
[40] Upadhyaya M, Jardine P, Maynard J, *et al*. Molecular analysis of British facioscapulohumeral dystrophy families for 4q DNA rearrangements. Hum Mol Genet 1993; 2: 981-7.
[41] Wijmenga C, van Deutekom JC, Hewitt JE, *et al*. Pulsed-field gel electrophoresis of the D4F104S1 locus reveals the size and the parental origin of the facioscapulohumeral muscular dystrophy (FSHD)-associated deletions. Genomics 1994; 19: 21-6.
[42] Lunt PW, Jardine PE, Koch MC, *et al*. Phenotypic-genotypic correlation will assist genetic counseling in 4q35 facioscapulohumeral muscular dystrophy. Muscle Nerve 1995; 2: S103-109.
[43] Winokur ST, Bengtsson U, Feddersen J, *et al*. The DNA rearrangement associated with facioscapulohumeral muscular dystrophy involves a heterochromatin-associated repetitive element: implications for a role of chromatin structure in the pathogenesis of the disease. Chromosome Res 1994; 2: 225-34.
[44] Lyle R, Wright TJ, Clark LN, Hewitt JE. The FSHD-associated repeat, D4Z4, is a member of a dispersed family of homeobox-containing repeats, subsets of which are clustered on the short arms of the acrocentric chromosomes. Genomics 1995; 28: 389-97.
[45] Deidda G, Cacurri S, Grisanti P, Vigneti E, Piazzo N, Felicetti L. Physical mapping evidence for a duplicated region on chromosome 10qter showing high homology with the facioscapulohumeral muscular dystrophy locus on chromosome 4qter. Eur J Hum Genet 1995; 3: 155-67.
[46] van Geel M, Dickson MC, Beck AF, *et al*. Genomic analysis of human chromosome 10q and 4q telomeres suggests a common origin. Genomics 2002; 79: 210-7.
[47] Deidda G, Cacurri S, Piazzo N, Felicetti L. Direct detection of 4q35 rearrangements implicated in facioscapulohumeral muscular dystrophy (FSHD). J Med Genet 1996; 33: 361-5.
[48] van Deutekom JC, Bakker E, Lemmers RJ, *et al*. Evidence for subtelomeric exchange of 3.3 kb tandemly repeated units between chromosomes 4q35 and 10q26: implications for genetic counselling and etiology of FSHD1. Hum Mol Genet 1996; 5: 1997-2003.
[49] van Overveld PG, Lemmers RJ, Deidda G, *et al*. Interchromosomal repeat array interactions between chromosomes 4 and 10: a model for subtelomeric plasticity. Hum Mol Genet 2000 22; 9: 2879-84.
[50] Matsumura T, Goto K, Yamanaka G, *et al*. Chromosome 4q;10q translocations; comparison with different ethnic populations and FSHD patients. BMC Neurol 2002; 2: 7.
[51] Rossi M, Ricci E, Colantoni L, *et al*. The Facioscapulohumeral muscular dystrophy region on 4qter and the homologous locus on 10qter evolved independently under different evolutionary pressure. BMC Med Genet 2007; 8: 8.
[52] Zatz M, Marie SK, Passos-Bueno MR, *et al*. High proportion of new mutations and possible anticipation in Brazilian facioscapulohumeral muscular dystrophy families. Am J Hum Genet 1995; 56: 99-105.
[53] Padberg GW, Frants RR, Brouwer OF, Wijmenga C, Bakker E, Sandkuijl LA. Facioscapulohumeral muscular dystrophy in the Dutch population. Muscle Nerve 1995; 2: S81-84.
[54] Upadhyaya M, Maynard J, Osborn M, Jardine P, Harper PS, Lunt P. Germinal mosaicism in facioscapulohumeral muscular dystrophy (FSHD). Muscle Nerve 1995; 2: S45-49.
[55] Kohler J, Rupilius B, Otto M, Bathke K, Koch MC. Germline mosaicism in 4q35 facioscapulohumeral muscular dystrophy (FSHD1A) occurring predominantly in oogenesis. Hum Genet 1996; 98: 485-90.
[56] Lemmers RJ, van der Wielen MJ, Bakker E, Padberg GW, Frants RR, van der Maarel SM. Somatic mosaicism in FSHD often goes undetected. Ann Neurol 2004; 55: 845-50.
[57] van der Maarel SM, Deidda G, Lemmers RJ, *et al*. De novo facioscapulohumeral muscular dystrophy: frequent somatic mosaicism, sex-dependent phenotype, and the role of mitotic transchromosomal repeat interaction between chromosomes 4 and 10. Am J Hum Genet 2000; 66: 26-35.
[58] Gabellini D, Green MR, Tupler R. Inappropriate gene activation in FSHD: a repressor complex binds a chromosomal repeat deleted in dystrophic muscle. Cell 2002; 110: 339-48.
[59] de Greef JC, Frants RR, van der Maarel SM. Epigenetic mechanisms of facioscapulohumeral muscular dystrophy. Mutat Res 2008; 647: 94-102.
[60] Zeng W, de Greef JC, Chen YY, *et al*. Specific loss of histone H3 lysine 9 trimethylation and HP1gamma/cohesin binding at D4Z4 repeats is associated with facioscapulohumeral dystrophy (FSHD). PLoS Genet 2009; 5: e1000559.
[61] Petrov A, Pirozhkova I, Carnac G, Laoudj D, Lipinski M, Vassetzky YS. Chromatin loop domain organization within the 4q35 locus in facioscapulohumeral dystrophy patients

versus normal human myoblasts. Proc Natl Acad Sci U S A 2006; 10: 6982-7.
[62] Cabianca DS, Casa V, Gabellini D. A novel molecular mechanism in human genetic disease: a DNA repeat-derived lncRNA. RNA Biol 2012; 9: 211-17.
[63] Gabellini D, D'Antona G, Moggio M, *et al*. Facioscapulohumeral muscular dystrophy in mice overexpressing FRG1. Nature 2006; 439: 973-7.
[64] Rijkers T, Deidda G, van Koningsbruggen S, *et al*. FRG2, an FSHD candidate gene, is transcriptionally upregulated in differentiating primary myoblast cultures of FSHD patients. J Med Genet 2004; 41: 826-36.
[65] Bodega B, Ramirez GD, Grasser F, *et al*. Remodeling of the chromatin structure of the facioscapulohumeral muscular dystrophy (FSHD) locus and upregulation of FSHD-related gene 1 (FRG1) expression during human myogenic differentiation. BMC Biol 2009; 7: 41.
[66] Broucqsault N, Morere J, Gaillard MC, *et al*. Dysregulation of 4q35- and muscle-specific genes in fetuses with a short D4Z4 array linked to facio-scapulo-humeral dystrophy. Hum Mol Genet 2013; 22: 4206-14.
[67] Winokur ST, Chen YW, Masny PS, *et al*. Expression profiling of FSHD muscle supports a defect in specific stages of myogenic differentiation. Hum Mol Genet 2003; 12: 2895-907.
[68] Osborne RJ, Welle S, Venance SL, Thornton CA, Tawil R. Expression profile of FSHD supports a link between retinal vasculopathy and muscular dystrophy. Neurology 2007; 68: 569-77.
[69] Masny PS, Chan OY, de Greef JC, *et al*. Analysis of allele-specific RNA transcription in FSHD by RNA-DNA FISH in single myonuclei. Eur J Hum Genet 2010; 18: 448-56.
[70] Gabriëls J, Beckers MC, Ding H, *et al*. Nucleotide sequence of the partially deleted D4Z4 locus in a patient with FSHD identifies a putative gene within each 3.3 kb element. Gene 1999; 236: 25-32.
[71] van der Maarel SM, Tawil R, Tapscott SJ. Facioscapulohumeral muscular dystrophy and DUX4: breaking the silence. Trends Mol Med 2011; 17: 252-8.
[72] Dixit M, Ansseau E, Tassin A, *et al*. DUX4, a candidate gene of facioscapulohumeral muscular dystrophy, encodes a transcriptional activator of PITX1. Proc Natl Acad Sci U S A 2007; 104: 18157-62.
[73] Kowaljow V, Marcowycz A, Ansseau E, *et al*. The DUX4 gene at the FSHD1A locus encodes a pro-apoptotic protein. Neuromuscul Disord 2007; 17: 611-23.
[74] Celegato B, Capitanio D, Pescatori M, *et al*. Parallel protein and transcript profiles of FSHD patient muscles correlate to the D4Z4 arrangement and reveal a common impairment of slow to fast fibre differentiation and a general deregulation of MyoD-dependent genes. Proteomics 2006; 6: 5303-21.
[75] Lemmers RJ, de Kievit P, Sandkuijl L, *et al*. Facioscapulohumeral muscular dystrophy is uniquely associated with one of the two variants of the 4q subtelomere. Nat Genet 2002; 32: 235-36.
[76] Lemmers RJ, Wohlgemuth M, van der Gaag KJ, *et al*. Specific sequence variations within the 4q35 region are associated with facioscapulohumeral muscular dystrophy. Am J Hum Genet 2007; 81: 884-94.
[77] Lemmers RJ, van der Vliet PJ, Klooster R, *et al*. A unifying genetic model for facioscapulohumeral muscular dystrophy. Science 2010; 329: 1650-53.
[78] Tonini MM, Pavanello RC, Gurgel-Giannetti J, *et al*. Homozygosity for autosomal dominant facioscapulohumeral muscular dystrophy (FSHD) does not result in a more severe phenotype. J Med Genet 2004; 41: e17.
[79] Olckers A, van der Merwe A, Wayne Towers G, Retief CF, Honey E, Schutte CM. Compound heterozygosity in a South African patient with facioscapulohumeral muscular dystrophy. Neuromuscul Disord 2012; 22: 728-34.
[80] Scionti I, Fabbri G, Fiorillo C, *et al*. Facioscapulohumeral muscular dystrophy: new insights from compound heterozygotes and implication for prenatal genetic counseling. J Med Genet 2012; 49: 171-78.
[81] Vitelli F, Villanova M, Malandrini A, *et al*. Inheritance of a 38-kb fragment in apparently sporadic facioscapulohumeral muscular dystrophy. Muscle Nerve 1999; 22: 1437-41.
[82] Upadhyaya M, Cooper DN. Molecular diagnosis of facioscapulohumeral muscular dystrophy. Expert Rev Mol Diagn 2002; 2: 160-71.
[83] Butz M, Koch MC, Muller-Felber W, Lemmers RJ, van der Maarel SM, Schreiber H. Facioscapulohumeral muscular dystrophy. Phenotype-genotype correlation in patients with borderline D4Z4 repeat numbers. J Neurol 2003; 250: 932-37.
[84] de Greef JC, Lemmers RJ, Camaño P, *et al*. Clinical features of facioscapulohumeral muscular dystrophy 2. Neurology 2010; 75: 1548-54.
[85] Zatz M, Marie SK, Cerqueira A, Vainzof M, Pavanello RC, Passos-Bueno MR. The facioscapulohumeral muscular dystrophy (FSHD1) gene affects males more severely and more frequently than females. Am J Med Genet 1998; 77: 155-61.
[86] Tonini MM, Passos-Bueno MR, Cerqueira A, Matioli SR, Pavanello R, Zatz M. Asymptomatic carriers and gender differences in facioscapulohumeral muscular dystrophy (FSHD). Neuromuscul Disord 2004; 14: 33-8.
[87] Ricci G, Scionti I, Sera F, *et al*. Large scale genotype-phenotype analyses indicate that novel prognostic tools are required for fascioscapulohumeral muscular dystrophy families. Brain 2013; 136(Pt 11): 3408-17.
[88] de Greef JC, Lemmers RJ, van Engelen BG, *et al*. Common epigenetic changes of D4Z4 in contraction-dependent and contraction-independent FSHD. Hum Mutat 2009; 30: 1449-59.
[89] Lemmers RJ, Tawil R, Petek LM, *et al*. Digenic inheritance of an SMCHD1 mutation and an FSHD-permissive D4Z4 allele causes facioscapulohumeral muscular dystrophy type 2. Nat Genet 2012; 44: 1370-74.
[90] Bailey RO, Marzulo DC, Hans MB. Infantile facioscapulohumeral muscular dystrophy: new observations. Acta Neurol Scand 1986; 74: 51-8.
[91] Tyler FH, Stephens FE. Studies in disorders of muscle. II Clinical manifestations and inheritance of facioscapulohumeral dystrophy in a large family. Ann Intern Med 1950; 32: 640-60.
[92] Chung CS, Morton NE. Discrimination of genetic entities in muscular dystrophy. Am J Hum Genet 1959; 11: 339-59.
[93] Griggs RC, Tawil R, Storvick D, Mendell JR, Altherr MR. Genetics of facioscapulohumeral muscular dystrophy: new mutations in sporadic cases. Neurology 1993; 43: 2369-72.
[94] Ricci E, Galluzzi G, Deidda G, *et al*. Progress in the molecular diagnosis of facioscapulohumeral muscular dystrophy and correlation between the number of KpnI repeats at the 4q35 locus and clinical phenotype. Ann Neurol 1999; 45: 751-57.
[95] Goto K, Nishino I, Hayashi YK. Very low penetrance in 85 Japanese families with facioscapulohumeral muscular dystrophy 1A. J Med Genet 2004; 41: e12.
[96] Lamperti C, Fabbri G, Vercelli L, *et al*. A standardized clinical evaluation of patients affected by facioscapulohumeral muscular dystrophy: The FSHD clinical score. Muscle Nerve 2010; 42: 213-17.
[97] Jardine PE, Upadhyaya M, Maynard J, Harper P, Lunt PW. A scapular onset muscular dystrophy without facial involvement: possible allelism with facioscapulohumeral muscular dystrophy. Neuromuscul Disord 1994; 4: 477-82.
[98] Laforêt P, de Toma C, Eymard B, *et al*. Cardiac involvement in genetically confirmed facioscapulohumeral muscular dystrophy. Neurology 1998; 51: 1454-56.
[99] Finsterer J, Stöllberger C, Meng G. Cardiac involvement in facioscapulohumeral muscular dystrophy. Cardiology 2005; 103: 81-3.
[100] Emmrich P, Ogunlade V, Gradistanac T, Daneschnejad S, Koch MC, Schober R. Facioscapulohumeral muscle dystrophy and heart disease. Z Kardiol 2005; 94: 348-54.
[101] Tsuji M, Kinoshita M, Imai Y, Kawamoto M, Kohara N. Facioscapulohumeral muscular dystrophy presenting with

hypertrophic cardiomyopathy: a case study. Neuromuscul Disord 2009; 19: 140-2.
[102] van der Kooi AJ, Visser MC, Rosenberg N, *et al*. Extension of the clinical range of facioscapulohumeral dystrophy: report of six cases. J Neurol Neurosurg Psychiatry 2000; 69: 114-16.
[103] Felice KJ, North WA, Moore SA, Mathews KD. FSH dystrophy 4q35 deletion in patients presenting with facial-sparing scapular myopathy. Neurology 2000 23; 54: 1927-31.
[104] Felice KJ, Moore SA. Unusual clinical presentations in patients harboring the facioscapulohumeral dystrophy 4q35 deletion. Muscle Nerve 2001; 24: 352-6.
[105] Yamanaka G, Goto K, Matsumura T, *et al*. Tongue atrophy in facioscapulohumeral muscular dystrophy. Neurology 2001; 57; 733-35.
[106] Uncini A, Galluzzi G, Di Muzio A, *et al*. Facioscapulohumeral muscular dystrophy presenting isolated monomelic lower limb atrophy. Report of two patients with and without 4q35 rearrangement. Neuromuscul Disord 2002; 12: 874-7.
[107] Umapathi T, Chaudhry V, Cornblath D, Drachman D, Griffin J, Kuncl R. Head drop and camptocormia. J Neurol Neurosurg Psychiatry 2002; 73: 1-7.
[108] Zeevaert B, Sadzot B, Deprez M, Wang FC. Scapulo-peroneal weakness with tardive symptoms of facio-scapulo-humeral muscular dystrophy, connected to 4q35 chromosomal deletion. Rev Neurol (Paris) 2002; 158: 1210-13.
[109] Krasnianski M, Eger K, Neudecker S, Jakubiczka S, Zierz S. Atypical phenotypes in patients with facioscapulohumeral muscular dystrophy 4q35 deletion. Arch Neurol 2003; 60: 1421-25.
[110] Wood-Allum C, Brennan P, Hewitt M, Lowe J, Tyfield L, Wills A. Clinical and histopathological heterogeneity in patients with 4q35 facioscapulohumeral muscular dystrophy (FSHD). Neuropathol Appl Neurobiol 2004; 30: 188-91.
[111] Sugie K, Hayashi YK, Kin T, Goto K, Nishino I, Ueno S. Teaching NeuroImages: Hemiatrophy as a clinical presentation in facioscapulohumeral muscular dystrophy. Neurology 2009; 73: e24.
[112] Zouvelou V, Manta P, Kalfakis N, Evdokimidis I, Vassilopoulos D. Asymptomatic elevation of serum creatine kinase leading to the diagnosis of 4q35 facioscapulohumeral muscular dystrophy. J Clin Neurosci 2009; 16: 1218-9.
[113] Reilich P, Schramm N, Schoser B, *et al*. Facioscapulohumeral muscular dystrophy presenting with unusual phenotypes and atypical morphological features of vacuolar myopathy. J Neurol 2010; 257: 1108-18.
[114] Figueroa JJ, Chapin JE. Isolated facial diplegia and very late-onset myopathy in two siblings: atypical presentations of facioscapulohumeral dystrophy. J Neurol 2010; 257: 444-6.
[115] Kottlors M, Kress W, Meng G, Glocker FX. Facioscapulohumeral muscular dystrophy presenting with isolated axial myopathy and bent spine syndrome. Muscle Nerve 2010; 42: 273-75.
[116] Jordan B, Eger K, Koesling S, Zierz S. Camptocormia phenotype of FSHD: a clinical and MRI study on six patients. J Neurol 2011; 258: 866-73.
[117] Papadopoulos C, Papadimas GK, Spengos K, Manta P. Bent spine syndrome in facioscapulohumeral muscular dystrophy. Muscle Nerve 2011; 43: 615; author reply 615-6.
[118] Hassan A, Jones LK Jr, Milone M, Kumar N. Focal and other unusual presentations of facioscapulohumeral muscular dystrophy. Muscle Nerve 2012; 46: 421-5.
[119] Fee DB. Elderly onset of weakness in facioscapulohumeral muscular dystrophy. Case Rep Neurol Med 2012; 2012: 726984.
[120] Tonini MM, Passos-Bueno MR, Cerqueira A, *et al*. Facioscapulohumeral (FSHD1) and other forms of muscular dystrophy in the same family: is there more in muscular dystrophy than meets the eye? Neuromuscul Disord 2002; 6: 554-57.
[121] Filosto M, Tonin P, Scarpelli M, *et al*. Novel mitochondrial tRNA Leu(CUN) transition and D4Z4 partial deletion in a patient with a facioscapulohumeral phenotype. Neuromuscul Disord 2008; 18: 204-09.
[122] Rudnik-Schöneborn S, Weis J, Kress W, Häusler M, Zerres K. Becker's muscular dystrophy aggravating facioscapulohumeral muscular dystrophy--double trouble as an explanation for an atypical phenotype. Neuromuscul Disord 2008; 18: 881-85.
[123] Lecky BR, MacKenzie JM, Read AP, Wilcox DE. X-linked and FSH dystrophies in one family. Neuromuscul Disord 1991; 1: 275-78.
[124] Korngut L, Siu VM, Venance SL, *et al*. Phenotype of combined Duchenne and facioscapulohumeral muscular dystrophy. Neuromuscul Disord 2008; 18: 579-82.
[125] Chuenkongkaew WL, Lertrit P, Limwongse C, *et al*. An unusual family with Leber's hereditary optic neuropathy and facioscapulohumeral muscular dystrophy. Eur J Neurol 2005; 12: 388-91.
[126] Ricci G, Scionti I, Alì G, *et al*. Rippling muscle disease and facioscapulohumeral dystrophy-like phenotype in a patient carrying a heterozygous CAV3 T78M mutation and a D4Z4 partial deletion: further evidence for "double trouble" overlapping syndromes. Neuromuscul Disord 2012; 22: 534-40.
[127] Masciullo M, Iannaccone E, Bianchi ML, *et al*. Myotonic dystrophy type 1 and de novo FSHD mutation double trouble: a clinical and muscle MRI study. Neuromuscul Disord 2013; 23: 427-31.
[128] McGonigal A, Thomas AM, Petty RK. Facioscapulohumeral muscular dystrophy and myasthenia gravis co-existing in the same patient: a case report. J Neurol 2002; 249: 219-20.
[129] Chadwick BP. Macrosatellite epigenetics: the two faces of DXZ4 and D4Z4. Chromosoma 2009; 118: 675-81.
[130] Siskind CE, Panchal S, Smith CO, *et al*. A review of genetic counseling for Charcot Marie Tooth disease (CMT). J Genet Couns 2013; 22: 422-36.
[131] Takahashi M, Iwashita T, Santoro M, Lyonnet S, Lenoir GM, Billaud M. Co-segregation of MEN2 and Hirschsprung's disease: the same mutation of RET with both gain and loss-of-function? Hum Mutat 1999; 13: 331-6.
[132] Moore SW, Zaahl MG. Multiple endocrine neoplasia syndromes, children, Hirschsprung's disease and RET. Pediatr Surg Int 2008; 24: 521-30.
[133] Bertrand AT, Chikhaoui K, Yaou RB, Bonne G. Clinical and genetic heterogeneity in laminopathies. Biochem Soc Trans 2011; 39: 1687-92.
[134] Kanagawa M, Toda T. The genetic and molecular basis of muscular dystrophy: roles of cell-matrix linkage in the pathogenesis. J Hum Genet 2006; 51: 915-26.
[135] Chahwan R, Wontakal SN, Roa S. The multidimensional nature of epigenetic information and its role in disease. Discov Med 2011; 11: 233-43.

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Annex 2

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Large scale genotype–phenotype analyses indicate that novel prognostic tools are required for families with facioscapulohumeral muscular dystrophy

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Facioscapulohumeral muscular dystrophy has been genetically linked to reduced numbers (⩽8) of D4Z4 repeats at 4q35 combined with 4A(159/161/168) *DUX4* polyadenylation signal haplotype. However, we have recently reported that 1.3% of healthy individuals carry this molecular signature and 19% of subjects affected by facioscapulohumeral muscular dystrophy do not carry alleles with eight or fewer D4Z4 repeats. Therefore, prognosis for subjects carrying or at risk of carrying D4Z4 reduced alleles has become more complicated. To test for additional prognostic factors, we measured the degree of motor impairment in a large group of patients affected by facioscapulohumeral muscular dystrophy and their relatives who are carrying D4Z4 reduced alleles. The clinical expression of motor impairment was assessed in 530 subjects, 163 probands and 367 relatives, from 176 unrelated families according to a standardized clinical score. The associations between clinical severity and size of D4Z4 allele, degree of kinship, gender, age and 4q haplotype were evaluated. Overall, 32.2% of relatives did not display any muscle functional impairment. This phenotype was influenced by the degree of relation with proband, because 47.1% of second- through fifth-degree relatives were unaffected, whereas only 27.5% of first-degree family members did not show motor impairment. The estimated risk of developing motor impairment by age 50 for relatives carrying a D4Z4 reduced allele with 1–3 repeats or 4–8 repeats was 88.7% and 55%, respectively. Male relatives had a mean score significantly higher than females (5.4 versus 4.0, $P = 0.003$). No 4q haplotype was exclusively associated with the presence of disease. In 13% of families in which D4Z4 alleles with 4–8 repeats segregate, the diagnosis of facioscapulohumeral muscular dystrophy was reported only in one generation. In conclusion, this large-scale analysis provides further information that should be taken into account when counselling families in which a reduced allele with 4–8 D4Z4 repeats segregates. In addition, the reduced expression of disease observed in distant relatives suggests that a family's genetic background plays a role in the occurrence of facioscapulohumeral muscular dystrophy. These results indicate that the identification of new susceptibility factors for this disease will require an accurate classification of families.

Keywords: facioscapulohumeral muscular dystrophy; D4Z4 reduced allele; genotype–phenotype correlations; penetrance; disease expression

Abbreviations: DRA = D4Z4 alleles of reduced size; FSHD = facioscapulohumeral muscular dystrophy

Introduction

Facioscapulohumeral muscular dystrophy (FSHD, OMIM #158900) is the third most common hereditary myopathy with prevalence of 1 in 20 000 (Mostacciuolo *et al.*, 2009). FSHD is characterized by progressive, asymmetric atrophy and weakness of a highly selective set of muscle groups (Padberg *et al.*, 1991; Lamperti *et al.*, 2010; Tawil *et al.*, 2010) and wide inter- and intra-familial variability of clinical expression (Padberg, 1982; Tawil and van der Maarel, 2006). Age-dependent penetrance based on the presence of the characteristic clinical signs was estimated >95% by age 20 (Lunt *et al.*, 1989; Tawil *et al.*, 2010). The mode of inheritance is considered autosomal dominant (Flanigan, 2004).

A large majority of patients with FSHD carry rearrangements occurring in a 3.3 kb tandemly repeated sequence (D4Z4) located at the 4q subtelomeric region (Wijmenga *et al.*, 1992; Lunt *et al.*, 1995*a*; Upadhyaya *et al.*, 1997). These rearrangements result in polymorphic EcoRI alleles detected by the p13E-11 probe (Wijmenga *et al.*, 1992; Upadhyaya *et al.*, 1997). Early studies of small numbers of individuals observed that both *de novo* and familial patients with FSHD carry p13E-11 EcoRI alleles of 35 kb, corresponding to eight D4Z4 units, or shorter (Griggs *et al.*, 1993; Lunt *et al.*, 1995*a*; van Deutekom *et al.*, 1996). For the last 20 years the clinical diagnosis of FSHD has been supported by this type of D4Z4 DNA testing, which has been considered highly sensitive and specific for disease (Lunt *et al.*, 1995*a*, *b*; Lunt, 1998; Tawil *et al.*, 2010). However, several studies on FSHD families describe subjects carrying D4Z4 alleles of reduced size (DRA) and no signs of the disease, defined as non-penetrant carriers (Tawil *et al.*, 1996; Zatz *et al.*, 1998; Ricci *et al.*, 1999; van Overveld *et al.*, 2000; Goto *et al.*, 2004; Tonini *et al.*, 2004; Sakellariou *et al.*, 2012; Scionti *et al.*, 2012*a*). As a possible explanation of some non-penetrant cases, it has been proposed that reduction of D4Z4 repeats on chromosome 4q35 is pathogenic only in certain chromosomal backgrounds, defined by 'permissive' specific haplotypes, namely (i) reduction of D4Z4 elements; (ii) presence of the 4A(159/161/168) haplotype; and (iii) a single nucleotide polymorphism that provides a polyadenylation signal (PAS) for the *DUX4* transcript (Lemmers *et al.*, 2002, 2007, 2010).

Nonetheless, our most recent studies (Scionti *et al.*, 2012*b*) showed that although the majority of FSHD index cases (70%) carry DRA with 4–8 units, this size range is also carried by 3% of healthy subjects from the general population. Additionally, our work raised the possibility that there is little predictive value of the 4A161PAS haplotype in the absence of family history because 1.3% of healthy subjects carry this haplotype, which is a frequency similar to other common polymorphisms. Finally, we found that 19% of FSHD probands do not carry D4Z4 alleles with 1–8 repeats and only 50% of the probands carry the 4A161PAS permissive haplotype associated with DRA (Scionti *et al.*, 2012*b*). These observations suggest that the genetic factors leading to FSHD might be incompletely described. Consistent with this idea, Lemmers *et al.* (2012) recently described mutations in *SMCHD1* gene in patients with FSHD and hypothesized that these mutations influence the disease penetrance (Lemmers *et al.*, 2012).

Here, we evaluate FSHD occurrence among relatives carrying DRA in relation to D4Z4 reduced allele size, gender, age, degree of kinship and 4q haplotype.

Materials and methods

Study design and subjects selection

The study has been performed on FSHD families accrued through the Italian National Registry for FSHD (INRF), established in 2007 by the Italian Clinical Network for FSHD (ICNF) (www.fshd.it) (Lamperti *et al.*, 2010). The ICNF includes two diagnostic laboratories at the University di Modena and Reggio Emilia and at the Fondazione Santa Lucia in Rome and 14 clinical centres, networked within the Italian Association of Myology (www.miologia.org) and distributed across all of Italy, from northern to southern regions, including the islands.

The study was conducted from 2008 to 2012. As outlined in Fig. 1, initially the selection process regarded 418 index cases carrying DRA with 1–8 repeats and fulfilling the clinical features of FSHD (Padberg *et al.*, 1991). One hundred and eighty-six cases were considered not eligible because they had no available relatives. Fourteen compound heterozygotes for DRA alleles were excluded from this study and analysed separately (Scionti *et al.*, 2012*a*). Forty-two *de novo* cases, defined as single subjects with neither parent carrying DRA, were excluded because they would not be informative for this study. For each proband the clinical and molecular examinations were extended to the available relatives at various degrees of kinship. Among the 645 relatives identified, 367 were found to be carriers of DRA. All clinical and molecular data were collected in the INRF database at Miogen Laboratory of University of Modena for data analysis.

Clinical examination

Each subject recruited during the time of the study was examined by a trained neurologist of the ICNF using the standardized FSHD clinical protocol with validated inter-rater reliability (Lamperti *et al.*, 2010). The FSHD clinical protocol was developed by the ICNF in order to numerically define the clinical severity of the motor impairment, and is not to be used to diagnose FSHD. The FSHD scale quantifies muscle weakness through the functional evaluation of six muscle groups specifically affected in FSHD, belonging to (i) face (score 0–2); (ii) shoulder girdle (score 0–3); (iii) upper limbs (score 0–2); (iv) distal legs (score 0–2); (v) pelvic girdle (score 0–5); and (vi) abdominal muscles (score 0–1). The FSHD score, which translates disability into a number, ranges from zero, when no objective evidence of muscle functional impairment is present, to 15, when all the muscle groups tested are severely impaired (www.fshd.it) (Lamperti *et al.*, 2010). DRA carriers who did not show an objective motor impairment received an FSHD score equal to zero and were considered clinically unaffected at the time of examination. On the basis of the FSHD score, subjects were classified as affected by mild (FSHD score 1–2), moderate (FSHD score 3–6), or severe (FSHD score 7–15) motor impairment.

Probands from 13 families were not re-evaluated as they were not alive at the time of this study.

Age at onset was estimated on the basis of patient records. When subjects did not complain of motor impairment, but a mild muscle weakness was observed, the age at examination was set as the age at onset, according to previous reports (Lunt *et al.*, 1995*b*). In six subjects it was not possible to obtain information about the age at onset of motor impairment due to their poor compliance.

The study was approved by the Local Ethics Committees of all participating Institutions. Informed consent, according to the Declaration of Helsinki, was obtained from each subject enrolled in the study.

Molecular characterization

Allele sizes were estimated by Southern hybridization using probe p13E-11. Genomic DNA extracted from peripheral blood lymphocytes was digested with EcoRI, EcoRI/BlnI or XapI, electrophoresed in a 0.4% agarose gel for 45–48 h at 35 V alongside an 8–48 kb marker (Bio-Rad) as previously described (Scionti *et al.*, 2012*b*). To assess the chromosomal origin of D4Z4-reduced alleles, DNA from each subject was analysed by NotI digestion and hybridization with the B31 probe (Scionti *et al.*, 2012*b*). Restriction fragments were detected by autoradiography or using a Typhoon Trio system (GE Healthcare). 4qA/4qB allelic variants were defined in all 530 subjects included in the study, using HindIII-digested DNA, pulsed field gel electrophoresis electrophoresis and Southern blot hybridization with radiolabeled 4qB and 4qA probes according to standard procedures (Scionti *et al.*, 2012*b*).

The Simple Sequence Length Polymorphism (SSLP) and the pLAM Single Nucleotide Polymorphism (SNP) [AT(T/C)AAA] sequences flanking the D4Z4 repeat units were defined in 294 relatives according to published procedures (Scionti *et al.*, 2012*b*).

Statistical analysis

The association between the risk of being asymptomatic (FSHD score equal to zero) and D4Z4 allele size and age was evaluated by using the multivariate logistic regression model. The association between age at onset or FSHD score and D4Z4 allele size and sex among symptomatic relatives was assessed by using a general linear model. Association estimates and their relative 95% confidence intervals (CI) were also reported.

The prevalence of FSHD score, classified into two categories (0 versus 1–15), among relatives was estimated and its association with D4Z4 allele sizes was also evaluated. Univariate and multivariate logistic regression models were fitted with D4Z4 allele size, sex and family degree as predictors.

An interaction test was also carried out to assess whether the difference in terms of FSHD scores between probands and relatives varied in relation to D4Z4 allele sizes.

In order to minimize any ascertainment bias, all the genotype–phenotype correlation analyses were performed on relatives and probands separately.

In all general and generalized linear models estimated, the sandwich estimator of covariance matrix of parameters was used to take into account any clustering effect within families (Williams, 2000). Wald tests were used to evaluate the effect of predictors and to evaluate the effects of predictors on outcomes (McCullagh and Nelder, 1989).

For the cohorts of probands and family members, Kaplan-Meier survival analysis (Kaplan and Meier, 1958) was used to estimate the age-specific cumulative motor impairment incidence, with the corresponding 95% CI. For each individual, time from birth to the earliest age at onset of motor impairment was estimated. The analysis was stratified by D4Z4 allele size only for relatives, and also by gender for relatives and probands.

The risk prediction algorithm was developed and validated using established methods (Hippisley-Cox *et al.*, 2007). The original cohort of 367 relatives was randomly split in the derivation and validation

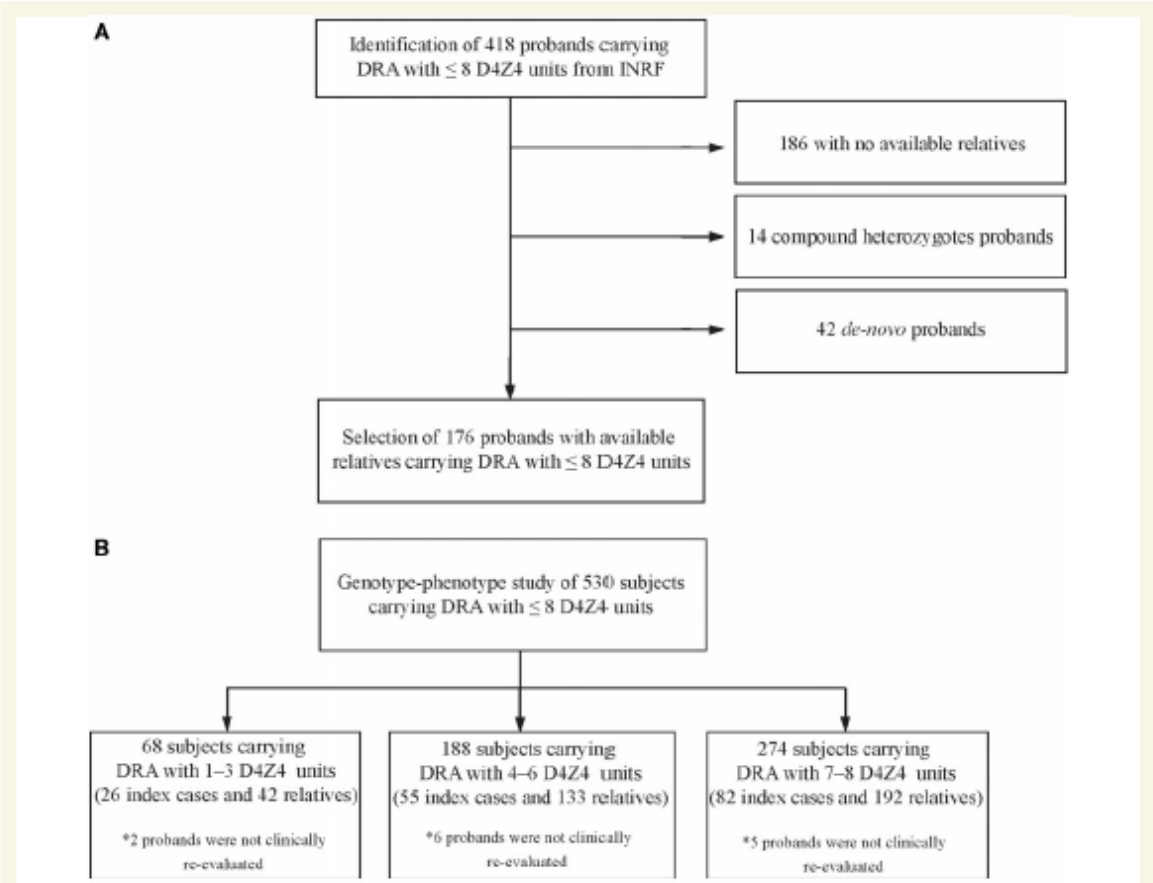

Figure 1 (**A**) Preliminary selection of probands/families from the Italian National Registry for FSHD (INRF). (**B**) Selection of the cohort of probands and their relatives for genotype–phenotype correlation analysis.

samples. The coefficients for D4Z4 allele size, sex and family degree were estimated by using the Cox proportional hazard model. The coefficients were used as weights, which were combined with the baseline survivor function to derive risk equations at age 50 years. The risk equation was applied to the validation sample and measures of discrimination were calculated (R^2, D statistics and area under the receiver operating characteristic curve).

Results

We examined 530 carriers of DRA (367 relatives and 163 index cases) from 176 unrelated families, in which at least one subject was affected by FSHD (Fig. 1B). Considering the cohort of relatives carrying DRA as a whole (367 subjects, 152 males and 215 females, mean age 46.4 ± 17.2, Supplementary Fig. 1), we observed that 118 (32.2%) did not show any functional motor impairment (FSHD score equal to zero) and 249 (67.8%) displayed muscle impairment to various degrees (FSHD score ⩾1) (Table 1).

The distribution of asymptomatic relatives was also analysed based on the size of DRA. We divided subjects in three groups: subjects carrying DRA with 1–3 D4Z4 repeats; subjects carrying DRA with 4–6 D4Z4 repeats; subjects carrying DRA with 7–8 D4Z4 repeats (Supplementary Table 1 and Supplementary Fig. 1). Table 1 also shows that 9.5% (4 out of 42) of all carriers of DRA with 1–3 repeats displayed no motor impairment. This percentage increases among carriers of DRA with 4–6 and 7–8 repeats (28.6% and 39.6%, respectively).

We then calculated the distribution of asymptomatic carriers based on age at examination, separately analysing four classes: those aged 18–30 years, 31–55 years, 56–70 years, and those over 70 years of age (Supplementary Table 2). Four classes were formed. As shown in Table 1, asymptomatic carriers were found in all classes up to 70 years. In particular, almost one-third of carriers of DRA with 4–6 and 7–8 repeats (27.6% and 35.9%, respectively) were asymptomatic between 56 and 70 years of age. Since the percentage of asymptomatic carriers varies among relatives carrying DRA of different sizes, the age-related risk of developing

Table 1 Distribution of unaffected relatives according to D4Z4 allele size and age at examination

D4Z4 units	Age (years)						Total		
	18–30		31–55		56–70				
	n subjects	% Score = 0 (*n*)	*n* subjects	% Score = 0 (*n*)	*n* subjects	% Score = 0 (*n*)	*n* subjects	% Score = 0 (*n*)	*P*-value*
1–3	8	12.5 (1)	23	8.7 (2)	9	11.1 (1)	42	9.5 (4)	0.707
4–6	31	25.8 (8)	65	33.8 (22)	29	27.6 (8)	133	28.6 (38)	0.461
7–8	42	54.8 (23)	85	40.0 (34)	39	35.9 (14)	192	39.6 (76)	0.013
Total							367	32.2 (118)	

*Wald test of the age's coefficient as ordinal predictor in the logistic model adjusted by sex.

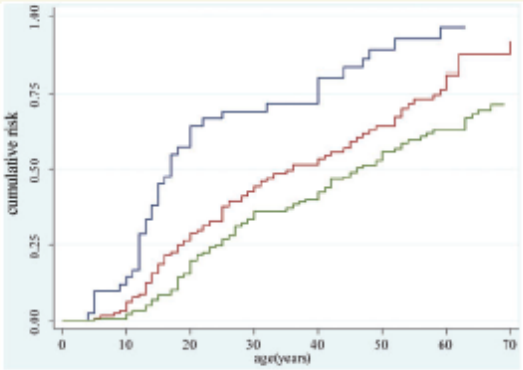

Figure 2 Age-specific cumulative risk of reported muscle impairment according to D4Z4 allele size. Estimates obtained on 361 relatives using the Kaplan-Meier analysis. Blue line refers to carriers of 1–3 D4Z4 units; red line refers to carriers of 4–6 D4Z4 units; green line refers to carriers of 7–8 D4Z4 units. Carriers of 7–8 versus 4–6 units Log rank test *P* value = 0.002.

Table 2 Estimates of the age-specific cumulative risk obtained using the Kaplan-Meyer analysis

Age (years)	Carriers of 1–3 D4Z4 units		Carriers of 4–6 D4Z4 units		Carriers of 7–8 D4Z4 units	
	Risk	95% CI	Risk	95% CI	Risk	95% CI
20	64.3	(50.1; 78.3)	21.8	(21.8; 37.5)	19.6	(14.6; 26.0)
30	69.1	(55.0; 82.2)	36.1	(36.1; 53.4)	35.9	(29.4; 43.5)
40	80.1	(66.5; 90.8)	44.8	(44.8; 62.5)	42.5	(35.5; 50.3)
50	88.7	(76.3; 96.3)	55.0	(55.0; 73.3)	55.7	(47.9; 63.8)
60	96.2	(84.6; 99.7)	71.5	(71.5; 88.8)	62.9	(54.7; 71.2)
70			80.3	(80.3; 97.6)	71.3	(62.3; 79.7)
80					82.2	(72.1; 90.4)

Table 3 Distribution of mean age at onset among affected relatives according to D4Z4 allele size

D4Z4 units	Relatives			
	Number of subjects	Mean age at onset (years)	95% CI	*P*-value*
1–3	38	20.3	15.5–25.2	
4–6	91	29.2	25.6–32.7	
7–8	114	34.6	30.1–39.1	0.0002
Total	243			

*Wald test of equality to zero of D4Z4 allele size's coefficients parametrized as categorical variable in a general linear model with age at onset as dependent variable and sex and D4Z4 allele size as predictors.

motor impairment was evaluated in correlation with D4Z4 size on the basis of data obtained from 361 relatives. Figure 2 and Table 2 show the penetrance estimates for DRA carriers calculated with the Kaplan-Meier method. Among subjects carrying DRA with 1–3 units the risk of developing motor impairment is 64.3% by age 20, 80.1% by age 40 and 96.2% by age 60. Among subjects carrying DRA with 4–6 and 7–8 D4Z4 units these risks are 21.8% and 19.6%, respectively, by age 20, 44.8% and 42.5% by age 40, and 71.5% and 62.9% by age 60. Therefore, FSHD penetrance is almost complete by age 60 only for carriers of DRA with 1–3 units.

We tested whether the size of DRA correlates with age at onset and disease severity. Table 3 shows that the mean age at onset is statistically lower among subjects carrying DRA with 1–3 units (20.3 years) in comparison with those carrying DRA with 4–6 and 7–8 D4Z4 repeats (29.2 and 34.6 years, respectively) ($P = 0.0002$). Overall, we found that 60.6% of affected relatives experienced scapular girdle onset, 19.0% facial muscle onset, 16.7% lower limbs onset, 0.9% upper limbs onset and 2.8% abdominal muscle onset (Supplementary Table 3).

Severity is also increased among carriers of DRA with 1–3 repeats. Indeed, as shown in Table 4, affected relatives carrying DRA with 1–3 repeats had a mean FSHD score of 7.2. By contrast, individuals carrying DRA with 4–6 and 7–8 D4Z4 units had mean FSHD score of 4.4 and 4.1, respectively. This association was statistically significant ($P = 0.0006$).

The degree of motor impairment among relatives was also evaluated in relationship to D4Z4 allele size and age at examination. Figure 3A shows that ~40% of relatives carrying DRA with 1–3 units are severely affected (FSHD score ≥ 7) by age 30. In contrast, no relatives carrying DRA with 4–8 units had a FSHD score

higher than 6 in this age window. Figure 3B and C shows that between age 31–55 and 56–70 a high percentage of relatives carrying DRA with 4–8 units were asymptomatic (FSHD score 0) or displayed minimal signs of functional motor impairment (FSHD score 1–2).

Table 4 Distribution of FSHD score calculated on affected relatives according to D4Z4 allele size and age at examination

D4Z4 units	Relatives			
	Number of subjects	FSHD score mean	95% CI	*P*-value[†]
1–3	38	7.2	5.8–8.6	
4–6	95	4.4	3.8–5.1	
7–8	116	4.1	3.5–4.7	0.0006

[†]Wald test of equality to zero of D4Z4 allele size and age at examination coefficients parametrized as categorical variable in a general linear model with FSHD score as dependent variable and sex, D4Z4 allele size and age at examination as predictors.

We then evaluated whether there is a correlation between the clinical status of the proband and his/her relatives. As shown in Table 5, intra-familial analysis on 163 families with 217 affected relatives revealed a positive correlation [0.72 (95% CI 0.40–1.04)] in families in which D4Z4 alleles with 1–3 D4Z4 repeats segregate. In contrast, in families with 4–6 and 7–8 D4Z4 alleles, a lower degree of correlation between the clinical status of the proband and his/her relatives was observed [0.01 (95% CI −0.23–0.26) and −0.14 (95% CI −0.35–0.07), respectively].

Remarkably, in 19 of 148 families (13%) in which 4–8 D4Z4 alleles segregate, we found affected individuals only within a single generation, and with older unaffected relatives carrying the DRA (Supplementary Fig. 2). In each of these 19 families, molecular testing excluded the presence of somatic mosaicism in the unaffected parent carrying the DRA. The finding of affected subjects in only one generation or the presence of only one affected subject in the entire family suggests that a complex mode of inheritance might be at the basis of FSHD development in these families.

To test this hypothesis we assessed whether the prevalence of disease varies among relatives according to degree of kinship with

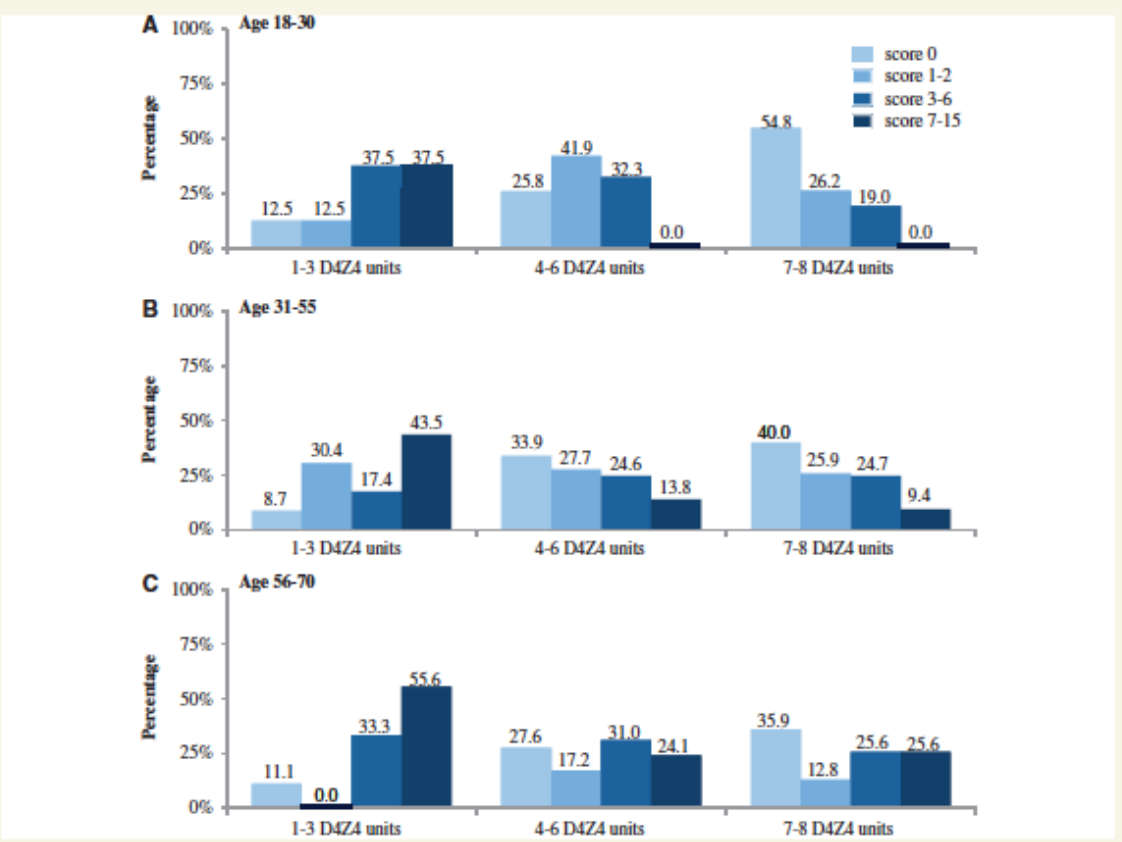

Figure 3 Distribution of clinical severity among relatives carrying D4Z4 reduced allele according to D4Z4 allele size and age. Subjects were subdivided by age: (A) 18–30 years, (B) 31–55 years, (C) 56–70 years and by D4Z4 allele size: 1–3, 4–6 and 7–8 units. In each subgroup, percentages of subjects who received FSHD score equal to 0, 1–2, 3–6 and ⩾ 7 are reported.

Table 5 Standardized regression coefficient between FSHD score of probands and relatives

Number of subjects	D4Z4-allele size 1–3		D4Z4-allele size 4–6		D4Z4-allele size 7–8		*P*-value
	Correlation coefficient	95% CI	Correlation coefficient	95% CI	Correlation coefficient	95% CI	
217	0.72	0.40–1.04	0.01	−0.23–0.26	−0.14	−0.35–0.07	<0.0001

General linear models with FSHD score of the relative as outcome and probands' FSHD score, age at examination, D4Z4 allele size and sex as predictors. Interaction test between proband's FSHD score and D4Z4 allele sizes.

the proband (distribution is reported in Supplementary Table 4). Table 6 shows that 72.5% of first-degree relatives are affected. This percentage significantly decreases to 52.9% among relatives with lower degree of kinship (from second- to fifth-degree), irrespective of D4Z4 size allele, sex and age at examination ($P = 0.018$), supporting the hypothesis that beside DRA, additional genetic factors may be necessary to develop FSHD. Conversely 47.1% of second- through fifth-degree relatives was unaffected, while only 27.5% of first-degree family members did not show any motor impairment.

It has also been observed that FSHD affects males more severely and more frequently than females (Zatz *et al.*, 1998; Tonini *et al.*, 2004; Sakellariou *et al.*, 2012). We thus evaluated whether gender influences expression and severity of motor impairment. We observed that the percentage of asymptomatic carriers does not significantly differ between genders (data not shown). Instead, as shown in Table 7, male relatives had a significantly higher mean FSHD score (5.4 versus 4.0, $P = 0.003$) and they developed motor impairment on average 7.3 years before than females ($P = 0.003$). Thus male relatives who develop motor impairment had a more severe disease than affected female relatives. We then calculated the risk of developing motor impairment between 20–50 years in females and males separately using the Kaplan-Meier method. As shown in Fig. 4A, the risk is higher in male relatives than females, although the difference is not statistically significant (log rank test *P*-value 0.113). Among probands the risk of developing motor impairment after age 20 is higher in males than in females (log rank test *P*-value = 0.028) (Fig. 4B). Remarkably, the risk becomes similar between genders after age 50.

All index cases and their relatives recruited in the present study carried the 4qA allele. As it has been recently proposed that FSHD occurs only when DRA at 4q35 are in combination with the 4A(159/161/168)PAS haplotype (Lemmers *et al.*, 2010), we further characterized DNA polymorphisms flanking the D4Z4 reduced array in 294 subjects (203 affected and 91 unaffected) belonging to 133 families from the cohort selected for this study. Table 8 reports the various haplotypes detected. All were associated with the polyadenylation signal (ATTAAA) that stabilized transcripts from *DUX4* gene. Notably, the 4A161PAS haplotype previously considered 'permissive' and the 4A166PAS haplotype previously considered 'non-permissive' for FSHD disease were detected in both DRA carriers with motor impairment (FSHD score ≥ 1) and without motor impairment (FSHD score 0). On this basis we conclude that no specific 4q haplotype can be considered as predictive of disease.

Collectively, the statistical analysis conducted on the entire cohort of relatives carrying DRA with 1–3 or 4–8 repeats indicates that individuals carrying DRA with 1–3 repeats have a high risk of developing motor impairment by age 50 (83–93%), regardless of sex or degree of kinship. In contrast, in the group with 4–8 repeats the reduced risk of becoming symptomatic (55-63% by age 50) is also modulated by sex (males show a higher risk than females) and degree of kinship (first degree relatives show a higher risk than second-fifth degree relatives).

Table 6 Prevalence of FSHD scores according to degree of kinship

Degree of kinship	FSHD score				*P*-value[†]
	0		1–15		
	Number of subjects	%	Number of subjects	%	
First	77	27.5	203	72.5	
Second/Fifth	41	47.1	46	52.9	0.018

[†]Wald test of coefficients associated to second or third degree of kinship in logistic models adjusted by D4Z4 allele size, sex and age at examination.

Discussion

Before the discovery of rearranged D4Z4 alleles, the diagnosis and counselling of FSHD families was entirely based on clinical evidence (Lunt *et al.*, 1989). Over the years, DNA testing of the D4Z4 locus and flanking polymorphisms has been considered highly sensitive and specific and extensively used to diagnose FSHD (Tawil *et al.*, 2010). However, two recent discoveries have challenged the current understanding of the prognostic value of D4Z4 reduced alleles (DRA) in FSHD families: (i) alleles with reduced numbers (≤ 8) of D4Z4 repeats at 4q35 combined with 4A(159/161/168)PAS haplotype, have a frequency of 1.3% among healthy subjects from the general population; and (ii) only 50% of FSHD probands carry the 4A161PAS permissive haplotype associated with DRA (Scionti *et al.*, 2012*b*). Therefore, our understanding of the factors that cause FSHD is incomplete and we conclude that it is crucial for clinical practice to define further elements that can influence motor impairment and can support the interpretation of molecular testing in FSHD families.

The present results rely on a population-based study involving index cases recruited from all regions of Italy. In order to minimize any ascertainment bias the analyses were performed excluding index cases and the evaluation of motor impairment was based

Table 7 Distribution of FSHD score and age at onset calculated on affected relatives according to sex

Sex	FSHD Score				Age at onset			
	Number of subjects	FSHD score mean	95% CI	P-value†	Number of subjects	Mean age at onset (years)	95% CI	P-value§
Male	102	5.4	4.7–6.1		99	26.8	(23.2; 30.5)	
Female	147	4.0	3.5–4.5	0.003	144	34.1	(30.5; 37.7)	0.003
Total	249				243			

†Wald test of equality to zero of female sex coefficients in a general linear model with FSHD score as dependent variable sex, D4Z4 allele size and age at examination as predictors.
§Wald test of equality to zero of female sex coefficients in a general linear model with age at onset as dependent variable and sex and D4Z4 allele size as predictors.

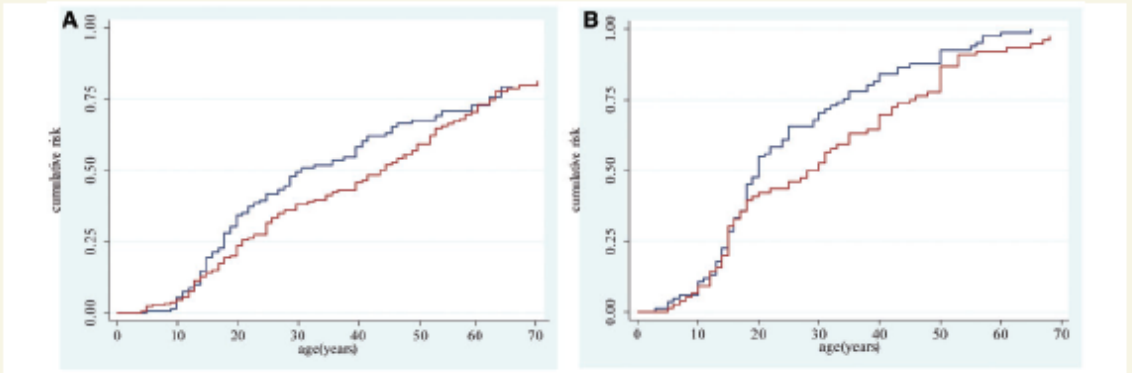

Figure 4 Age-specific cumulative risk of reported muscle impairment according to sex. (**A**) Estimates obtained on 361 relatives using the Kaplan-Meier analysis. Log-rank test *P*-value = 0.113. (**B**) Estimates obtained on 160 probands using the Kaplan-Meier analysis. Blue line refers to male; red line refers to female. Log-rank test *P*-value = 0.028.

Table 8 Distribution of haplotypes on 294 relatives

	Relatives					
	4A161 (*n* = 204)	4A162 (*n* = 14)	4A163 (*n* = 5)	4A164 (*n* = 1)	4A166 (*n* = 69)	4A167 (*n* = 1)
FSHD score = 0						
Number of subjects (%)	72 (79.1)	5 (5.5)	2 (2.2)	0 (0.0)	12 (13.2)	0 (0.0)
FSHD score ⩾1						
Number of subjects (%)	132 (65.0)	9 (4.4)	3 (1.5)	1 (0.5)	57 (28.1)	1 (0.5)

on a standardized protocol shared within the ICNF. However, beside these strengths, the study has some limitations. First, the genetic background and the socio-demographic characteristics of the Italian population might restrict the external validity of the study results. Second, even though the study has a good coverage of index cases, the involvement of relatives might be due to the presence of any symptoms with the consequence that the healthy ones might be under-represented in the study. In that case the true estimated prevalence of disease among relatives would be lower. Third, given that FSHD is a rare disease and no routinely collected diagnosis records are available (Lunt *et al.*, 1989) the age at onset was collected retrospectively. Therefore we cannot rule out the presence of recall bias. Indeed, the perception of disease onset may be subjective and could depend on the specific motor skills required in daily activities. It is thus possible that in a number of subjects the motor impairment of limbs may be perceived as early symptom because more disabling. According to this possibility and consistent with previous works (Tawil and van der Maarel, 2006; Pastorello *et al.*, 2012), in our cohort the most frequently complained symptom at onset was also the impairment of upper girdle (Supplementary Table 3). Nevertheless, we considered that patient's complaints provide a reliable estimate of the time of functional disability onset related to disease. When subjects did not refer any motor impairment, but a mild

muscle weakness was observed at the clinical evaluation, the age at examination was arbitrarily set as the age at onset (Lunt *et al.*, 1995*b*).

Given these premises, our study shows that FSHD penetrance in DRA carriers is not complete by age 20, as previously proposed (Tawil *et al.*, 2010), as asymptomatic carriers in all the classes of ages up to 70 years were found.

The present analysis highlights different prognostic values of DRA with 1–3 units when compared with DRA with 4–8 units. First, among carriers of DRA with 1–3 units FSHD penetrance is almost complete; in contrast, ~30% of carriers of DRA with 4–8 units older than 55 years display no muscle weakness (Table 1). Second, the estimated risk of developing motor impairment by age 50 differs between the two classes of alleles. Carriers of DRA with 1–3 units have a risk of 88.5% of developing motor impairment by age 50; instead the risk among carriers of DRA with 4–8 units by the same age is 55% (Fig. 2). Third, 44% of carriers of DRA with 1–3 units develop severe FSHD (FSHD score $\geq$7) by age 55; whereas only 24% of carriers of DRA with 4–8 units develop disease with high degree of severity by the same age (Fig. 3B). Fourth, the clinical phenotype is more homogeneous in families with DRA with 1–3 units, as shown by the intra-familial analysis (Table 5). In contrast, the clinical status of probands does not seem to be predictive of disease severity in relatives carrying DRA with 4–8. Importantly, in these families with DRA with 4–8 units, the penetrance of FSHD is lower as the degree of relationship to the affected individual becomes more distant, indicating that the genetic background can affect the disease outcome.

Our study also shows that gender influences disease expression, because males are characterized by a lower mean age at onset of motor impairment (26.8 years in males versus 34.1 years in females, Table 7) and by a more severe disability in terms of FSHD score (5.4 in males versus 4.0 in females). Interestingly, the risk of developing motor impairment is higher in male relatives during adult age (range 18–55 years), whereas it is similar between males and females in childhood/teens and elderly age (Fig. 4). Overall, these data indicate that variables related to gender, including genetic, hormonal, and/or lifestyle factors, may be considered and should be further investigated. Finally, our study suggests that the predictive value of 4q haplotypes must be carefully considered because no specific 4q haplotype was exclusively associated with the presence of disease.

In summary, the genotype-phenotype correlation study presented here confirms that DRAs with 4–8 repeats have no definitive prognostic value, and that other prognostic parameters, beside DRAs, such as sex and degree of kinship with the proband should be considered. We estimated that the risk of developing the motor impairment by age 50 in FSHD family members is higher (83–93%) in subjects carrying DRA with 1–3 repeats. Instead, considering the cohort of relatives carrying DRA with 4–8 repeats, the risk of developing motor impairment is 48% for females and/or subjects with lower degree of kinship and raises to 55–63% for males and/or subjects with first degree of kinship with the proband. None of the various 4q haplotypes detected in FSHD families studied here were exclusively associated with the presence of disease, as reported in Table 8.

Interestingly, in our cohort, 19 of 148 FSHD families (13%) in which a DRA with 4–8 units segregates presented affected subjects only in one generation (Supplementary Fig. 2). In these cases the lack of autosomal dominant inheritance should prompt us to consider whether the disease develops because of the presence of additional genetic defect(s). This possibility is supported by recent observation that mutations in the *SMCHD1* gene segregate independently from the FSHD permissive D4Z4 allele on chromosome 4 in FSHD subjects that do not carry a DRA, also defined as patients with FSHD2 (Lemmers *et al.*, 2012). Therefore searches for secondary FSHD loci should be considered in all cases in which the ratio between affected and unaffected individuals expected for an autosomal dominant disease is not observed and random association between the DRA and FSHD cannot be excluded.

For all of these reasons, to define the predictive value of DRA, it is necessary to carry out clinical evaluation and collection of DNA samples of all of the proband's family members, not only in a research setting but also in clinical practice. We believe that broadening the analysis of FSHD families may facilitate genetic counselling of patients and families with FSHD in particular when interpreting the data for prenatal diagnosis.

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Supplementary material

Supplementary material is available at *Brain* online.

References

Flanigan KM. Facioscapulohumeral muscular dystrophy and scapulohumeral syndrome. In: Engel AG, Franzini-Armstrong C, editors. Myology. New York: McGraw-Hill; 2004. p. 123–34.

Goto K, Nishino I, Hayashi YK. Very low penetrance in 85 Japanese families with facioscapulohumeral muscular dystrophy 1A. J Med Genet 2004; 41: e12.

Griggs RC, Tawil R, Storvick D, Mendell JR, Altherr MR. Genetics of facioscapulohumeral muscular dystrophy: new mutations in sporadic cases. Neurology 1993; 43: 2369–72.

Hippisley-Cox J, Coupland C, Vinogradova Y, Robson J, May M, Brindle P. Derivation and validation of QRISK, a new cardiovascular

disease risk score for the United Kingdom: prospective open cohort study. BMJ 2007; 335: 136.
Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. J Am Stat Assoc 1958; 53: 457–81.
Lamperti C, Fabbri G, Vercelli L, D'Amico R, Frusciante R, Bonifazi E, et al. A standardized clinical evaluation of patients affected by facioscapulohumeral muscular dystrophy: the FSHD clinical score. Muscle Nerve 2010; 42: 213–17.
Lemmers RJ, de Kievit P, Sandkuijl L, Padberg GW, van Ommen GJ, Frants RR, et al. Facioscapulohumeral muscular dystrophy is uniquely associated with one of the two variants of the 4q subtelomere. Nat Genet 2002; 32: 235–6.
Lemmers RJ, Tawil R, Petek LM, Balog J, Block GJ, Santen GW, et al. Digenic inheritance of an SMCHD1 mutation and an FSHD-permissive D4Z4 allele causes facioscapulohumeral muscular dystrophy type 2. Nat Genet 2012; 44: 1370–4.
Lemmers RJ, van der Vliet PJ, Klooster R, Sacconi S, Camaño P, Dauwerse JG, et al. A unifying genetic model for facioscapulohumeral muscular dystrophy. Science 2010; 329: 1650–3.
Lemmers RJ, Wohlgemuth M, van der Gaag KJ, van der Vliet PJ, van Teijlingen CM, de Knijff P, et al. Specific sequence variations within the 4q35 region are associated with facioscapulohumeral muscular dystrophy. Am J Hum Genet 2007; 81: 884–94.
Lunt PW, Compston DA, Harper PS. Estimation of age dependent penetrance in facioscapulohumeral muscular dystrophy by minimising ascertainment bias. J Med Genet 1989; 26: 755–60.
Lunt PW, Jardine PE, Koch MC, Maynard J, Osborn M, Williams M, et al. Correlation between fragment size at D4F104S1 and age at onset or at wheelchair use, with a possible generational effect, accounts for much phenotypic variation in 4q35-facioscapulohumeral muscular dystrophy (FSHD). Hum Mol Genet 1995a; 4: 951–8.
Lunt PW, Jardine PE, Koch MC, Maynard J, Osborn M, Williams M, et al. Phenotypic-genotypic correlation will assist genetic counseling in 4q35-facioscapulohumeral muscular dystrophy. Muscle Nerve 1995b; 2: S103–9.
Lunt PW. 44th ENMC international workshop: facioscapulohumeral muscular dystrophy: molecular studies 19–21 July 1996, Naarden, The Netherlands. Neuromuscul Disord 1998; 8: 126–30.
McCullagh P, Nelder JA. Generalized linear models. 2nd edn. New York: Chapman and Hall, Inc; 1989.
Mostacciuolo ML, Pastorello E, Vazza G, Miorin M, Angelini C, Tomelleri G, et al. Facioscapulohumeral muscular dystrophy: epidemiological and molecular study in a north-east Italian population sample. Clin Genet 2009; 75: 550–55.
Padberg G. Facioscapulohumeral disease. MD thesis. University of Leiden; 1982.
Padberg GW, Lunt PW, Koch M, Fardeau M. Diagnostic criteria for facioscapulohumeral muscular dystrophy. Neuromuscul Disord 1991; 1: 231–4.
Pastorello E, Cao M, Trevisan CP. Atypical onset in a series of 122 cases with facioscapulohumeral muscular dystrophy. Clin Neurol Neurosurg 2012; 114: 230–4.
Ricci E, Galluzzi G, Deidda G, Cacurri S, Colantoni L, Merico B, et al. Progress in the molecular diagnosis of facioscapulohumeral muscular dystrophy and correlation between the number of KpnI repeats at the 4q35 locus and clinical phenotype. Ann Neurol 1999; 45: 751–7.
Sakellariou P, Kekou K, Fryssira H, Sofocleous C, Manta P, Panousopoulou A, et al. Mutation spectrum and phenotypic manifestation in FSHD Greek patients. Neuromuscul Disord 2012; 22: 339–49.
Scionti I, Fabbri G, Fiorillo C, Ricci G, Greco F, D'Amico R, et al. Facioscapulohumeral muscular dystrophy: new insights from compound heterozygotes and implication for prenatal genetic counselling. J Med Genet 2012a; 49: 171–8.
Scionti I, Greco F, Ricci G, Govi M, Arashiro P, Vercelli L, et al. Large scale population analysis challenges the current criteria for the molecular diagnosis of fascioscapulohumeral muscular dystrophy (FSHD). Am J Hum Genet 2012b; 90: 628–65.
Tawil R, Forrester J, Griggs RC, Mendell J, Kissel J, McDermott M, et al. Evidence for anticipation and association of deletion size with severity in facioscapulohumeral muscular dystrophy. The FSH-DY Group. Ann Neurol 1996; 39: 744–48.
Tawil R, van der Maarel SM. Facioscapulohumeral muscular dystrophy. Muscle Nerve 2006; 34: 1–15.
Tawil R, van der Maarel S, Padberg GW, van Engelen BG. 171st ENMC international workshop: standards of care and management of facioscapulohumeral muscular dystrophy. Neuromuscul Disord 2010; 20: 471–5.
Tonini MM, Passos-Bueno MR, Cerqueira A, Matioli SR, Pavanello R, Zatz M. Asymptomatic carriers and gender differences in facioscapulohumeral muscular dystrophy (FSHD). Neuromuscul Disord 2004; 14: 33–8.
Upadhyaya M, Maynard J, Rogers MT, Lunt PW, Jardine P, Ravine D, et al. Improved molecular diagnosis of facioscapulohumeral muscular dystrophy (FSHD): validation of the differential double digestion for FSHD. J. Med. Genet 1997; 34: 476–9.
van Deutekom JC, Bakker E, Lemmers RJ, van der Wielen MJ, Bik E, Hofker MH, et al. Evidence for subtelomeric exchange of 3.3 kb tandemly repeated units between chromosomes 4q35 and 10q26: implications for genetic counselling and etiology of FSHD1. Hum Mol Genet 1996; 5: 1997–2003.
van Overveld PG, Lemmers RJ, Deidda G, Sandkuijl L, Padberg GW, Frants RR, van der Maarel SM. Interchromosomal repeat array interactions between chromosomes 4 and 10: a model for subtelomeric plasticity. Hum Mol Genet 2000; 9: 2879–84.
Wijmenga C, Hewitt JE, Sandkuijl LA, Clark LN, Wright TJ, Dauwerse HG, et al. Chromosome 4q DNA rearrangements associated with facioscapulohumeral muscular dystrophy. Nat Genet 1992; 2: 26–30.
Williams RL. A note on robust variance estimation for cluster-correlated data. Biometrics 2000; 56: 645–6.
Zatz M, Marie SK, Cerqueira A, Vainzof M, Pavanello RC, Passos-Bueno MR. The facioscapulohumeral muscular dystrophy (FSHD1) gene affects males more severely and more frequently than females. Am J Med Genet 1998; 77: 155–61.

Annex 3

Infantile Anamnestic Questionnaire (IAQ)

Referring Hospital:
Referring physician:
Date:
Patient's code: *Initials:*
Date of birth: *Sex:* □ M □ F

Pregnancy
Active fetal movements □ Normal □ Reduced
Diseases/Infections in pregnancy: _____
Therapy during pregnancy: _____

Birth
Partum □ Eutocic □ Dystocic week:_____

Fetal position	□ Normal	□ Podalic
Weight	□ Normal	□ Low
Revived	□ Yes	□ No
Clubfoot	□ Yes	□ No
Neonatal jaundice	□ Yes	□ No

Perinatal period/ months after

Suction	□ Normal	□ Reduced
Facial nerve palsy's diagnosis	□ Yes	□ No
Moebius syndrome's diagnosis	□ Yes	□ No
Hip dysplasia	□ Yes	□ No
Facial hypomimia	□ Yes	□ No
Floppy infant	□ Yes	□ No

Psychomotor development
Social smile □ Yes age (months) □ No
Sitting alone: age (months)
Walking alone: age (months)

After the first year of life

Data collection

Non-invasive ventilation	□ Yes (age:)	□ No
Coats' retinopathy	□ Yes (age:)	□ No
Sensorineural deafness	□ Yes	□ No
Academic success	□ Yes	□ No
Cognitive impairment	□ Yes	□ No
Loss of independent walking	□ Yes (age:)	□ No

Annex 4

Open Access Research

BMJ Open

Clinical expression of facioscapulohumeral muscular dystrophy in carriers of 1–3 D4Z4 reduced alleles: experience of the FSHD Italian National Registry

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ABSTRACT

Objectives: Facioscapulohumeral muscular dystrophy type 1 (FSHD1) has been genetically linked to reduced numbers (≤8) of D4Z4 repeats at 4q35. Particularly severe FSHD cases, characterised by an infantile onset and presence of additional extra-muscular features, have been associated with the shortest D4Z4 reduced alleles with 1–3 repeats (1–3 DRA). We searched for signs of perinatal onset and evaluated disease outcome through the systematic collection of clinical and anamnestic records of de novo and familial index cases and their relatives, carrying 1–3 DRA.

Setting: Italy.

Participants: 66 index cases and 33 relatives carrying 1–3 DRA.

Outcomes: The clinical examination was performed using the standardised FSHD evaluation form with validated inter-rater reliability. To investigate the earliest signs of disease, we designed the Infantile Anamnestic Questionnaire (IAQ). Comparison of age at onset was performed using the non-parametric Wilcoxon rank-sum or Kruskal-Wallis test. Comparison of the FSHD score was performed using a general linear model and Wald test. Kaplan-Meier survival analysis was used to estimate the age-specific cumulative motor impairment risk.

Results: No patients had perinatal onset. Among index cases, 36 (54.5%) showed the first signs by 10 years of age. The large majority of patients with early disease onset (26 out of 36, 72.2%) were de novo; whereas the majority of patients with disease onset after 10 years of age were familial (16, 53.3%). Comparison of the disease severity outcome between index cases with age at onset before and over 10 years of age, failed to detect statistical significance (Wald test p value=0.064). Of 61 index cases, only 17 (27.9%) presented extra-muscular conditions. Relatives carrying 1–3 DRA showed a large clinical variability ranging from healthy subjects, to patients with severe motor impairment.

Conclusions: The size of the D4Z4 allele is not always predictive of severe clinical outcome. The high degree of clinical variability suggests that additional factors contribute to the phenotype complexity.

Strengths and limitations of this study

- This is the most comprehensive survey of the clinical status of patients carrying a 1–3 D4Z4 reduced allele (DRA). Data were acquired through the Italian National Registry for facioscapulohumeral muscular dystrophy (FSHD) (INRF), which systematically collects clinical and molecular data from the whole Italian territory.
- The Infantile Anamnestic Questionnaire (IAQ) form was designed to acquire retrospective information about pregnancy, delivery, birth and perinatal period of carriers of 1–3 DRA.
- Mixed methods were used to obtain a standardised clinical assessment of all participants, including questionnaire, interview data and a structured medical assessment undertaken by the Italian Clinical Network for FSHD (ICNF).
- Data were usually self-reported for the medical history, without access being sought to individuals' health records.
- The data sets were derived only from Italy and have their own limitations.

INTRODUCTION

Facioscapulohumeral muscular dystrophy (FSHD (MIM 158900)) is the third most common muscular dystrophy with an

estimated prevalence of 1:20 000.[1] FSHD is considered an autosomal dominant disorder, with a typical onset within the second decade of life.[2 3] The disease presents a remarkably wide variety of phenotypic expressions, ranging from almost asymptomatic subjects to severe wheelchair-dependent patients.[4–6] The classical FSHD phenotype, first described as an independent nosological entity in 1884, by Landouzy and Dejerine,[7] is characterised by progressive facial, shoulder girdle and pectoral muscle weakness and atrophy. Disease progression may lead to involvement of abdominal and pelvic muscles, causing lumbar hyperlordosis and a waddling gait. Weakness of anterior leg muscles results in a steppage gait.

Several genotype–phenotype correlation studies reported a rough inverse correlation between the number of D4Z4 repeats and the severity of FSHD.[5 8–10] It has thus been suggested that alleles of extremely short size (1–3 D4Z4 repeats) are associated with the most severe form of disease.[5 6 8 11] A number of reports described cases carrying very short D4Z4 alleles with 1–3 D4Z4 repeats characterised by childhood onset, rapid progression of muscle weakness and extra-muscular clinical features.[12–20] In 1994, Brouwer *et al*[21] introduced the concept of Infantile Onset FSHD, based on the following diagnostic criteria: (1) signs or symptoms of facial weakness by 5 years of age; (2) signs or symptoms of scapular girdle weakness by 10 years of age.

However, several exceptions to these general trends have been found since the molecular analysis of the D4Z4 region became part of clinical diagnoses. Differences of clinical expression have been documented between participants carrying shorter alleles, varying from very severe forms of disease and complex phenotypes starting in infancy,[12–20] to milder form or asymptomatic carriers.[6 22–25] Based on the results presented in these studies, it was not possible to establish whether a congenital form of FSHD exists and whether detection of a 1–3 D4Z4 reduced allele (DRA) is always predictive of a severe phenotype with infantile onset. Furthermore, it is unclear whether additional clinical features observed in some patients with FSHD represent the extreme of the FSHD clinical spectrum or if they result from random associations.

In the present study, we investigated the prognostic significance of very short 4q35 alleles (1–3 DRA), through the clinical evaluation of 66 index cases and 33 relatives. Moreover, we searched for signs of perinatal onset through the systematic collection of anamnestic records of 80 patients carrying 1–3 DRA. Our study aimed to examine the clinical variability in the cohort of the participants carrying the shortest 4q35 alleles, presented in earlier clinical studies,[6 26–28] supporting the hypothesis that additional factors must contribute to FSHD disease.

METHODS

Study design and subject selection

The study was performed on FSHD families accrued through the Italian Clinical Network for FSHD (ICNF) (http://www.fshd.it).[29] The INCF is distributed across all of Italy, and includes a diagnostic laboratory at the University of Modena and Reggio Emilia, and 14 clinical centres, networking with the Italian Association of Myology (http://www.miologia.org). All clinical and molecular data were collected in the Italian National Registry for FSHD (INRF) database at the Miogen Laboratory at the University of Modena. The present study was conducted from January 2008 to December 2013. Of 850 index cases from the INRF, we identified 114 index cases carrying DRA with 1–3 repeats (figure 1A) and fulfilling the clinical diagnostic criteria defined for FSHD.[30] Family studies were conducted in 66 index cases, in which clinical and molecular analysis was extended to all available relatives willing to participate. Screening for 1–3 DRA was performed in 226 relatives. We defined de novo cases as single participant with neither parent carrying DRA; when the DRA was detected in one of the parents and/or other family members (ie, sibs), we classified the participant as familial. We considered participants as not informative when it was not possible to examine their parents and/or other informative family members.

Informed consent, according to the Declaration of Helsinki, was obtained from each participant enrolled in the study.

Clinical examination

The clinical examination was performed using the standardised FSHD clinical protocol with validated inter-rater reliability.[29] The FSHD clinical protocol was developed by the ICNF in order to numerically define the clinical severity of the motor impairment. The FSHD score, which translates disability into a number, ranges from zero, when no objective evidence of muscle functional impairment is present, to 15, when all the muscle groups tested are severely impaired (http://www.fshd.it). Age at onset was estimated on the basis of patients' records. To obtain a more objective evaluation of facial weakness onset, we asked specific questions, such as, 'Have your relatives ever noticed that you were sleeping with half-closed eyes?'; 'Have you ever been able to drink with a straw?'; 'Have you ever noticed difficulty in blowing out candles?' (see online supplementary figure S1). When participants did not report of motor impairment, but a mild muscle weakness was observed during clinical examination, we arbitrarily considered the age at examination as the age at onset, according to previous reports.[6 31]

Data about age at onset were available for all index cases, while the clinical re-evaluation using FSHD clinical score was performed in the 61 index cases and 33 relatives recruited for the family study (figure 1A,B).

In order to investigate the earliest signs of disease and to rule out pre- or perinatal events as possible causes of delayed achieving of motor milestones, we designed the Infantile Anamnestic Questionnaire (IAQ) (see online supplementary figure S1). All data about: (1) pregnancy,

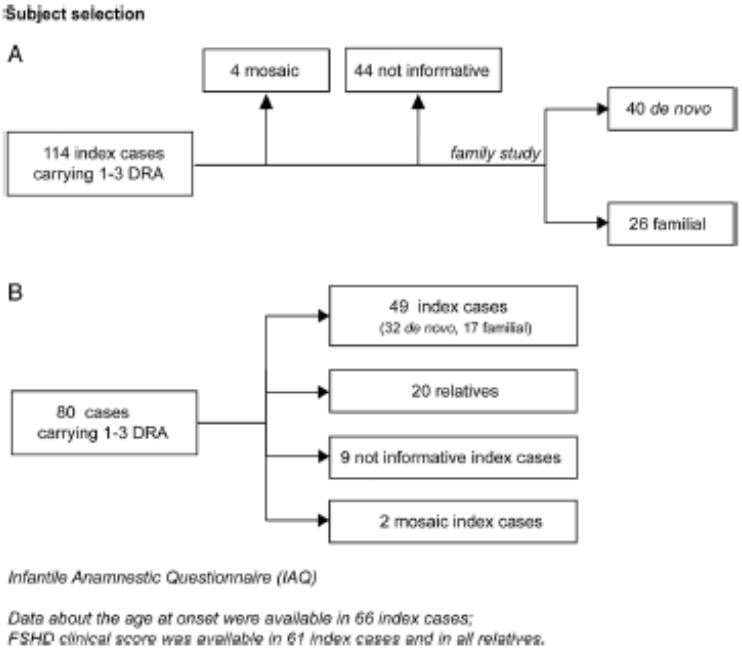

Figure 1 Selection of probands from the Italian National Registry of facioscapulohumeral muscular dystrophy (FSHD) for clinical and molecular study. (A) Data on age at onset were available for all index cases carrying 1–3 D4Z4 reduced alleles (DRA); FSHD clinical score was assessed in 61 index cases and 33 relatives carrying 1–3 DRA, recruited for the family study. (B) The Infantile Anamnestic Questionnaire was administered in 80 cases carrying 1–3 DRA.

(2) birth, (3) the prenatal period and first month of life and (4) psychomotor and language development were collected in a retrospective manner. Items related to each section were scored as normal/altered. We collected anamnestic reports about neurological examinations in the first year of life, together with clinical and instrumental data in the following years, whenever possible, in 80 cases carrying 1–3 DRA (figure 1B).

Molecular characterisation

DNA was prepared from isolated lymphocytes according to standard procedures. In brief, restriction endonuclease digestion of DNA was performed in agarose plugs with the appropriate restriction enzyme: EcoRI, EcoRI/BlnI. Digested DNA was separated by pulsed field gel electrophoresis (PFGE) in 1% agarose gels, as previously described.[28]

Allele sizes were estimated by Southern hybridisation with probe p13E-11 of 7 µg of EcoRI-digested, EcoRI/BlnI-digested genomic DNA extracted from peripheral blood lymphocytes, electrophoresed in a 0.4% agarose gel, for 45–48 h at 35 V, alongside an 8–48 kb marker (Bio-Rad) (see online supplementary figure S2A). Participants carrying alleles of 11–19 kb (1–3 D4Z4 units) in size were included in the study (see online supplementary figure S2B). To distinguish whether the DRA came from chromosome 10q or from 4q, DNA from each proband was analysed by NotI digestion and hybridisation with the B31 probe, to confirm the chromosome 4q origin of the 11–19 kb EcoRI allele.[28] Restriction fragments were detected by autoradiography or by using a Typhoon Trio system (GE Healthcare).

Statistical analysis

Comparison of age at onset was performed in de novo, familial and relatives of index cases using the non-parametric Wilcoxon rank-sum (for comparison between two groups) or Kruskal-Wallis (for comparison among three groups) test. To calculate the prevalence of infantile onset, we subdivided the patients on the basis of the age at onset, before 10 years and after 10 years of age. The prevalence of infantile onset was then compared across de novo, familial and relatives of index cases using χ^2 test.

Comparison of the FSHD score among de novo, familial and relatives of index cases was performed using a general linear model adjusted by sex and age at examination. Wald tests were used to evaluate if FSHD score differs between de novo, familial and relatives of index cases.

For the cohorts of probands and family members, time before developing disease was estimated from birth to the earliest age at onset of motor impairment. Kaplan-Meier survival analysis was used to estimate the age-specific cumulative motor impairment risk and loss of independent walking,[32] with the corresponding 95% CI. The differences in cumulative risk between de novo and familial index cases, carriers of 1–3 DRA, were evaluated using the Log rank test.

RESULTS

High percentage of de novo rearrangements among carriers of 1–3 DRA

Molecular analysis of 114 index cases from the INRF revealed that four participants were carriers of a somatic mosaicism for DRA. Forty-four cases were considered as not informative, since parents and/or other family members were not available for the molecular

investigation. In 66 unrelated index cases, we extended molecular characterisation to parents and/or other relatives and found that 26 probands were familial (39.4%) and 40 probands were de novo (60.6%). We also established the parental origin of de novo rearrangements in 31 cases and found that 48.4% were from the father and 52.6% were from the mother. Parental age at conception ranged from 18 to 43 years (maternal mean age at conception: 28.1±5.1 years; paternal mean age at conception: 31.3±5.3). In neither de novo cases nor in familial cases did we observe any correlation between the detection of a de novo D4Z4 rearrangement at 4q with the parent's age at conception.

Our analysis shows that the percentage of de novo cases in our cohort is higher (60.6%) than that previously described in the whole FSHD1 population (10–30%).[9 10 55–57] However, when we considered the 246 probands with 4–8 DRA we found that only 14 cases carried a de novo DRA (5.7%). Therefore, the overall percentage of de novo cases (17.3%) observed in the Italian cohort of 312 FSHD1 probands does not differ from published records. Instead, our analysis shows a skewed percentage of carriers of de novo rearrangement in the cohort of 1–3 DRA carriers, rather than in the cohort of 4–8 DRA carriers.

FSHD onset before 10 years of age is more prevalent among de novo carriers

Several studies suggest that carriers of 1–3 DRA develop a severe form of disease. In particular, the age at onset has been considered the clinical feature discriminating different FSHD phenotypic entities,[21] and has been used as a prognostic parameter for defining phenotype severity.[31] In a previous study, we found that FSHD occurs earlier in families in which 1–3 DRA segregate.[6] In the present study, we extended our analysis to all probands carrying 1–3 DRA, subdivided in two groups, de novo and familial. Table 1A shows that the mean age at onset observed among de novo probands appears significantly earlier than in familial.

To verify the prevalence of infantile onset, we then subdivided these two groups of patients on the basis of the age at onset, before 10 years and after 10 years of age (table 1B). In 54.5% of index cases carrying 1–3 DRA, the disease onset was in the first decade of life. A higher percentage of these cases with infantile onset reported facial weakness as the most common sign of disease (76.5%), while shoulder girdle weakness at onset resulted most commonly (69%) among patients developing FSHD after 10 years of age. Notably, the majority of patients with early disease onset were de novo. In contrast, the majority of patients with disease onset after 10 years of age were familial. We then estimated the age-specific cumulative motor impairment incidence (see online supplementary figure S3A), with the corresponding 95% CI in the two cohorts of de novo and familial probands by Kaplan-Meier survival analysis. For each individual, we assessed the interval of time from birth to the earliest age at onset of motor impairment. Supplementary figure S3A shows that, among participants carrying a de novo 1–3 DRA, the risk of developing motor impairment is 65% by age 10 years, 88% by age 15 years and 98% by age 20 years. Among participants carrying a familial 1–3 DRA the risk is 38% by age 10 years, 77% by age 15 years and 88% by age 20 years. Therefore the risk of developing FSHD before 10 years of age is significantly higher in participants carrying a de novo 1–3 DRA.

In our previous work[6] on the large cohort of FSHD1 families, we showed that the risk of developing motor impairment is higher in male probands/relatives during adulthood (18–55 years), and is similar between males and females in childhood-teens and in the elderly. In this cohort of patients carrying 1–3 DRA, the cumulative risk of motor impairment according to sex did not show

Table 1 Age at onset among de novo and familial index cases carrying 1–3 DRA

(A)
Mean age at onset

1–3 DRA index cases	Number of participants	Years	SD	p Value*
De novo	40	8.1	6.0	
Familial	26	13.1	10.1	0.020
Total	66			

(B)
Age at onset

		0–10 years		>10 years		
1–3 DRA index cases	Number of participants	Number of participants	Per cent	Number of participants	Per cent	p Value†
De novo	40	26	65.5	14	35.5	
Familial	26	10	38.5	16	61.5	0.045
Total	66	36		30		

*Wilcoxon rank-sum test p value.
†χ^2 Test p value.
DRA, D4Z4 reduced allele.

significant difference among males and females (log rank test p value=0.305). We hypothesise that the absence of gender difference is due to the fact that, in this group, the disease onset occurs, on average, before patients reach 20 years of age.

Infantile onset does not always predict a very severe clinical outcome

It has been reported that extremely short 4q35 alleles (1–3 D4Z4 units) are associated with more severe forms of FSHD.[5 6 8 11] Considering the earlier average onset in de novo cases compared to familial 1–3 DRA carriers, we tested whether de novo index cases present a more severe disease expression, comparing the degrees of motor disability indicated by the FSHD score.[29] Statistical evaluation failed to detect any significant difference in the mean FSHD score adjusted by age and sex between the two groups (10.6 in de novo index cases vs 9.5 in familial index cases; 95% CI, respectively, (8.0 to 11.3), (9.3 to 12.0); Wald test p value=0.280).

We then calculated the age-specific relative risk of loss of independent walking ability in de novo familial index cases, using Kaplan-Meier statistics. This analysis did not detect a statistically significant difference between the two groups (see online supplementary figure S3B), although the percentage of wheelchair-bound patients under 40 years of age is higher in de novo index cases (70%) versus familial index cases (35%). Finally, comparison of the severity of disease expression between index cases (both de novo and familial) with early age at onset (0–10 years; age and sex adjusted mean FSHD score=11.4) versus probands with age at onset over 10 years (age and sex adjusted mean FSHD score=9.5) failed to detect statistically significant more severe FSHD disease in probands with infantile onset (Wald test p value=0.064). Overall, the lack of significant differences in each of these three comparisons argues against the idea that early FSHD onset is necessarily associated with a more disabling outcome.

Relatives carrying 1–3 DRA are not always severely affected

In eight families, we were able to extend molecular analysis to three generations, and found that the molecular defect appeared de novo and was transmitted to offspring in six members. Three carriers of de novo mutation were probands, whereas three other carriers were discovered because of the appearance of FSHD in one child. Importantly, two of them were unaffected (see online supplementary figure S4) at the time of examination (41 and 45 years, respectively) and one suffered from a mild form of disease (FSHD score 3 at 38 years of age). These observations indicate that the presence of a de novo 1–3 DRA does not always associate with a severe phenotype.

We compared the age at disease onset detected in the group of familial probands with that recorded in the group of 33 relatives carrying a DRA. This comparison displayed that affected relatives had significantly later onset of FSHD than the probands (table 2A).

We also compared the degree of motor impairment, recorded as FSHD score, detected in the two groups. The age and sex adjusted mean FSHD score received by relatives was significantly lower than that recorded in the probands (4.7 vs 9.5; Wald test p value <0.0001). Notably, four relatives (12.1%), respectively aged 33, 42, 47 and 50 years, presented no muscle weakness (table 2B). Overall, similarly to what we observed among carriers of 4–8 DRA,[6] we found a reduced severity of clinical expression also in the group of relatives carrying very short D4Z4 allele in comparison with probands, including participants with no signs of disease after the age of 40 years.

Carriers of 1–3 DRA did not show signs of prenatal and neonatal FSHD onset

To systematically obtain information about the perinatal period and the appearance of the first signs and/or symptoms of disease, we designed the IAQ (see online

Table 2 Age at onset in familial index cases and their relatives carrying 1–3 DRA

(A)
Mean age at onset

1–3 DRA carriers	Number of participants	Years	SD	p Value*
Probands	26	13.1	10.1	
Relatives	29	17.1	14.2	0.019
Total	55			

(B)
Age at onset

		0–10 years		>10 years		Unaffected	
1–3 DRA index cases	Number of participants	Number of participants	Per cent	Number of participants	Per cent	Number of participants	Per cent
Probands	26	12	38.5	14	61.5	0	0.0
Relatives	33	13	39.4	16	48.5	4	12.1

*Wilcoxon rank-sum test p value.

supplementary figure S1). We gathered anamnestic data about pregnancy, delivery and birth from all participants who were able to respond to this questionnaire. We interviewed 80 cases carrying 1–3 DRA (figure 2). Figure 2 shows that no significant alterations in pregnancy, delivery and birth were reported. There was no report of any floppy infant at birth. In 72 of 80 participants (90%), psychomotor development milestones were reached appropriately.

This analysis shows that children carrying 1–3 DRA do not display signs of muscle weakness prenatally or at birth. Moreover, signs that can possibly be attributed to early onset of muscle weakness are reported only in a small percentage of participants. Therefore we conclude that very early onset is not a frequent feature of FSHD.

Carriers of 1–3 DRA with extra-muscular clinical conditions

In some reports, severe FSHD is associated with extra-muscular features such as sensorineural deafness, Coats' retinopathy, epilepsy and mental retardation. We assessed the frequency of additional clinical conditions in the cohort of 61 index cases carrying 1–3 DRA, summarised in figure 3.

Thirteen participants suffered from sensorineural deafness (21.3%). In eight cases, it was isolated, with no other recognisable medical condition, and in five cases we detected additional extra-muscular manifestations.

In four cases, we observed Coats' retinopathy (6.6%). In one it was found as an isolated condition, whereas in three other cases it was associated with sensorineural deafness or cognitive impairment. Cognitive impairment was reported in six cases (9.8%), and two of these also suffered from epilepsy. All cases with mental retardation showed a very severe form of disease.

We also investigated the presence of restrictive respiratory disease requiring intervention, previously described in about 1% of patients with FSHD1, typically in patients with severe muscle weakness.[5] In our cohort of 61 index cases with 1–3 DRA, we identified 7 cases (11.5%) with respiratory insufficiency requiring non-invasive ventilation (NIV). Two of these showed a complex phenotype with additional extra-muscular manifestations (figure 3), while all the others suffered from a disabling form of disease and required use of NIV since the ages of 22, 29, 30, 42 and 53 years, respectively.

DISCUSSION

Since molecular analysis of the D4Z4 region was introduced to study FSHD, it has been suggested that very severe forms of disease are associated with a very short DRA.[5 6 8 11] This notion has supported the idea that a rough inverse correlation exists between the size of D4Z4 allele and disease severity. Moreover, it has been long debated whether 'infantile FSHD' might exist as a distinct nosological entity, characterised by specific peculiarities that distinguish it from classical FSHD with onset in the second decade of life.[21 22] 'Infantile FSHD' has been defined by childhood onset and severe muscle impairment, associated with high-frequency hearing loss, retinal vascular abnormalities, mental retardation and epilepsy.[14 15 18 20] By revising the literature, as summarised in figure 4, we found that not all severe cases had an infantile onset, and not all carried a very reduced size D4Z4 allele. However, the different designs used in each of these previous studies prevented the possibility of pooling various observations to obtain a complete or more defined picture of clinical features of participants carrying 'very short' D4Z4 allele.

Here, the large number of index cases carrying DRA with 1–3 units accrued through the INRF allowed us to obtain more precise information about this group of patients. In particular, we aimed at verifying whether perinatal onset of FSHD exists and whether presence of a 1–3 DRA is always predictive of a severe phenotype associated with infantile onset. In addition, we searched

Figure 2 Infantile anamnestic records of 80 carriers of 1–3 D4Z4 reduced alleles (DRA) (NA, not applicable).

Period	Features	N of subjects		N of subjects		N of subjects	
prenatal	active fetal movements	*normal*	71	*reduced*	2	*NA*	7
delivery	partum	*eutocic*	63	*dystocic*	15	*NA*	2
	fetal position	*cephalic*	62	*podalic*	6	*NA*	12
birth	weight	*normal*	73	*low*	0	*NA*	7
	revived	*no*	74	*yes*	2	*NA*	4
	clubfoot	"	74	"	1	*NA*	5
perinatal	reduced suction	*no*	72	*yes*	7	*NA*	1
	facial nerve palsy	"	76	"	2	*NA*	2
	Moebius syndrome	"	76	"	1	*NA*	3
	hip dysplasia	"	74	"	2	*NA*	4
	facial hypomimia	"	76	"	3	*NA*	1
	floppy	"	76	"	0	*NA*	4
psychomotor development	social smile	*normal*	72	*altered*	5	*NA*	3
	walk independently		<15 months	69			
			15-18 months	7			
			>18 months	3			
			NA	1			

ID	Sex	Index case	DRA	Age at examination	FSHD score	Age at onset	Mode of onset	Loss of independent walking (since age)	Respiratory insufficiency (since age)	Additional features/ extra-muscular comorbidities
Patient 1	F	de novo	16 kb	11 yrs	10	3 yrs	facial weakness	22 yrs	---	sensorineural hypoacusia
Patient 2	F	de novo	14 kb	42 yrs	12	2 yrs	facial weakness	37 yrs	---	sensorineural hypoacusia
Patient 3	F	de novo	12 kb	40 yrs	9	8 yrs	facial weakness	---	---	sensorineural hypoacusia
Patient 4	M	de novo	14 kb	50 yrs	14	12 yrs	shoulder girdle weakness	34 yrs	---	sensorineural hypoacusia
Patient 5	F	de novo	14 kb	34 yrs	12	8 yrs	facial weakness	---	---	sensorineural hypoacusia
Patient 6	M	de novo	14 kb	37 yrs	14	11 yrs	facial weakness	30 yrs	---	sensorineural hypoacusia
Patient 7	M	de novo	12 kb	22 yrs	14	<1 yrs	facial weakness	20 yrs	---	sensorineural hypoacusia, cognitive impairment
Patient 8	M	de novo	14 kb	21 yrs	7	4 yrs	facial weakness	---	---	sensorineural hypoacusia
Patient 9	M	familial	17 kb	53 yrs	14	8 yrs	shoulder girdle weakness	20 yrs	---	sensorineural hypoacusia, Coats' retinopathy
Patient 10	F	de novo	17 kb	28 yrs	14	3 yrs	facial weakness	18 yrs	---	sensorineural hypoacusia, cognitive impairment
Patient 11	M	familial	17 kb	7 yrs	2	<1 yrs	visual problems	---	---	sensorineural hypoacusia, Coats' retinopathy
Patient 12	M	de novo	14 kb	64 yrs	15	15 yrs	shoulder girdle weakness	45 yrs	---	sensorineural hypoacusia, Coats' retinopathy, cognitive impairment
Patient 13	F	de novo	14 kb	32 yrs	14	< 1 yrs	facial weakness	18 yrs	32 yrs	sensorineural hypoacusia
Patient 14	M	de novo	12 kb	29 yrs	13	10 yrs	shoulder girdle weakness	29 yrs	---	Coats' retinopathy
Patient 15	M	de novo	17 kb	10 yrs	10	2 yrs	facial weakness	---	---	cognitive impairment, epilepsy
Patient 16	M	familial	14 kb	27 yrs	10	7 yrs	lower limbs weakness	---	---	cognitive impairment
Patient 17	M	de novo	11 kb	33 yrs	11	5 yrs	shoulder girdle weakness	29 yrs	29 yrs	cognitive impairment, epilepsy

Figure 3 Clinical features of 1–3 D4Z4 reduced alleles (DRA) carriers with extra-muscular comorbidities (FSHD, facioscapulohumeral muscular dystrophy; M, male; F, female).

this cohort of patients for the presence of additional clinical conditions determining complex phenotypes.

First, our analyses showed that the majority of 1–3 DRA carriers (60.6%) are de novo. These data, together with the observation that 1–3 DRAs have never been detected in the normal population,[28] support the notion that the D4Z4 repeat array is highly recombinogenic and therefore prone to a high mutation rate.[35 37]

Second, the majority (72.2%) of cases presenting disease onset before 10 years of age are isolated and carry a de novo rearranged DRA; in contrast, the majority (53.3%) of familial FSHD1 cases develop around the second decade of life (table 1B). However, we did not find a statistically significant difference in disease outcome between de novo and familial probands, even though there is a trend towards a more severe progression among de novo cases (age and sex adjusted mean FSHD score in de novo vs familial probands, is 10.6 vs 9.5). We confirm a more severe phenotype in index cases carrying 1–3 DRA in comparison with participants carrying longer alleles (4–10 DRA).[6 38] Our data also confirmed that, among index cases with infantile onset, the majority (76.5%) reported facial weakness as the first sign of disease, presenting difficulty in closing eyes and puffing cheeks, or progressive facial hypomimia. In three participants, we collected anamnestic records of abnormalities in pronouncing some phonemes at the age of 3–4 years, and interpreted these difficulties as possibly due to the onset of facial muscle weakness. Two cases were initially misdiagnosed as affected by Moebius syndrome, most likely because of the very early onset of severe facial muscle weakness. Instead index cases with FSHD onset after 10 years reported shoulder girdle weakness as the most common first sign of the disease, according to the previous reports.[6 39 40]

Third, even when signs or symptoms of muscle weakness are detected within the first year of life, the long-term disease outcome does not differ from cases with later onset.

Fourth, the use of the IAQ confirmed that pre- and perinatal onset is not present in the group of 1–3 DRA carriers.

Authors/year	N of subjects	DRA	Age at onset	Family history
Jardine et al, [11] *Arch Dis Child* 1994	27 *de novo* cases	13 → 29 kb	-in 19 cases ≤10 yrs (17 of them with ≤19 kb DRA) -8 cases >10 yrs (5 of them with ≤19 kb DRA)	—
Brouwer et al, [22] *Muscle Nerve Suppl* 1995	10 cases, *de novo* and familial (3 of them no molecularly characterized)	-5 cases with ≤19 kb DRA -1 case with 22 kb DRA -1 case without DRA	≤10 yrs	Relatives of familial cases carrying the same DRA showed a mild form of disease
Nakagawa et al, [13] *Acta Neurol Scand* 1996	2 familial cases (the proband and her mother)	13 kb	-2 yrs (daughter) -23 yrs (mother)	Mother with 13 kb DRA showed an adult form of disease, with prevalent limb girdle involvement
Okinaga et al, [23] *Brain Dev* 1997	-1 familial case -1 *de novo* case	-15 kb -13 kb	early onset (<1 yrs)	Father with 15 kb DRA did not show muscle weakness
Miura et al, [14] *Neuropediatrics* 1998	2 sporadic cases with mental retardation and epilepsy	10 kb	early onset (<5 yrs)	—
Funakoshi et al, [15] *Neurology* 1998	-9 familial cases -12 *de novo* cases	11 → 20 kb	early onset (<5 yrs)	—
Dorobek and Kabzińska, [17] *Eur J Paediatr Neurol* 2004	1 case	8 kb	5 months	Father with somatic mosaicism was asymptomatic
Trevisan et al, [18] *Eur J Neurol* 2008	-4 *de novo* cases -2 familial cases	10 → 13 kb	<10 yrs	—
Wang et al, [20] *Neuromuscul Disord* 2012	7 cases	11 → 14 kb	early onset (≤8 yrs)	—
Chen et al, [25] *Neuromuscul Disord* 2013	-6 familial cases -3 sporadic cases	10 → 13 kb	≤5 yrs	Relatives of familial cases, carrying the same DRA, showed an adult/subtle form of FSHD. Relatives with somatic mosaicism were asymptomatic
Dorobek et al, [26] *J Child Neurol* 2014	-7 familial cases -12 sporadic cases (9 *de novo*) -3 not informative cases	9 → 19 kb	≤5 yrs (17 cases) ≤10 yrs (5 cases)	—

Figure 4 Revisited literature: 1–3 D4Z4 reduced alleles (DRA) case reports comorbidities (FSHD, facioscapulohumeral muscular dystrophy).

Fifth, among the 33 relatives carrying 1–3 D4Z4 alleles, 4 (12.1%) were unaffected, confirming the incomplete penetrance also in the cohort of participants carrying shorter DRA.[6] Moreover, relatives displayed a milder phenotype than family proband, supporting the notion that in this subgroup of patients, the genetic background also plays a role in modulating the disease expression.[6]

Finally, in contrast with the previous reports,[11–19] our investigations demonstrated that only seven cases (11.5%; 5 de novo and 2 familial) displayed extramuscular comorbidities (Coats' retinopathy, sensorineural deafness, mental retardation, epilepsy) in various combinations (figure 4). We thus conclude that extramuscular clinical features are not part of a specific nosological entity associated with 1–3 DRA.

In summary, our study shows that a high variability of FSHD clinical expression is also present among participants carrying 1–3 DRA, with some healthy relatives carrying the same DRA as the affected ones. The observation that the majority of 1–3 DRA cases carry 'de novo' rearrangements, confirms the high frequency of recombination events within the D4Z4 region. Importantly, our comparisons of probands and relatives disclose that the presence of a de novo 1–3 DRA is not always associated with a disease phenotype, and emphasises the possibility that a more disabling phenotype might have a negative influence on reproductive fitness. Accordingly, in our cohort de novo rearrangements in unaffected individuals or in patients displaying mild phenotypes had normal reproductive fitness with transmission of the DRA to the offspring.

Our analysis, conducted on the largest cohort of 1–3 DRA carriers to date, shows that 1–3 DRAs are not always predictive of infantile onset or severe disease outcome. Importantly, the finding that only 27.9% of 1–3 DRA carriers present extra-muscular clinical conditions supports the notion that additional defects contribute to a more complex clinical phenotype.

Overall, our study indicated that an important future goal of FSHD clinical research is the selection of patients with homogeneous clinical features, regardless of the size of D4Z4 alleles, to provide the appropriate background for molecular studies aimed at dissecting the complex pathogenesis of this disease.

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REFERENCES

1. Mostacciuolo ML, Pastorello E, Vazza G, *et al*. Facioscapulohumeral muscular dystrophy: epidemiological and molecular study in a north-east Italian population sample. *Clin Genet* 2009;75:550–5.
2. Lunt PW, Compston DA, Harper PS. Estimation of age dependent penetrance in facioscapulohumeral muscular dystrophy by minimising ascertainment bias. *J Med Genet* 1989;26:755–60.
3. Tawil R, van der Maarel S, Padberg GW, *et al*. 171st ENMC international workshop: standards of care and management of facioscapulohumeral muscular dystrophy. *Neuromuscul Disord* 2010;20:471–5.
4. Lunt PW, Harper PS. Genetic counselling in facioscapulohumeral muscular dystrophy. *J Med Genet* 1991;28:655–64.
5. Ricci E, Galluzzi G, Deidda G, *et al*. Progress in the molecular diagnosis of facioscapulohumeral muscular dystrophy and correlation between the number of KpnI repeats at the 4q35 locus and clinical phenotype. *Ann Neurol* 1999;45:751–7.
6. Ricci G, Scionti I, Sera F, *et al*. Large scale genotype-phenotype analyses indicate that novel prognostic tools are required for families with facioscapulohumeral muscular dystrophy. *Brain* 2013;136:3408–17.
7. Landouzy L, Dejerine J. De la myopathie atrophique progressive. *Rev Med Francaise* 1885;5:81.
8. Lunt PW, Jardine PE, Koch MC, *et al*. Correlation between fragment size at D4F104S1 and age at onset or at wheelchair use, with a possible generational effect, accounts for much phenotypic variation in 4q35-facioscapulohumeral muscular dystrophy (FSHD). *Hum Mol Genet* 1995;4:951–8.
9. Tawil R, Forrester J, Griggs RC, *et al*. Evidence for anticipation and association of deletion size with severity in facioscapulohumeral muscular dystrophy. The FSH-DY Group. *Ann Neurol* 1996;39:744–8.
10. Zatz M, Marie SK, Cerqueira A, *et al*. The facioscapulohumeral muscular dystrophy (FSHD1) gene affects males more severely and more frequently than females. *Am J Med Genet* 1998;77:155–61.
11. Jardine PE, Koch MC, Lunt PW, *et al*. De novo facioscapulohumeral muscular dystrophy defined by DNA probe p13E-11 (D4F104S1). *Arch Dis Child* 1994;71:221–7.
12. Okinaga A, Matsuoka T, Umeda J, *et al*. Early-onset facioscapulohumeral muscular dystrophy: two case reports. *Brain Dev* 1997;19:563–7.
13. Nakagawa M, Matsuzaki T, Higuchi I, *et al*. Facioscapulohumeral muscular dystrophy: clinical diversity and genetic abnormalities in Japanese patients. *Intern Med* 1997;36:333–9.
14. Miura K, Kumagai T, Matsumoto A, *et al*. Two cases of chromosome 4q35-linked early onset facioscapulohumeral muscular dystrophy with mental retardation and epilepsy. *Neuropediatrics* 1998;29:239–41.
15. Funakoshi M, Goto K, Arahata K. Epilepsy and mental retardation in a subset of early onset 4q35-facioscapulohumeral muscular dystrophy. *Neurology* 1998;50:1791–4.
16. Yamanaka G, Goto K, Hayashi YK, *et al*. Clinical and genetical features of Japanese early-onset facioscapulohumeral muscular dystrophy. *No To Hattatsu* 2002;34:318–24.
17. Dorobek M, Kabzińska D. A severe case of facioscapulohumeral muscular dystrophy (FSHD) with some uncommon clinical features and a short 4q35 fragment. *Eur J Paediatr Neurol* 2004;8:313–16.
18. Trevisan CP, Pastorello E, Tomelleri G, *et al*. Facioscapulohumeral muscular dystrophy: hearing loss and other atypical features of patients with large 4q35 deletions. *Eur J Neurol* 2008;15:1353–8.
19. Grosso S, Mostardini R, Di Bartolo RM, *et al*. Epilepsy, speech delay, and mental retardation in facioscapulohumeral muscular dystrophy. *Eur J Paediatr Neurol* 2011;15:456–60.
20. Wang CH, Leung M, Liang WC, *et al*. Correlation between muscle involvement, phenotype and D4Z4 fragment size in facioscapulohumeral muscular dystrophy. *Neuromuscul Disord* 2012;22:331–8.
21. Brouwer OF, Padberg GW, Wijmenga C, *et al*. Facioscapulohumeral muscular dystrophy in early childhood. *Arch Neurol* 1994;51:387–94.
22. Brouwer OF, Padberg GW, Bakker E, *et al*. Early onset facioscapulohumeral muscular dystrophy. *Muscle Nerve Suppl* 1995;2:S67–72.
23. Sakellariou P, Kekou K, Fryssira H, *et al*. Mutation spectrum and phenotypic manifestation in FSHD Greek patients. *Neuromuscul Disord* 2012;22:339–49.
24. Chen TH, Lai YH, Lee PL, *et al*. Infantile facioscapulohumeral muscular dystrophy revisited: expansion of clinical phenotypes in patients with a very short EcoRI fragment. *Neuromuscul Disord* 2013;23:298–305.
25. Dorobek M, van der Maarel SM, Lemmers RJ, *et al*. Early-onset facioscapulohumeral muscular dystrophy type 1 with some atypical features. *J Child Neurol* 2015;30:580–7.
26. Tonini MM, Passos-Bueno MR, Cerqueira A, *et al*. Asymptomatic carriers and gender differences in facioscapulohumeral muscular dystrophy (FSHD). *Neuromuscul Disord* 2004;14:33–8.

27. Yamanaka G, Goto K, Ishihara T, *et al*. FSHD-like patients without 4q35 deletion. *J Neurol Sci* 2004;219:89–93.
28. Scionti I, Greco F, Ricci G, *et al*. Large-scale population analysis challenges the current criteria for the molecular diagnosis of fascioscapulohumeral muscular dystrophy. *Am J Hum Genet* 2012;90:628–35.
29. Lamperti C, Fabbri G, Vercelli L, *et al*. A standardized clinical evaluation of patients affected by facioscapulohumeral muscular dystrophy: the FSHD disability score. *Muscle Nerve* 2010;42:213–17.
30. Padberg GW, Lunt PW, Koch M, *et al*. Diagnostic criteria for facioscapulohumeral muscular dystrophy. *Neuromuscul Disord* 1991;1:231–4.
31. Lunt PW, Jardine PE, Koch M, *et al*. Phenotypic-genotypic correlation will assist genetic counseling in 4q35-facioscapulohumeral muscular dystrophy. *Muscle Nerve* 1995;2:S103–9.
32. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 1958;53:457–81.
33. Flanigan KM. Facioscapulohumeral muscular dystrophy and scapulohumeral syndrome. In: Engel AG, Franzini-Armstrong C, eds. *Myology*. New York: McGraw-Hill, 2004:123–34.
34. Padberg GW, Frants RR, Brouwer OF, *et al*. Facioscapulohumeral muscular dystrophy in the Dutch population. *Muscle Nerve Suppl* 1995;2:S81–4.
35. Zatz M, Marie SK, Passos-Bueno MR, *et al*. High proportion of new mutations and possible anticipation in Brazilian facioscapulohumeral muscular dystrophy families. *Am J Hum Genet* 1995;56:99–105.
36. Lunt PW. 44th ENMC International Workshop: Facioscapulohumeral Muscular Dystrophy: Molecular Studies 19–21 July 1996, Naarden, The Netherlands. *Neuromuscul Disord* 1998;8:126–30.
37. van der Maarel SM, Deidda G, Lemmers RJ, *et al*. De novo facioscapulohumeral muscular dystrophy: frequent somatic mosaicism, sex-dependent phenotype, and the role of mitotic transchromosomal repeat interaction between chromosomes 4 and 10. *Am J Hum Genet* 2000;66:26–35.
38. Salort-Campana E, Nguyen K, Bernard R, *et al*. Low penetrance in facioscapulohumeral muscular dystrophy type 1 with large pathological D4Z4 alleles: a cross-sectional multicenter study. *Orphanet J Rare Dis* 2015;10:2.
39. Tawil R, Van Der Maarel SM. Facioscapulohumeral muscular dystrophy. *Muscle Nerve* 2006;34:1–15.
40. Pastorello E, Cao M, Trevisan CP. Atypical onset in a series of 122 cases with facioscapulohumeral muscular dystrophy. *Clin Neurol Neurosurg* 2012;114:230–4.

Annex 5

Part A

Referring Hospital: _____ Referring Physician: _____

Compilation Date: _ _/_ _/_ _ _ _

Patient Initials: _____ Patient code: _____

Date of birth: _ _/_ _/_ _ _ _ Sex: ○ M ○ F

Handedness: ○ Left ○ Right

Weight:_____kg

Height:_____cm

Family Ancestry - geographic origins:

Maternal: _____ Mother Surname: _____

Paternal: _____

Consanguinity: ○ Yes ○ No

Current profession: _____ Since year: _ _ _ _

If you were previously employed:

Previous profession(s) :

_____ From year: _ _ _ _ to year _ _ _ _

_____ From year: _ _ _ _ to year _ _ _ _

_____ From year: _ _ _ _ to year _ _ _ _

Highest degree: ○ University degree ○ High-school diploma ○ Primary school diploma ○ None

(years of education: _____)

Clinical history

Previous evaluation in other center(s): ○ Yes ○ No If yes, centre: (1) _____

(2) _____

FSHD score at last clinical examination: _ _ Date: _ _/_ _ /_ _ _ _

Comorbidities:

Diabetes mellitus: ○ Yes ○ No ○ Not evaluated

If Yes, ○ type I ○ type II Age at diagnosis: _ _

Therapy, Drugs :

_____ Dose: _ _ _ _ unit _ _ _ _ From year: _ _ _ _ to year _ _ _ _

_____

_____

Thyroid hormones alterations: ○ Yes ○ No ○ Not evaluated

If Yes, ○ hypothyroidism ○ hyperthyroidism Age at diagnosis: _ _

Therapy, Drugs :

_____ Dose: _ _ _ _ unit _ _ _ _ From year: _ _ _ _ to year _ _ _ _

_____

Hepatitis: ○ Yes ○ No ○ Not evaluated

If Yes, ○ HBV ○ HCV ○ Toxic Age at diagnosis: _ _

Therapy, Drugs :

_____ Dose: _ _ _ _ unit _ _ _ _ From year: _ _ _ _ to year _ _ _ _

_____

Diagnosis of cancer: ○ Yes ○ No

If yes, specify:_____ Age at diagnosis: _ _

Therapy, Drugs :

_____ Dose: _ _ _ _ unit _ _ _ _ From year: _ _ _ _ to year _ _ _ _

_____

Retinal vasculopathy: ○ Yes ○ No ○ Not evaluated

Sensorineural deafness: ○ Yes ○ No

Audiometry: ○ Alterated ○ Normal ○ Not performed

Epilepsy: ○ Yes ○ No

Cognitive impairment: ○ Yes ○ No

Other disease(s)

Other diseases ○ Yes ○ No

If yes, specify: _____

_____ _____

_____

Previous trauma: joint, bone fractures: ○ Yes ○ No If yes, specify site and age_ _

Drugs:

Statins: ○ Yes ○ No If yes, Type: _____

Dose: _ _ _ _ unit _ _ _ _ From year: _ _ _ _ to year _ _ _ _

Others chronic treatments: ○ Yes ○ No

If yes

Drug: _____ Dose: _____ From year: _____to year _____

_____

_____

Reproductive History:

Have you ever been pregnant? ○ Yes ○ No

Are you pregnant now? ○ Yes ○ No

How many times have you been pregnant: _ _

Spontaneous abortion: ○ Yes ○ No if yes, Number of spontaneous abortion: _ _

How many vaginal deliveries have you had? {Please count stillbirths as well as live births}: _ _

How many cesarean deliveries have you had? {Please count stillbirths as well as live births}: _ _

How many of the deliveries resulted in a live birth? : _ _

How old were you at the time of your first live birth? age _ _

How old were you at the time of your last live birth? age _ _

Prenatal diagnosis ○ Yes (N°_____) ○ No If yes, result: _____

Modification of the disease after the pregnancy: ○ None ○ Worsening ○ Improvement

Menopause: ○ Yes ○ No If yes, physiological menopause: ○ Yes ○ No age _ _

Hormonal therapy: ○ Yes ○ No Modification of the disease: ○ None ○ Worsening ○ Improvement

Physical activity: Have you ever regularly played a sport? ○ Yes ○ No

If yes, report the two most played sports:

Sport (1): _____ ○ Professional ○ Amateur From age: _ _ to age _ _

Modification of the disease: ○ None ○ Worsening ○ Improvement

Sport (2): _____ ○ Professional ○ Amateur From age: _ _ to age _ _

Modification of the disease: ○ None ○ Worsening ○ Improvement

_____

_____

_____

Physiokinesitherapy (PKT): ○ Yes ○ No

If yes, Duration of PKT treatment: From year: _ _ _ _ to year _ _ _ _

Modification of the disease: ○ None ○ Worsening ○ Improvement

Surgery: ○ Yes ○ No

If yes, operation (1): _____ year: _ _ _ _

Anesthesia: ○ General ○ Local ○ Epidural

Modification of the disease: ○ None ○ Worsening ○ Improvement

If yes, operation (2): _____ year: _____

Anesthesia: ○ General ○ Local ○ Epidural

Modification of the disease: ○ None ○ Worsening ○ Improvement

_____

_____

Family history *(information from at least three generations should be collected)*:

"Was/is any of your relatives wheelchair bound?"

"Did/does any of your relatives have a posture like yours?"

"Was any of your relatives sleeping with half-open eyes?"

Other considerations_____

_____

(pedigree attached)

Part B *NEUROLOGICAL EXAMINATION*

Age at Onset of motor impairment

Subjective age at onset (**when subject has noticed the appearance of motor impairment in his/her daily activities**):_____ years old

Site of muscle weakness reported by patient at onset

Muscle group:

Facial muscles:	○ Yes ○ No	Shoulder girdle muscles:	○ Yes ○ No
Abdominal muscles:	○ Yes ○ No	Distal lower limb muscles:	○ Yes ○ No
Pelvic girdle muscles:	○ Yes ○ No	Distal upper limb muscles:	○ Yes ○ No
Asymmetry at onset:	○ Yes ○ No	If yes, ○ Right ○ Left	
Triggering events	○ Yes ○ No	If yes, event: (1) _____	
		(2) _____	

Objective evaluation of age at onset by specific questions:

Have your relatives never noticed that you were sleeping with half-open eyes? ○ Yes ○ No

If yes, since age _____

Can you drink with a straw? ○ Yes ○ No

If no, since what age have you been unable to drink with a straw? _____

Can you to puff your cheeks? ○ Yes ○ No

If no, since what age have you been unable to puff your cheeks ?_____

Have you always been able to whistle? ○ Yes ○ No

If no, since age_____

Have you noticed the appearance of winged scapula? ○ Yes ○ No

If yes, since age _____

Have you ever noticed thinness of upper arms or a dropped shoulder? ○ Yes ○ No

If yes, since age _____

Have you ever noticed asymmetry of the mouth or smile when looking in a mirror or in past photographs from childhood? ○ Yes ○ No

If yes, since age _____

Other observations:_____

Duration (years) from onset_____

Recurrent/chronic pain: ○ Yes ○ No If yes, since age _ _

Specify location_____

Precocious muscle fatigue during the common daily activities,before the onset of muscle impairment:

○ Yes ○ No If yes, since age _ _

Specify location_____

Other observations ○ Yes ○ No

_____

Face:

Presence of:

Widened palpebral fissures: ..○ Yes ○ No

Puckered lips: ○ Yes ○ No

Horizontal smile: ○ Yes ○ No

Orbiculari oris hypokinesia during speech: ○ Yes ○ No

Dysartria: ○ Yes ○ No

Orbicularis oculi evaluation: ○ Normal (able to close heavily eyes)

○ Partial (able to close eyes but incapable to close heavily eyes)

○ Unable (unable to completely close eyes)

Ability to protrude lips: ○ Normal ○ Partial ○ Unable

Ability to puff out cheeks (against no resistance): ○ Normal ○ Partial ○ Unable

Asymmetric involvement of facial muscle: ○ Yes ○ No

if yes, specify side_____

Scapular girdle:

Ability to abduct arms: ○ Whole (180°)

○ Complete but abnormal (patient can rise arms above head but only by flexing the elbow or using the accessory muscle)

○ Incomplete: >45° but <180° (specify if: ○ ≥90° or ○ <90°)

○ Incomplete: ≤45°

Pelvic girdle:

Ability to climb 4 stairs: ○ Without support

○ Without support but abnormally

○ With support (since age_ _)

○ Unable (since age_ _)

Ability to walk: ○ Without support

○ With support (since age_ _)

○ Unable (since age_ _)

Gait: ○ Normal ○ Waddling ○ Hyperlordotic ○ Steppage

Ability to stand up from a chair: ○ Without support

○ With support (since age _ _)

○ Unable (since age _ _)

Ability to rise from the floor: ○ Without support

○ With support (since age _ _)

○ Unable (since age _ _)

Use of wheelchair: ○ Not necessary ○ With manual control ○ With electric control ○ Bed bound

Legs:

Ability to walk on tiptoes and/or heels: ○ Normal ○ On tiptoes only ○ On heels only ○ Unable

Beevor's sign: ○ Positive ○ Negative

Part C

Medical Research Council (MRC) score:

Scores range from 0 to 5, with .5 increments (e.g. 3, 3.5, 4, 4.5, etc)

MUSCLE	***RIGHT MRC score***	***LEFT MRC score***	***ATROPHY Yes (right or left) /no***
Extrarotator muscles of upper limb*			
Triceps*			
Biceps*			
Common finger extensors*			
Wrist extensors*			
Long fingers flexors*			
Wrist flexors*			
Gluteus maximus			
Iliopsoas			
Biceps femoris			
Quadriceps			
Triceps surae			
Tibialis anterior			

(* *Muscles to be considered for FSHD score "Upper limbs involvement"*)

Strength of neck extensors muscles: MRC score_____

Weakness of pectoralis muscles: ○ Yes ○ No If yes, ○ Right ○ Left

Pectoralis muscles atrophy: ○ Yes ○ No If yes, ○ Right ○ Left

PRESENCE OF FOLLOWING TYPICAL FEATURES:

Scapular winging at rest: ○ Yes ○ No

(if yes, specify: ○ Symmetric winging, or ○ Asymmetric winging ○ > right; ○ > left)

Scapular winging on attempted shoulder abduction or forward flexion: ○ Yes ○ No

(if yes, specify: ○ Symmetric winging, or ○ Asymmetric winging ○ > right; ○ > left)

Horizontal clavicles: ○ Yes ○ No

Forward sloping of shoulders at rest: ○ Yes ○ No

Atrophy of pectoral muscles/ axillary creases: ○ Yes (○ > right; ○ > left) ○ No

Sunken or flattened appearance of the chest: ○ Yes ○ No

"Poly-hill sign" with neck, shoulders, and arms observed from behind in

fullest possible abduction (70–90°), with external rotation of the shoulders : ○ Yes ○ No

Hyperlordosis: ○ Yes ○ No

Orbiculari oris hypokinesia during speech: ○ Yes ○ No

PRESENCE OF UNCOMMON FEATURES:

Myotonic phenomenon: ○ Yes ○ No

Rippling phenomenon: ○ Yes ○ No

Eyelid ptosis: ○ Yes ○ No

Extra-ocular weakness: ○ Yes ○ No

Pharyngeal and lingual muscle weakness (persistent dysphagia): ○ Yes ○ No

Bent syndrome: ○ Yes ○ No

Early contractures: ○ Yes ○ No

(If yes, specify site_____)

Dropped head: ○ Yes ○ No

Pes cavus: ○ Yes ○ No

Myoglobinuria: ○ Yes ○ No

Ogival palatus: ○ Yes ○ No

Others:_____

Creatine phosphokinase (CPK) (value of two blood assays separated by at least one month):

- ○ Normal range
- ○ < 4x normal value (<1000 U/L)
- ○ > 4x normal value (>1000 U/L)

Instrumental evaluation

Cardiac involvement (ECG, echocardiogram):

Last ECG's report_____ (date: _ _/_ _/_ _ _ _)

Last echocardiogram's report_____ (date: _ _/_ _/_ _ _ _)

Electromyographic pattern of four limbs (detail the examinated muscles) (date: _ _/_ _/_ _ _ _)

- ○ Myopathic pattern (○ Proximal ; ○ Distal)
- ○ Neurogenic pattern (○ Proximal ; ○ Distal)
- ○ Mixed pattern (○ Proximal ; ○ Distal)

Electroneurography of four limbs (detail the examinated nerves) (date: _ _/_ _ /_ _ _ _)

- ○ Normal ○ Abnormal

Report of last pulmonary function tests (FVC, MIP, MEP, Cough peak flow) (date_ _/_ _ /_ _ _ _):

_____

_____

Report of muscle biopsy (if available; please specify date and biopsied muscle)*: (date_ _/_ _ /_ _ _ _)

Biopsied muscle: _____

Report: _____

_____

Other genetic test previously performed (if available):_____

_____

*(*please attach reports*)

Name of the Examiner: _____

I - Facial weakness

0 - no weakness

1 - moderate weakness;

partial ability to do at least one of the following tasks:

- to close eyes
- to protrude lips
- to puff out cheeks

2 - severe weakness;

unable to do at least one of the following tasks:

- to close eyes
- to protrude lips
- to puff out cheeks

II - Scapular girdle involvement

0 - no involvement

1 - mild involvement with no limitation of arm abduction

2 - arm abduction > 45°

3 - arm abduction < 45°

III - Upper limbs involvement *

0 - no involvement

1 - at least two muscles affected with MRC >3

2 - at least two muscles with MRC ≤ 3

**The following 4 muscles will be assessed on each side: 1. triceps; 2. biceps; 3. common fnger extensors and wrist extensors; 4. long finger flexors and wrist extensors. Only the weaker muscles will be considered for evaluation.*

IV - Legs involvement

The ability to walk on tiptoes and heels will be assessed on each side:

0 - no involvement

1 - unable to walk on tiptoes or heels (only one task impaired)

2 - unable to walk on tiptoes and heels (two tasks impaired)

V - Pelvic girdle involvement

0 - no involvement

1 - able to walk and to climb stairs without support but abnormally/ because of posterior leg muscle hypotrophy

2 - able to walk unaided, to climb stairs or to stand up from a chair with support

3 - able to walk unaided but unable to stand up from a chair or to climb stairs without support/ more than 12 seconds

4 - able to walk with support

5 - wheelchair bound

VI - Abdominal muscle involvement

0 - no involvement

1 - presence of Beevor's sign

FSHD clinical score:_____

	TYPICAL FEATURES	**UNCOMMON FEATURES**
1. ONSET OF MUSCLE WEAKNESS	☐Facial weakness of orbicularis oculi or oris ☐Scapular weakness with alterated ability to abduct arms ☐Humeral muscles (biceps/triceps)	☐Distal lower limbs onset with triceps surae weakness ☐Distal upper limbs onset ☐Pelvic girdle onset
2. AXIAL MUSCLES INVOLVEMENT	☐Hyperlordosis ☐Beevor's sign	☐Camptocormia ☐Dropped head
3. FACIAL INVOLVEMENT	☐Weakness of Orbicularis oculi (facial score ≥1) ☐Weakness of Orbiculari oris (facial score ≥1)	☐Weakness of extra-ocular muscles ☐Weakness of masticatory muscles (persistent dysphagia)
4. SCAPULAR GIRDLE INVOLVEMENT	☐Impairment of upper limb abduction with winged scapula or limitation of forward flexion (scapular FSHD score ≥1)	☐Isolated distal upper limb muscle weakness ☐Impairment of arms abduction (<90°) without winged scapula at rest and/or on attempted shoulder abduction or forward flexion
5. PELVIC GIRDLE INVOLVEMENT	------	☐Isolated and/or prevailing pelvic girdle muscle weakness
6. LOWER LIMBS INVOLVEMENT	☐Weakness of tibialis anterior muscles weakness	☐Early gastrocnemius and/or soleus atrophy/weakness
7. BLOOD CPK LEVEL (at least two samples 1 month apart)	☐Normal range ☐< 4x normal value (<1000 U/L)	☐Value > 4x normal value (>1000 U/L)
8. OTHER SIGNS	☐Shoulders winging on attempted shoulder abduction or forward flexion ☐Horizontal clavicles ☐Forward sloping of the shoulders at rest ☐Sunken or flattened appearance of the chest ☐Atrophy of pectoralis muscles ☐Orbiculari oris hypokinesia during speech	☐Myotonic phenomenon ☐Rippling ☐Eyelid ptosis ☐Extra-ocular muscle weakness ☐Early muscle contractures ☐Cardiomyopathy ☐Early respiratory insufficiency (Non Invasive Ventilation, NIV; FSHD score <12) ☐Pes cavus ☐Myoglobinuria

Importantly: Indicate the presence of comorbidities / results of previous injuries / illnesses that can possibly affect the neurological examination:_____
Extra-muscular involvement: ☐hearing loss, ☐epilepsy, ☐retinal involvement, ☐cognitive impairment

CATEGORY A

Category A1
Severe facial weakness (unable **both** to close eyes **and** to protrude lips) + impairment of upper limb abduction with winged scapula (scapular FSHD score ≥1) + absence of uncommon features

Category A2
Facial weakness (upper **and** lower facial weakness) + impairment of upper limb abduction with winged scapula (scapular FSHD score ≥1) + absence of uncommon features

Category A3
Facial weakness (upper **or** lower facial weakness) + impairment of upper limb abduction with winged scapula (scapular FSHD score ≥1) + absence of uncommon features

CATEGORY B

Category B1
Impairment of upper limb abduction with winged scapula (scapular FSHD score ≥1), no facial weakness + absence of uncommon features

Category B2
Facial weakness (facial FSHD score ≥1), no impairment of upper limb abduction + absence of uncommon features

CATEGORY C

Category C1
Subject with presence of at least one typical sign + FSHD score =0

Category C2
Subject without signs of muscle weakness + FSHD score =0

CATEGORY D

Category D1
Subject fulfilling criteria of categories A1, A2, A3, B1, B2 + at least one uncommon feature

Category D2
-Subject fulfilling criteria of categories C1 or C2 + at least one uncommon feature
-Subject no fulfilling criteria of any of the above categories

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Annex 6

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ORIGINAL COMMUNICATION

A novel clinical tool to classify facioscapulohumeral muscular dystrophy phenotypes

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Abstract Based on the 7-year experience of the Italian Clinical Network for FSHD, we revised the FSHD clinical form to describe, in a harmonized manner, the phenotypic spectrum observed in FSHD. The new Comprehensive Clinical Evaluation Form (CCEF) defines various clinical categories by the combination of different features. The inter-rater reproducibility of the CCEF was assessed between two examiners using kappa statistics by evaluating 56 subjects carrying the molecular marker used for FSHD diagnosis. The CCEF classifies: (1) subjects presenting facial and scapular girdle muscle weakness typical of FSHD (category A, subcategories A1–A3), (2) subjects with muscle weakness limited to scapular girdle or facial muscles (category B subcategories B1, B2), (3) asymptomatic/healthy subjects (category C, subcategories C1, C2), (4) subjects with myopathic phenotype presenting clinical features not consistent with FSHD canonical phenotype (D, subcategories D1, D2). The inter-rater reliability study showed an excellent concordance of the final four CCEF categories with a κ equal to 0.90; 95 % CI (0.71; 0.97). Absolute agreement was observed for cate-

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gories C and D, an excellent agreement for categories A [$\kappa = 0.88$; 95 % CI (0.75; 1.00)], and a good agreement for categories B [$\kappa = 0.79$; 95 % CI (0.57; 1.00)]. The CCEF supports the harmonized phenotypic classification of patients and families. The categories outlined by the CCEF may assist diagnosis, genetic counseling and natural history studies. Furthermore, the CCEF categories could support selection of patients in randomized clinical trials. This precise categorization might also promote the search of genetic factor(s) contributing to the phenotypic spectrum of disease.

Keywords FSHD · Clinical phenotype · Diagnostic criteria · Disease registry · Disease classification

Introduction

Facioscapulohumeral muscular dystrophy (FSHD) is one of the most common forms of hereditary myopathy [1]. The classical FSHD phenotype is rather distinctive, characterized by a progressive asymmetric facial, shoulder girdle and pectoral muscle weakness and atrophy, with a descending progression to involve the distal lower extremity muscles before affecting the hip girdle muscles [2]. However, a wide variability of clinical expression has been extensively documented [3].

At present, two genetically distinct disease subtypes, FSHD1 and FSHD2 are described. The molecular defect associated with FSHD1 resides in a stretch of tandemly arrayed 3.3 kb repetitive elements, named D4Z4, ranging from 11 to 150 repeat units in healthy subjects [4]. Alleles with 8 or fewer D4Z4 repeats on chromosome 4q have been found in the majority of FSHD patients. FSHD2 patients carry D4Z4 alleles of size at the lower end of the general healthy population range size [5]. In these patients, the disease is associated with heterozygous dominant mutations in the *SMCHD1* gene [6].

However, D4Z4 alleles in the size-range of FSHD1 patients (4–8 units, 20–35 kb EcoRI alleles) are carried by 3 % of healthy control population [7–9]. Thus, a D4Z4 allele of reduced size may be permissive but it is not sufficient to develop autosomal dominant disease. Consistently, in FSHD families, we found that almost 25 % of FSHD heterozygotes older than 55 years were asymptomatic [10]. Moreover, there are families in which the disease appears only in one generation or in a single subject [8, 10] with no other relatives with signs of disease. Besides, several reports describe atypical phenotypes in carriers of a D4Z4 reduced allele (DRA) [11].

Collectively, the extensive use of DNA analysis in FSHD has revealed an unanticipated complexity without a straightforward correlation between the clinical phenotype and molecular variations. Incomplete penetrance and wide clinical variability argue for the role of modifying loci or epigenetic mechanisms influencing the clinical expression of disease. This clinical and genetic variability, which is observed also in other hereditary neuromuscular diseases, represents an obstacle for the interpretation of clinical data, for genotype-phenotype correlations, appropriate genetic counseling and for the definition of a minimal dataset necessary for the stratification of patients eligible for therapeutic trials. Therefore, to formulate optimal diagnostic criteria, molecular analysis must be associated with standardized and harmonized clinical evaluation.

Here, in light of our 7-year experience, we present the FSHD Comprehensive Clinical Evaluation Form (CCEF), a modified version of the original FSHD Clinical Form [12] for the detailed description of all phenotypic features detected in FSHD patients and families.

Methods

Study design

Through the systematic use of the FSHD Clinical Form [10, 12, 13] we recognized that it assesses the severity of motor impairment by translating disability into a number (*FSHD Evaluation Scale*, CCEF Section 2, Supplementary Figure 1), but it does not capture clinical features that may describe various phenotypes. To overcome this limitation, we integrated several items including typical and atypical features on the basis of published reports describing the clinical phenotypes observed in carriers of a DRA (reviewed in [11]). Typical and atypical clinical features were combined in the new CCEF, which includes the *Evaluation Form* (CCEF Section 1, Supplementary Figure 1), the *FSHD Evaluation Scale* (CCEF Section 2, Supplementary Figure 1), the *Clinical Diagnostic Form* (CCEF Section 3, Fig. 1), and the *Clinical Categories* (CCEF Section 4, Fig. 2). The integral CCEF can be downloaded as Supplementary Figure 1 and at http://www.fshd.it. The definition and the validation of the CCEF were performed in two steps. We first recruited 106 subjects carrying a DRA with 1–9 units (11–38 kb) to test the clinical application of this new tool. The recruitment was based on 452 subjects examined by the Italian Clinical Network for FSHD (ICNF) in 2-year time-window (2008–2009). Subjects were summoned by consecutive phone calls following the order of the previous recruitment. We called those near the clinical centers of Modena, Turin and Naples. The latter choice was made to avoid people a long-distance trip. We organized three meetings dividing the 106 available subjects into three groups on the basis of their geographic

	TYPICAL FEATURES	UNCOMMON FEATURES
1. ONSET OF MUSCLE WEAKNESS	□ Facial weakness of orbicularis oculi or oris □ Scapular weakness with alterated ability to abduct arms □ Humeral muscles (biceps/triceps)	□ Distal lower limbs onset with triceps surae weakness □ Distal upper limbs onset □ Pelvic girdle onset
2. AXIAL MUSCLES INVOLVEMENT	□ Hyperlordosis □ Beevor's sign	□ Camptocormia □ Dropped head
3. FACIAL INVOLVEMENT	□ Weakness of Orbicularis oculi (facial score ≥1) □ Weakness of Orbiculari oris (facial score ≥1)	□ Weakness of extra-ocular muscles □ Weakness of masticatory muscles (persistent dysphagia)
4. SCAPULAR GIRDLE INVOLVEMENT	□ Impairment of upper limb abduction with winged scapula or limitation of forward flexion (scapular FSHD score ≥1)	□ Isolated distal upper limb muscle weakness □ Impairment of arms abduction (<90°) without winged scapula at rest and/or on attempted shoulder abduction or forward flexion
5. PELVIC GIRDLE INVOLVEMENT	------	□ Isolated and/or prevailing pelvic girdle muscle weakness
6. LOWER LIMBS INVOLVEMENT	□ Weakness of tibialis anterior muscles weakness	□ Early gastrocnemius and/or soleus atrophy/weakness
7. BLOOD CPK LEVEL (at least two samples 1 month apart)	□ Normal range □ < 4x normal value (<1000 U/L)	□ Value > 4x normal value (>1000 U/L)
8. OTHER SIGNS	□ Shoulders winging on attempted shoulder abduction or forward flexion □ Horizontal clavicles □ Forward sloping of the shoulders at rest □ Sunken or flattened appearance of the chest □ Atrophy of pectoralis muscles □ Orbiculari oris hypokinesia during speech	□ Myotonic phenomenon □ Rippling □ Eyelid ptosis □ Extra-ocular muscle weakness □ Early muscle contractures □ Cardiomyopathy □ Early respiratory insufficiency (Non Invasive Ventilation, NIV; FSHD score <12) □ Pes cavus □ Myoglobinuria

Importantly: Indicate the presence of comorbidities / results of previous injuries / illnesses that can possibly affect the neurological examination:_____

Extra-muscular involvement: □hearing loss, □epilepsy, □retinal involvement, □cognitive impairment

Fig. 1 CCEF Section 3: Clinical Diagnostic Form

location (Northern, Central and Southern Italy). Twelve experienced clinicians of the ICNF were selected according to their geographic location, so that four neurologists examined patients from each one of the three groups. The four selected neurologists used the CCEF to evaluate each subject of a single group independently. The results of this

CATEGORY A

Category A1
Severe facial weakness (unable **both** to close eyes **and** to protrude lips) **+** impairment of upper limb abduction with winged scapula (scapular FSHD score ≥1) **+** absence of uncommon features

Category A2
Facial weakness (upper **and** lower facial weakness) **+** impairment of upper limb abduction with winged scapula (scapular FSHD score ≥1) **+** absence of uncommon features

Category A3
Facial weakness (upper **or** lower facial weakness) **+** impairment of upper limb abduction with winged scapula (scapular FSHD score ≥1) **+** absence of uncommon features

CATEGORY B

Category B1
Impairment of upper limb abduction with winged scapula (scapular FSHD score ≥1), no facial weakness + absence of uncommon features

Category B2
Facial weakness (facial FSHD score ≥1), no impairment of upper limb abduction + absence of uncommon features

CATEGORY C

Category C1
Subject with presence of at least one typical sign + FSHD score =0

Category C2
Subject without signs of muscle weakness **+** FSHD score =0

CATEGORY D

Category D1
Subject fulfilling criteria of categories A1, A2, A3, B1, B2 **+** at least one uncommon feature

Category D2
-Subject fulfilling criteria of categories C1 or C2 + at least one uncommon feature
-Subject no fulfilling criteria of any of the above categories

Fig. 2 CCEF Section 4: Clinical Categories

first round of clinical applications were discussed in a subsequent meeting. We revised the emerged critical points, i.e. some difficulties in establishing mild facial weakness, and approved the final version of the CCEF (Supplementary Figure 1). Then, in a second round, the inter-rater reliability in assigning patients to different phenotypic categories using the new CCEF was tested. Two clinicians, selected by drawing lots, examined

additional 56 subjects (Supplementary Table 1) recruited from the cohort of 452 subjects as described above. The two clinicians administered the functional motor evaluation test of the *Evaluation Form* (Supplementary Figure 1, Section 1, parts b and c) to each subject and calculated the FSHD clinical score on the basis of the *FSHD Evaluation Scale*, previously validated [12]. Then, the two clinicians completed the *Clinical Diagnostic Form* (CCEF Section 3, Fig. 1) and assigned each subjects to one of the nine clinical subcategories (CCEF Section 4, Fig. 2) independently. A tutorial for the clinical assessment is available at http://www.fshd.it. It takes 20 min to collect clinical information and complete the neurologic evaluation.

The subject recruitment was approved by the Ethics Committee of Modena and all the participating centers. Signed informed consent from patients was obtained before inclusion in the study.

Statistical analysis

The inter-observer reproducibility between the two examiners respect to the four and nine CCEF categories was assessed using the kappa statistics [14]. κ value scores are interpreted as follows: κ value 1.0 = perfect agreement; κ value $\geq 0.75 < 1.0$ = excellent; κ value $\geq 0.40 < 0.75$ = good; κ value ≤ 0.40 = poor. The 95 % confidence intervals of kappa statistics were calculated using the (biased corrected) bootstrap resampling method [15].

Results

A tool to describe clinical variability

The CCEF consists of four sections. The first section, the *Evaluation Form* (Section 1, Supplementary Figure 1), investigates the subject's clinical history (part a), evaluates the patient's disability (part b) and assesses muscle segmental involvement using the Medical Research Council (MRC) scale (part c). The other sections include the *FSHD Evaluation Scale* (Section 2, Supplementary Figure 1), the *Clinical Diagnostic Form* (Section 3, Fig. 1) and the *Clinical Categories* (Section 4, Fig. 2).

Several items are examined in the *Evaluation Form* section.

Family history

Questions such as "did/does any of your relatives have a posture like yours?", "was any of your relatives sleeping with half-open eyes?" are asked to identify subjects with possible muscle weakness suggestive of FSHD.

Evaluation of age at onset

To obtain a more objective evaluation of age at onset and the type of muscle initially affected, we introduced specific questions, such as "have your relatives ever noticed that you were sleeping with half-open eyes?", "when have you noticed the appearance of winged scapula?", "have you ever noticed thinness of upper arms or a dropped shoulder?", "have you ever noticed asymmetry of the mouth or smile when looking in a mirror or in past photographs from childhood?".

Functional motor evaluation

For a precise description of the distribution of muscle weakness, the CCEF evaluates: (a) the presence of widened palpebral fissures; *orbicular oris* weakness, horizontal smile; inability to protrude lips, to puff out cheeks, to close eyes and bury the eyelashes (facial weakness); (b) the maximum degree in abducting arms (scapular girdle weakness); (c) the ability to climb 4 stair-steps, to stand up from a chair, to rise from the floor, to walk (pelvic girdle weakness); (d) the ability to walk on tiptoes and/or heels (distal legs weakness); (e) the presence of Beevor's sign (abdominal muscles weakness).

Evaluation of segmental muscle strength by MRC scale

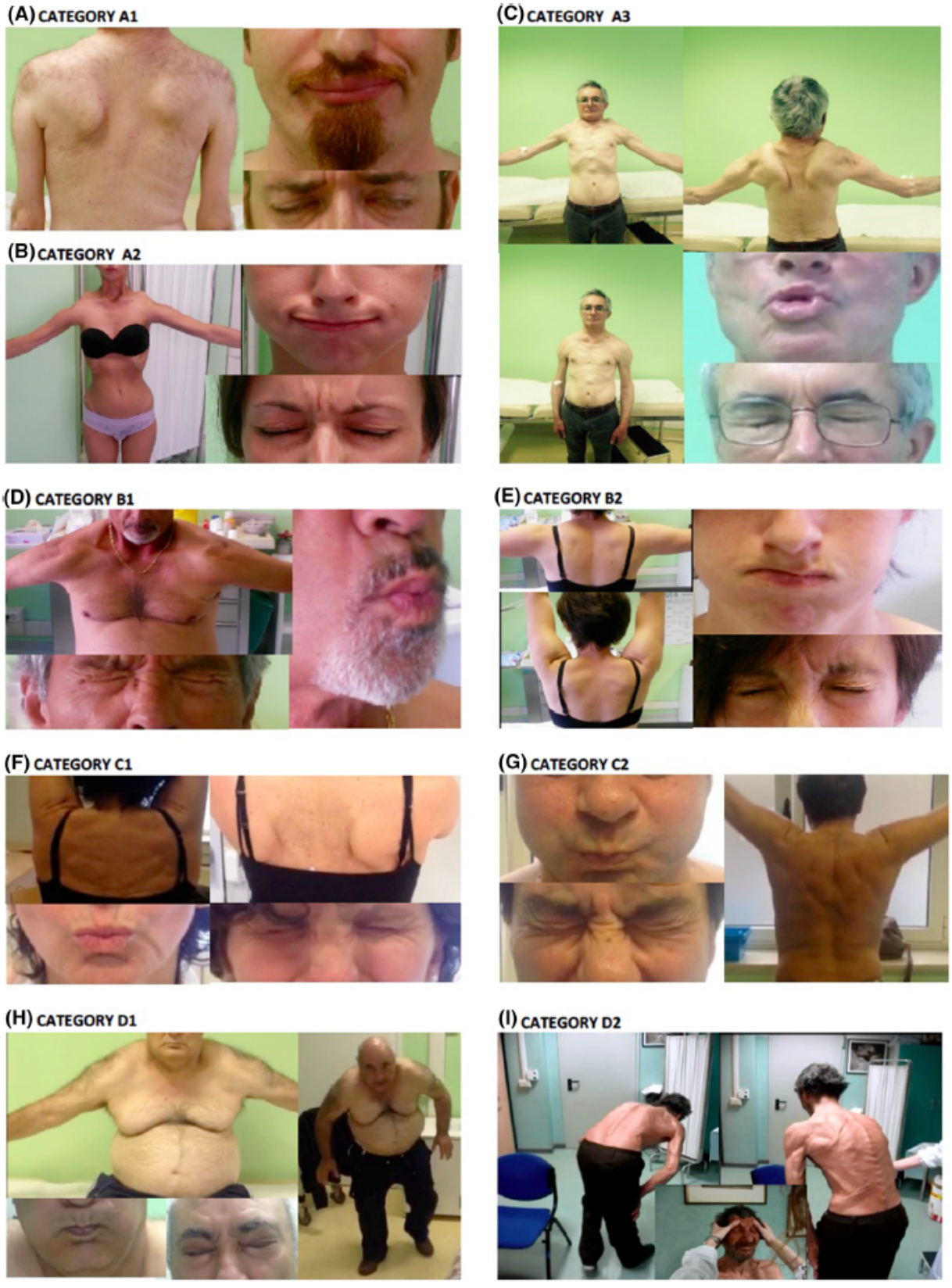
Fourteen muscle groups are examined. Neck extensors are evaluated as single muscle group; external-rotator muscles of upper limb, triceps, biceps, common finger extensors, wrist extensors, long fingers flexors, wrist flexors, gluteus maximum, iliopsoas, quadriceps, biceps femoris, triceps surae, tibialis anterior are evaluated on both sides.

Annotation of typical signs

Shoulders with symmetric/asymmetric winging on attempted shoulder abduction or forward flexion, straight clavicles, forward sloping of shoulders at rest, axillary creases reflecting pectoral muscle wasting, sunken or flattened appearance of the chest, "poly-hill sign" with neck, shoulders and arms observed from behind in fullest possible abduction (70°–90°), with external rotation of the shoulders, hyperlordosis.

Annotation of atypical signs

Palpebral ptosis [2], myotonic phenomenon [16], muscle rippling [17], weakness of extra-ocular [2], masticatory, pharyngeal and lingual muscles [2, 18], bent spine syndrome [19], early contractures [2], *pes cavus* [20], dropped


(A) CATEGORY A1
(B) CATEGORY A2
(C) CATEGORY A3
(D) CATEGORY B1
(E) CATEGORY B2
(F) CATEGORY C1
(G) CATEGORY C2
(H) CATEGORY D1
(I) CATEGORY D2

◀**Fig. 3** Examples of clinical categories: case reports. **a** Category A1: male, 38-year old, showing severe upper and lower facial weakness (unable to close both eyelids completely, puff cheeks and protrude lips), and impairment of upper limb abduction with winged scapula. **b** Category A2: female, 31-year old, with moderate upper (partial ability to close eyes, without the presence of widened palpebral fissures) and lower facial weakness (partial ability to puff out cheeks), impairment of upper limb abduction with winged scapula. **c** Category A3: male, 60-year old, with moderate lower facial weakness (partial ability to protrude lips), impairment of upper limb abduction with winged scapula. **d** Category B1: male, 66-year old, with impairment of upper limb abduction with winged scapula, no facial weakness. **e** Category B2: female, 34-year old, with moderate lower facial weakness (partial ability to puff out cheeks and to protrude lips), no scapular weakness. **f** Category C1: female, 55-year old, presenting asymmetric scapular winging on forward flexion without motor impairment (FSHD score 0). **g** Category C2: male, 56-year old, without motor impairment or other FSHD typical signs of muscle atrophy/weakness (FSHD score 0). **h** Category D1: male, 66-year old: onset after 50 age at shoulder girdle, without facial motor impairment and "bent spine". **i** Category D2: male, 75-year old, with isolated bent spine syndrome, without signs suggestive of FSHD

head, myoglobinuria and persistently high CK values above the level of 1000 U/L are [2] considered atypical signs. The presence of cardiomyopathy and a respiratory restrictive insufficiency at onset or in subjects still walking (FSHD score <12) is also considered an atypical sign [2, 21].

The *Evaluation Form* allows completing the *FSHD Evaluation Scale* to calculate the FSHD clinical score (Section 2, Supplementary Figure 1) [12]. The score considers the regional distribution of muscle weakness and the functionality of: (I) facial muscles (scored from 0 to 2); (II) scapular girdle muscles (scored from 0 to 3); (III) upper limb muscles (scored from 0 to 2); (IV) leg muscles (scored from 0 to 2); (V) pelvic girdle muscles (scored from 0 to 5); and (VI) abdominal muscles (scored from 0 to 1). Overall, the total FSHD score ranges from 0 to 15 and numerically defines the clinical severity of the motor impairment [10, 12, 13].

All sections of CCEF are used for the assessment and the classification of a patient. Based on the distribution of muscle weakness, scored by the *FSHD Evaluation Scale*, and the combination of the clinical features suggestive or not of FSHD, summarized in the *Clinical Diagnostic Form* (CCEF Section 3, Fig. 1), it is possible to assign patients to different phenotypic categories (CCEF Section 4, Fig. 2). In particular, we assigned (1) subjects with typical FSHD presenting facial and scapular girdle muscle weakness in category A; (2) subjects with muscle weakness limited to facial or scapular girdle muscles in category B; (3) asymptomatic subjects without motor impairment in category C; (4) subjects with myopathic phenotype presenting other anomalous clinical features not consistent with FSHD in category D.

Moreover, in view of our experience on FSHD phenotypes accrued through the past years in INRF [10, 13], we further described additional variants within each category (Fig. 2). Patients with typical phenotype were classified in three subcategories (A1–A3), on the basis of the severity of facial involvement, which seems to discriminate some classical phenotypes (Fig. 3a–c). This is because, we observed that some infantile forms or more severe phenotypes [13] are characterized by an early and prominent weakness of *orbicularis oculi* and *oris* with facial diplegia and dysartria. Thus, these patients were defined as category A1 to distinguish them from the vast majority of patients in which we observed a milder facial involvement (categories A2 and A3). This distinction should facilitate the identification of a specific clinical group deserving *ad hoc* studies.

Incomplete FSHD phenotype, not presenting a coexisting involvement of facial and scapular girdle muscles without other uncommon features, are considered category B1 or B2 (Fig. 3d, e). We identified these categories because, for instance, an isolated scapular girdle muscle weakness can be observed in FSHD relatives, but it can be also related to other myopathic disorders or nerve injuries.

Category D comprises myopathic subjects presenting some FSHD features in association with other uncommon characteristics suggestive of a possible comorbidity (D1) or patients that do not fulfill the diagnostic criteria for FSHD and can be affected by an alternative disease (D2) (Fig. 3h, i). Atypical features were chosen based on evidences from the literature [11]. This category may facilitate the discovery of factors that contribute to the disease expression or identify those subjects who are wrongly considered FSHD because of a diagnostic bias due to the random finding of DRA.

Finally, we decided to further differentiate non penetrant carriers: the asymptomatic subjects without motor impairment that present minor signs suggestive of FSHD ("typical features-other signs" Fig. 1) are described as category C1, whereas category C2 includes subjects with a neurologic examination completely normal (Fig. 3f, g). This distinction might be of particular importance for studying the natural history of disease (i.e. subjects described as C1 might develop clinical FSHD later or remain asymptomatic).

Overall, the categories we generated aim at describing different phenotypes thus capturing clinical diversity, regardless of the severity of motor impairment, otherwise reported as FSHD score.

Table 1 Agreement between Observer 1 and Observer 2 with respect to the nine CCEF categories classification

	CCEF categories	Observer 2									
		A1	A2	A3	B1	B2	C1	C2	D1	D2	Total
Observer 1	A1	6	2	0	0	0	0	0	0	0	8
	A2	1	18	2	0	0	0	0	0	0	21
	A3	0	2	4	2	0	0	0	0	0	8
	B1	0	0	1	5	0	0	0	0	0	6
	B2	0	0	0	0	2	0	0	0	0	2
	C1	0	0	0	0	0	2	0	0	0	2
	C2	0	0	0	0	0	1	4	0	0	5
	D1	0	0	0	0	0	0	0	2	0	2
	D2	0	0	0	0	0	0	0	0	2	2
	Total	7	22	7	7	2	3	4	2	2	56

$\kappa = 0.75$; 95 % CI (0.57; 0.87)

Table 2 Agreement between Observer 1 and Observer 2 with respect to the fourth CCEF categories classification

	CCEF categories	Observer 2				
		A	B	C	D	Total
Observer 1	A	35	2	0	0	37
	B	1	7	0	0	8
	C	0	0	7	0	7
	D	0	0	0	4	4
	Total	36	9	7	4	56

$\kappa = 0.90$; 95 % CI (0.71; 0.97)

Inter-rater reliability of phenotype subgroups

The characteristics of the 56 FSHD patients enrolled in the inter-rater reliability study are shown in Supplementary Table 1. The sample is almost balanced by sex, 34 % aged less than 40 years, 12.5 % had an FSHD score higher than 10, all but three carried a DRA with 8 or fewer repeats (p13E−11 EcoRI fragments ≤35 kb).

The concordance between the clinical assessments performed by the two neurologists was evaluated for the nine CCEF categories described in Fig. 2. As shown in Table 1, a good/excellent agreement [$\kappa = 0.75$; 95 % CI (0.57; 0.87)] was observed using the nine CCEF classifications. The overall kappa statistic combines the reliability of the nine categories with a perfect agreement observed for categories B2, C2, D1, D2; a good/excellent agreement for categories A1, A2, B1 and C2, and a good agreement observed for the category A3. The results of the concordance of the final four CCEF categories are presented in Table 2. As expected, the reliability increased with a κ equal to 0.90; 95 % CI (0.71; 0.97). A perfect agreement was observed for categories C and D, an excellent agreement for categories A [$\kappa = 0.88$; 95 % CI (0.75; 1.00)], and a good agreement for categories B [$\kappa = 0.79$; 95 % CI (0.57; 1.00)]. A lower level of κ, when compared with values obtained for each subcategory, is due to the increased number of categories taken into account in the final score and reflects the sensitivity of the test.

Discussion

The recently published Guidelines on FSHD of the American Academy of Neurology [22] represent an attempt toward the formulation of optimal standards of diagnosis and care for patients. In these recent Guidelines on FSHD, a relevant diagnostic significance is attributed to the detection of D4Z4 alleles associated with the 4qA polymorphism regardless of the phenotypic features. However, large-scale genotype-phenotype studies have revealed incomplete penetrance and wide variable expressivity in FSHD [8–11, 23] supporting the role of modifying loci or epigenetic mechanisms influencing the clinical expression of disease [5, 6]. Moreover, the FSHD molecular signature has a frequency of 1.3 % [7], which decreases the specificity of the molecular testing for FSHD. So, in our opinion, diagnosis of FSHD must be supported by the harmonized description of the observed clinical phenotypes and the family history.

Nowadays, studies suggest the role of epigenetic modifiers in FSHD onset and expression, including the level of 4q35 methylation and/or mutations in *SMCHD1* gene [5, 24]. Besides, a vast number of reports describe subjects with peculiar/atypical phenotypes carrying a DRA and suggest that mutations in other genes, i.e. gene associated with other neuromuscular diseases, might contribute to disease phenotype [11]. This genetic heterogeneity requires the harmonized classification of clinical phenotypes among patients and within families to serve clinical practice. In FSHD, intra-familial clinical variability is one of the most relevant challenges affecting clinical practice and genetic

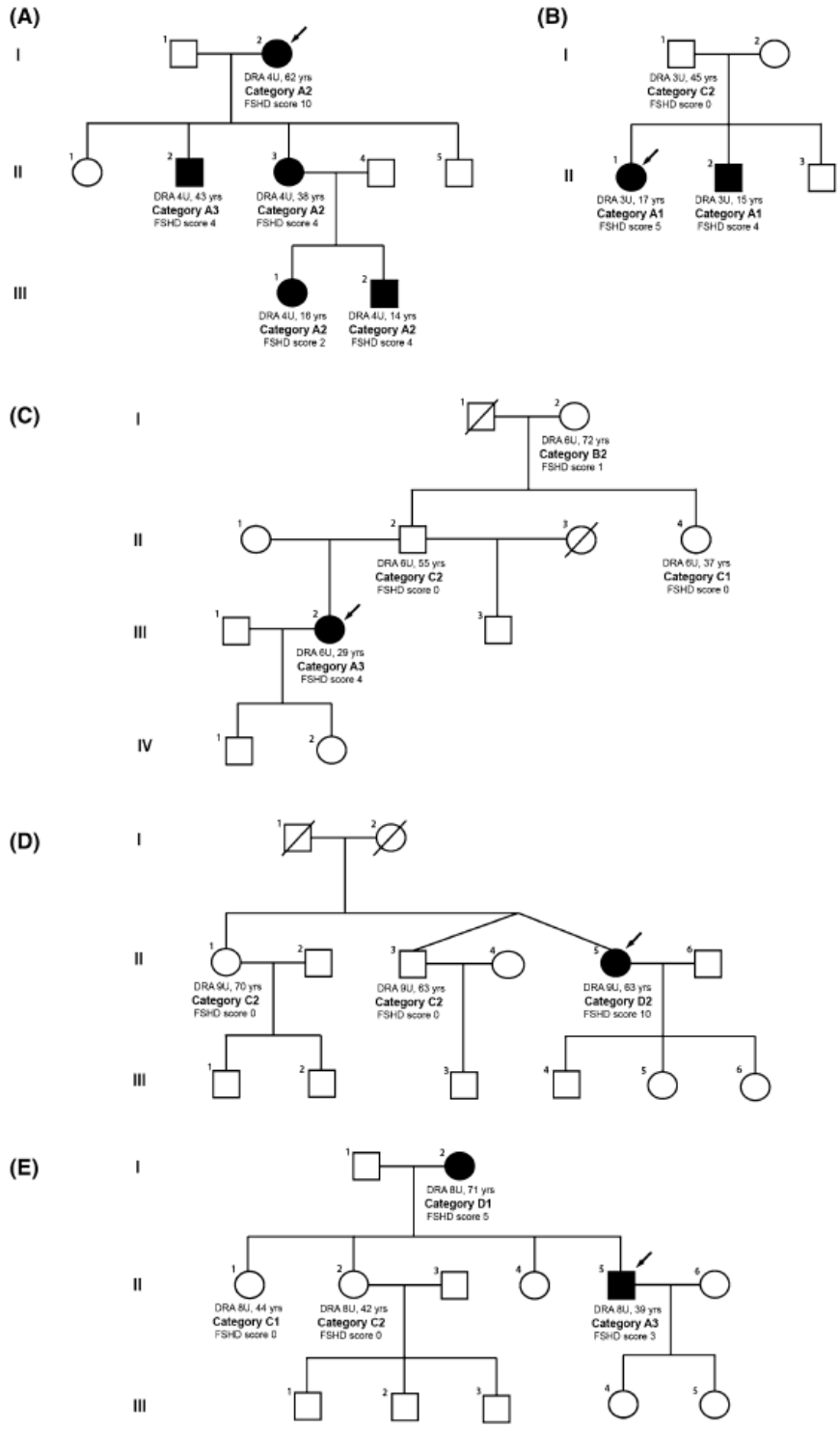


Fig. 4 Clinical characterization of families in which a DRA segregates. Five families are presented. For each subject carrying a 4qA-type DRA on a permissive haplotype, age at evaluation, size of the DRA, clinical category and FSHD score are reported

counseling. Our work shows that the CCEF is an easy clinical tool useful to capture various phenotypes from classic FSHD to individuals with incomplete phenotype, or asymptomatic carriers as well as subjects with atypical signs for which alternative diagnoses may be supposed. The choice of the nine categories responds to the necessity of describing the wide clinical spectrum of FSHD patients and their relatives with a simple and direct approach. Notably, the CCEF collects several items regarding anamnestic data, including onset, disease progression, distribution and degree of motor impairment (measured as the *FSHD Evaluation Scale*).

By applying the CCEF, it will be possible to quickly classify families on the basis of the harmonized description of genotypes and phenotypes. This classification will support genetic counseling taking into account disease penetrance and expression within a single family. Figure 4 shows some examples. Figure 4a displays a family with the canonical autosomal dominant pattern of inheritance. The disease is present in all three generations and all subjects, carrying a DRA, display facial and scapular girdle weakness typical of FSHD, categories A2 and A3. Figure 4b shows a family in which two sibs are severely affected (A1) whereas the father carrying the same 3U DRA (no somatic mosaicism of the DRA was detected) is healthy (C2). Figure 4c presents a four-generation pedigree in which a single 29-year-old subject, III.2, developed mild weakness of *orbicularis oris* and weakness of scapular girdle muscle (category A3). She carries a 6U DRA inherited by her healthy 55-year-old father, II.2 (category C2). The paternal 37-year-old aunt, carrying the 6U DRA, is asymptomatic with non-specific signs as horizontal clavicles and axillary creases (category C1) and the paternal 72-year-old grandmother, I.2, carrying the 6U DRA, presents only incomplete and mild weakness of facial muscle (category B2). Figure 4d describes a family with a single patient presenting severe myopathy with atypical phenotype (D2). The 63-year-old proband carries a DRA with 9 units as do the twin brother and the 70-year-old sister, both healthy (C2). Finally, Fig. 4e displays a family that may mimic an autosomal dominant inheritance. The proband (II.5), carrying a DRA, presents a typical FSHD phenotype (A3). His mother (I.2) carries the same DRA, but she displays an atypical phenotype (D1) without the facial muscle involvement, and with an early and predominant involvement of the pelvic girdle probably related to old age. Instead, his two older sisters (II.1 and II.2) are asymptomatic carriers. In our opinion, all these unexpected distribution of clinical phenotypes require particular attention in evaluating the risk of disease onset and expression, and the possible contribution of genetic modifiers. Indeed, the systematic application of the CCEF might support physicians in the identification of these critical families that might be suitable for further investigations and promote the understanding of disease pathophysiology.

Moreover, using the CCEF, it is possible to obtain the longitudinal trajectory of disease progression for each patient and describe the disease's natural history, including the follow-up of non-manifesting carriers.

Overall, the CCEF is a flexible tool that can assist novel strategies to study the etiology of rare diseases. It can support a catalog of the phenotypes observed among and within families facilitating the phenotypic stratification of FSHD patients, the search of genetic modifiers, and studies on the natural history of disease. Finally, the harmonized clinical classification of subjects is fundamental for the stratification of patients eligible for clinical trials. In this perspective, the CCEF can be an instrument for observational studies or randomized clinical trials.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

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References

1. Lunt PW, Jardine PE, Koch MC et al (1995) Correlation between fragment size at D4F104S1 and age at onset or at wheelchair use, with a possible generational effect, accounts for much phenotypic variation in 4q35-facioscapulohumeral muscular dystrophy (FSHD). Hum Mol Genet 4:951–958
2. Padberg GW, Lunt PW, Koch M, Fardeau M (1991) Diagnostic criteria for facioscapulohumeral muscular dystrophy. Neuromuscul Disord 1:231–234
3. Statland JM, Donlin-Smith CM, Tapscott SJ, Lemmers RJ, van der Maarel SM, Tawil R (2015) Milder phenotype in facioscapulohumeral dystrophy with 7-10 residual D4Z4 repeats. Neurology 85:2147–2150

4. Wijmenga C, Hewitt JE, Sandkuijl LA et al (1992) Chromosome 4q DNA rearrangements associated with facioscapulohumeral muscular dystrophy. Nat Genet 2:26–30
5. Lemmers RJ, Goeman JJ, van der Vliet PJ et al (2015) Inter-individual differences in CpG methylation at D4Z4 correlate with clinical variability in FSHD1 and FSHD2. Hum Mol Genet 24:659–669
6. Lemmers RJ, Tawil R, Petek LM et al (2012) Digenic inheritance of an SMCHD1 mutation and an FSHD-permissive D4Z4 allele causes facioscapulohumeral muscular dystrophy type 2. Nat Genet 44:1370–1374
7. Scionti I, Greco F, Ricci G et al (2012) Large-scale population analysis challenges the current criteria for the molecular diagnosis of fascioscapulohumeral muscular dystrophy. Am J Hum Genet 90:628–635
8. Goto K, Nishino I, Hayashi YK (2004) Very low penetrance in 85 Japanese families with facioscapulohumeral muscular dystrophy 1A. J Med Genet 41:e12
9. Sakellariou P, Kekou K, Fryssira H et al (2012) Mutation spectrum and phenotypic manifestation in FSHD Greek patients. Neuromuscul Disord 22:339–349
10. Ricci G, Scionti I, Sera F et al (2013) Large scale genotype-phenotype analyses indicate that novel prognostic tools are required for families with facioscapulohumeral muscular dystrophy. Brain 136:3408–3417
11. Ricci G, Zatz M, Tupler R (2014) Facioscapulohumeral muscular dystrophy: more complex than it appears. Curr Mol Med 14:1052–1068
12. Lamperti C, Fabbri G, Vercelli L et al (2010) A standardized clinical evaluation of patients affected by facioscapulohumeral muscular dystrophy: the FSHD clinical score. Muscle Nerve 42:213–217
13. Nikolic A, Ricci G, Sera F et al (2016) Clinical expression of facioscapulohumeral muscular dystrophy in carriers of 1-3 D4Z4 reduced alleles: experience of the FSHD Italian National Registry. BMJ Open 6(1):e007798
14. Fleiss JL (1981) Statistical Methods for Rates and proportions, 2nd edn. Wiley, New York
15. Lee J, Fung KP (1993) Confidence interval of the kappa coefficient by bootstrap resampling [letter]. Psychiatry Res 49:97–98
16. Masciullo M, Iannaccone E, Bianchi ML et al (2013) Myotonic dystrophy type 1 and de novo FSHD mutation double trouble: a clinical and muscle MRI study. Neuromuscul Disord 23:427–431
17. Ricci G, Scionti I, Alì G et al (2012) Rippling muscle disease and facioscapulohumeral dystrophy-like phenotype in a patient carrying a heterozygous CAV3 T78M mutation and a D4Z4 partial deletion: further evidence for "double trouble" overlapping syndromes. Neuromuscul Disord 22:534–540
18. Wohlgemuth M, de Swart BJ, Kalf JG et al (2006) Dysphagia in facioscapulohumeral muscular dystrophy. Neurology 66:1926–1928
19. Ghosh PS, Milone M (2015) Camptocormia as presenting manifestation of a spectrum of myopathic disorders. Muscle Nerve 52:1008–1012
20. Schreiber O, Schneiderat P, Kress W et al (2013) Facioscapulohumeral muscular dystrophy and Charcot-Marie-Tooth neuropathy 1A—evidence for "double trouble" overlapping syndromes. BMC Med Genet 14:92
21. Stübgen JP, Schultz C (2009) Lung and respiratory muscle function in facioscapulohumeral muscular dystrophy. Muscle Nerve 39:729–734
22. Tawil R, Kissel JT, Heatwole C et al (2015) Evidence-based guideline summary: evaluation, diagnosis, and management of facioscapulohumeral muscular dystrophy. Neurology 85:357–364
23. Salort-Campana E, Nguyen K, Bernard R et al (2015) Low penetrance in facioscapulohumeral muscular dystrophy type 1 with large pathological D4Z4 alleles: a cross-sectional multicenter study. Orphanet J Rare Dis 10:2
24. Sacconi S, Lemmers RJ, Balog J et al (2013) The FSHD2 gene SMCHD1 is a modifier of disease severity in families affected by FSHD1. Am J Hum Genet 93:744–751