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INVESTIGATION OF GENETIC AND CLINICAL COMPLEXITY OF FACIOSCAPULOHUMERAL DYSTROPHY: EXPERIENCE OF THE FSHD ITALIAN NATIONAL REGISTRY

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Summary and aim of the thesis

FSHD, an autosomal dominant disease, has been associated with reduced numbers (<11, alleles ≤41 kb) of D4Z4 repeats at 4q35. However, in the our previuos genetic studies, we observed that 3% of healthy individuals carry D4Z4 reduced alleles (DRA). Moreover, through a large genotype-phenotype study on a cohort of FSHD patients and families, we confirmed that DRAs correspond to several phenotypes with variable severity of the disease. We, also, observed that penetrance is incomplete and that the pattern of inheritance is not always autosomal dominant.

The aim of the present study is to test the predictive significance of molecular variations in FSHD.

For this, an accurate clinical research has been conducted in collaboration with the Italian Clinical Network for Facioscapulohmeral Muscular Dystrophy (FSHD). A detailed clinical characterization of probands and relatives, the analysis of disease penetrance and the study of inheritance mode in FSHD families from the Italian National Registry for FSHD (INRF) has been personally performed.

Initially we investigated patients with 1-3 D4Z4 repeats, which represent the lower extreme of the molecular diagnostic spectrum with the most relavant clinical expression. Notably, our detailed analysis highlighted clinical variability also in this subgroup of patients.

These observations have emphasized the concept that the molecular mechanisms leading to disease are still not clear and that the molecular markers proposed for FSHD diagnosis are not predictive of disease precense and/or severity.

To dissect these clinical complexity, we designed a new Comprehensive Clinical Evaluation Form (CCEF), a clinical tool useful in describing in a 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).

Through the use of CCEF we decided to reevaluate the clinical spectrum of 154 proband and 306 relatives carriers of 33-35 kb (7-8 rps) D4Z4 reduced alleles, which represent the higher extreme of the molecular diagnostic spectrum with a wide disease variability This study showed two main aspects: first, the majority of probands carrying 33-35 DRA (53%) had moderate phenotype with partial or mild facial involvement; in contrast about 40% showed a

prevalent shoulder girdle impairment or atypical phenotype. Therefore this clinical variability could be confounding for the differential diagnosis between the FSHD and a myopathy presenting with FHSD-like features.

Second, the majority of relatives were non penetrant carriers. This result highlighted the necessity of additional parameters to evaluate the risk to develop the desease in relatives and the importance of an accurate genetic counselling.

In conclusion in dissecting FSHD clinical complexity it is mandatory to associate clinical scales for the evaluation of the disability degree and a tool for the precise phenotypic classification.

Publications related to the thesis

Large scale population analysis challenges the current criteria for the molecular diagnosis of fascioscapulohumeral muscular dystrophy

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. [Am J Hum Genet. 2012]*

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

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. [Brain. 2013]*

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

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. [BMJ Open. 2016]*

A novel clinical tool to classify facioscapulohumeral muscular dystrophy phenotypes."

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. [J Neurol. 2016]*

Clinical expression of facioscapulohumeral muscular dystrophy in carriers of 33-35 kb D4Z4 reduced alleles: experience of the Italian National Registry for FSHD.

Ruggiero L*, Mele F , Ricci G, Vercelli L, Govi M; Nikolic A, Louise M, Sera F, Bruzzese D, Berardinelli A, Angelini C; Antonini G, Bucci E, Filosto M, Cao M, Giardina E, Pegoraro E, Di Muzio A, Telese R, Maggi L, Portaro S, Rodolico C, Villa L, Mongini T, Siciliano G, Tomelleri G, D'Angelo G, Maioli MA, Moggio M, Santoro L, Rossella Tupler. [To be submitted]*

SECTION 1: introduction

1 CLINICAL FEATURES

FSHD (OMIM #158900) is the third most common form of hereditary myopathy with a prevalence of 1 in 20.000 [Mostacciuolo et al, 2009]. 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, 1862]. Thereafter, Duchenne described the disease in the famous series of papers in Archives of General Medicine in 1869, which are often cited as the earliest reference of FSHD[Duchenne, 1869]. In 1885, Landouzy and Dejerine described in detail the clinical features of FSHD, which was also called "Landouzy-Dejerine form of muscular dystrophy". FSHD was characterized by initial progressive facial, shoulder girdle and pectoral muscle weakness and atrophy, followed by the involvement of abdominal muscles with lumbar hyperlordosis and anterior leg muscles with steppage gait [Landouzy et al, 1885]. Subsequently, in 1982, the thesis of Padberg provided the first modern clinical description of FSHD families. Padberg inves

tigated a group of 107 subjects from 19 families, including 73 subjects displaying clinical signs of FSHD, none of his patients had pelvic girdle or calf muscle weakness. This study provided the first evidence for wide clinical variability in FSHD patients, even within the same family [Padberg, 1982].

In 1991 an International Consortium established the clinical, laboratory and genetic criteria for FSHD diagnosis. 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
2) facial weakness in more than 50% of the affected family members
3) autosomal dominant inheritance in familial cases
4) evidence of myopathic disease in EMG and muscle biopsy in at least one affected member of a family

By contrast were considered suggestive of alternative diagnosis the:

1) involvement of extra-ocular, masticatory, pharyngeal and lingual muscles
2) Onset in pelvic girdle muscles

3) regression of symptoms and signs
4) presence of severe and diffuse contractures
5) involvement of myocardium with presence of cardiomyopathy
6) persistently high CK values above five times the upper limit

It was reported that the clinically recognizable age of onset is often very variable. However, the mean age of recognizable onset, at least by clinical examination, was in the second decade. The involvement of facial muscles was considered necessary for diagnosis. The FSHD phenotype (figure 1) is characterized by muscle weakness starting with the facial district followed by the progressive involvement of scapular fixator, humeral, truncal and lower extremity muscles, 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 [Awerbuch et al., 1990]. A notable distinctive feature of FSHD is that muscle weakness displays asymmetric distribution [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 be normal or can show muscles with abnormalities on T1-weighted MRI sequences, corresponding to areas of fatty fibrous replacement, or with areas characterized by increased signal on T2- short inversion recovery (T2-STIR) sequences, reflecting an increase in tissue water content, due to muscle oedema, without fat replacement [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, FSHD showed a fully penetrant autosomal dominant pattern with age-dependent penetrance estimated to be >95% by age 20. In the symptomatic cases, the disease is progressive, tough the rate of progression is variable in the majority of cases. Rarely, there can be long periods of apparent arrest of progression [Lunt et al., 1989].

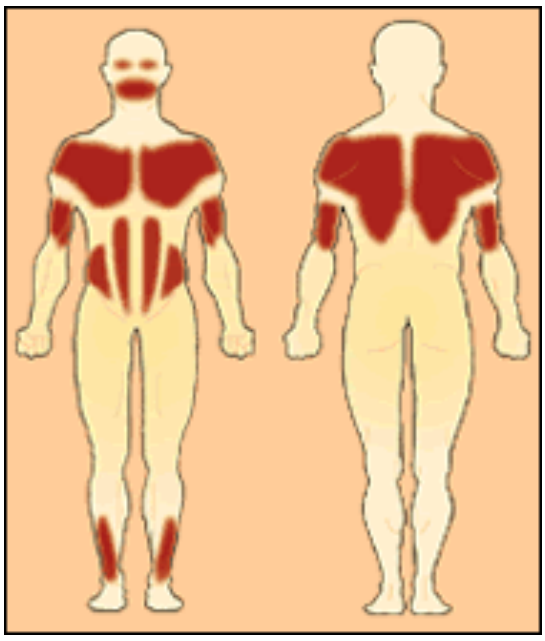


Figure 1: **Schematic representation of the FSHD phenotype**.

2 THE DISCOVERY OF DNA ALTERATIONS

An International Consortium was organized for FSHD linkage analysis. At the first meeting of the Consortium, in 1988, an initial exclusion map for FSHD was constructed [Sarfarazi et al, 1989; Jacobsen et al, 1990]. In 1990 the FSHD gene was assigned to chromosome 4 by positional mapping in 10 Dutch families [Wijmenga et al, 1990]; the confirmation of this location was performed in other families and with additional probes [Upadhyaya et al, 1990, 1991]. Wijmenga reported that a Variable Number Tandem Repeat structure (VNTR) locus, was the most closely linked to FSHD and established the location of the FSHD gene in the subtelomeric region of chromosome 4q [Wijmenga et al., 1991]. Later, Wijmenga 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 [Wijmenga et al., 1992]. 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 over-representation of fragments smaller than 28 kb. 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 [Wijmenga et al., 1992].

Based on restriction fragment mapping and DNA sequencing, van Deutekom and coworkers confirmed that the rearrangements associated with FSHD result in deletion of integral number of repeat KpnI fragments, designated D4Z4 [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, Deidda et al., 1996] (figure 2).

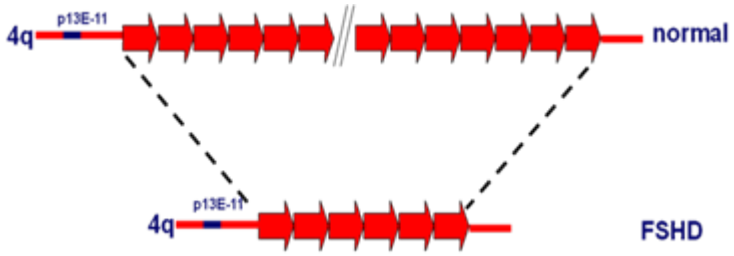


Figure 2: Deletion of a defined number of D4Z4 units on chromosome 4 in FSHD patients. Schematic representation of the D4Z4 repeats on chromosome 4 of a normal individual and a FSHD patient. Having less than 11 D4Z4 units is considered pathological.

2.1 Size of D4Z4 allele and clinical expression

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

In 1995, Lunt and coworkers [Lunt et al., 1995a] reported the genotype/phenotipe analysis on 14 FSHD families (carriers 19-30 kb DRA) and 25 clinically isolated cases (carriers 13-24 kb DRA). The study revealed a clear correlation between smaller fragment sizes and earlier age at onset. The median age at onset in sporadic cases resulted 6.9 years (range <1-16 years) and 18 years (range 8-23) in familial cases. Interestingly, the authors also observed within the families a anticipation of age onset. However, it was hypothesized that this trend might be more a reflection of ascertainment bias than a biological anticipation.

The authors proposed that FSHD families could be divided broadly into three groups:

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 of 19-30 kb;

iii) small families, often with a later onset presentation (median 15-23 years), or scapulohumeral presentation, in which a the 4q35-cosegregating fragment is of 30-38 kb size.

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

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 mild course of the disease, while those who had the smaller ones were more severely affected. However, when genders were 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 (9-11) and 102 healthy controls, in order to identify the molecular diagnostic cut-off point between FSHD cases and the control population. The results indicated that there was not a definite diagnostic cut-off point separating FSHD, FSHD-like myopathies and healthy controls. Therefore, the authors suggested the D4Z4 cut off of 8 repeats [Butz et al., 2003] In a more recent study of Statland and coworkers [Statland et al., 2015] the group of patients with milder phenotype seems to be expandinged, including also 7-8 DRA carriers.

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, 4-8 D4Z4 repeats are associated with the classical form of FSHD, and 9-10 D4Z4 repeats with a milder disease [Lunt et al., 1995b; 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].

3 *MOLECULAR BASIS OF FSHD: PATHOGENETIC HYPOTHESIS*

The D4Z4 units are members of a large family of 3.3 kb tandem repeat loci that are located on the short arm of the acrocentric chromosomes, the pericentromeric regions (especially on

chromosome 1), and the telomeric regions of the long arms of chromosomes 4 and 10 [Hewitt et al, 1994; Lyle et al, 1995].

The organization of the D4Z4 repeat is rather unusual (Figure 3), in particular the presence of two homeobox sequences within the same open reading frame. Homeobox genes, encoding homeodomain transcription factors, often play important roles in embryonic development.

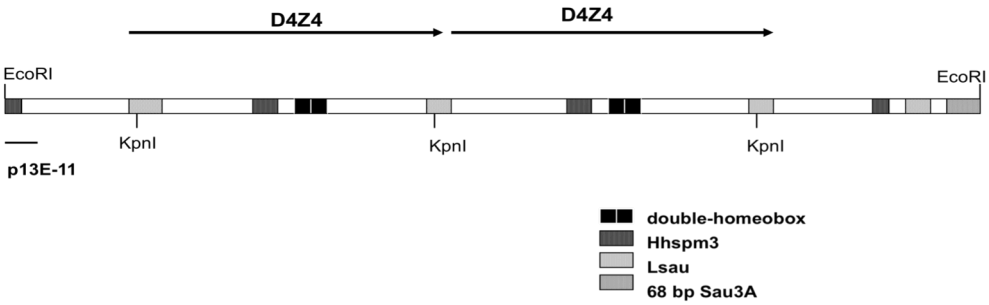


Figure 3: Schematic representation of the structure and the organization of the D4Z4 repeats. Each D4Z4 unit has LSau motifs, the hhspm3 motif and two homeobox sequences.

3.1 The role of DUX4 gene

Each D4Z4 unit contains a putative promoter and a single open redaing frame (ORF) encoding a putative double homeobox gene, named DUX4 (figure 4)[Gabriels et al, 1999; Hewitt et al, 1994; Lyle et al, 1995]. Homeodomain proteins are important for many early and late developmental processes. Thus, DUX4 is considered a strong candidate for FSHD pathogenesis. It was hypothesized that partial deletion of the D4Z4 repeat array resulted in destabilization of the D4Z4 heterochromatin and in the inappropriate upregulation of DUX4 [Hewitt et al, 1994; Gabriels et al, 1999]. DUX4 overexpression may induce cell death by apoptosis, induce caspase 3/7 activation and alter emerin distribution at the nuclear envelope. In addition, DUX4 overexpression may activate PITX1 (paired-like homeodomain transcription factor 1), as was determined for both a reporter gene fused to the Pitx1 promoter and the endogenous Pitx1 gene. Interestingly, upregulation of the PITX1 protein was also observed in muscle biopsies of patients with FSHD [Kowaljow et al, 2007; Dixit et al, 2007]. Nevertheless, for a long time, the functionality of the DUX4 gene was questioned, because of lack of introns and polyadenylation signals and absence of evidence for in vivo transcription [Hewitt et al, 1994; Gabriels et al, 1999; Winokur et al, 2003; Osborne et al, 2007; Alexiadis et al, 2007]. Instead, in the following years, D4Z4 homologues have been identified in several mammalian species and it was established that the DUX4 open reading frame (ORF) shows evolutionary conservation, disputing the non-functionality of DUX4 and suggesting a

coding role, possibly during development. However, the study by Lemmers and coworkers [Lemmers et al. 2010] has suggested a new developmental model for the disease, reporting the requirement of *DUX4* polyadenylation site for develop FSHD. The distal end of the repeat array and flanking pLAM1 sequences, which contains a poly(A) signal that presumably stabilizes this transcript, are thought to be crucially important for the development of FSHD.

More detailed analysis of DUX4 expression shows that the DUX4 pre-mRNA can be alternatively spliced and it has been suggested that the FSHD muscle expresses a different splice form of DUX4 mRNA compared to control muscle [Snider et al, 2010], However, Jones and coworkers [Jones et al., 2012] observed the DUX4 mRNA and protein expression also in muscle biopsies and myogenic cells from genetically unaffected relatives of the FSHD patients Recently, transgenic mouse models carrying human genomic constructs with the FSHD subtelomeric region permissive for somatic DUX4 expression were generated [Krom et al, 2013]. Data suggest that these mice maintain the transcriptional profile of the DUX4 retrogene as observed in FSHD patients and controls. However they do not show an obvious muscle pathological phenotype. Therefore the role of DUX4 in muscle disease still needs to be clarified.

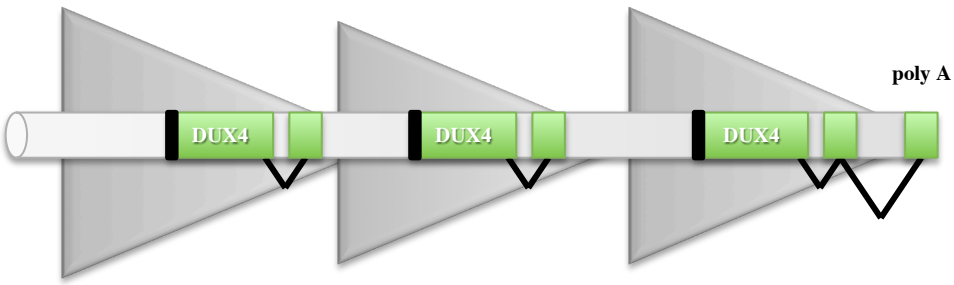


Figure 4: Localization of the DUX4 gene within each D4Z4 unit. On the permissive chromosomes the last copy of the DUX4 gene spices to the third region immediately flanking and stabilizing the transcript owing to the presence of the poly(A) signal (PAS).

3.2 *Expression of proximal genes controlled by D4Z4*

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 (figure 5).

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., 1996a; van Koningsbruggen et al., 2007; Bodega et al., 2009].

ii) FSHD-related gene 2 (*FRG2*), a predicted transcript with no significant homology to any known protein [Rijkers T et al, 2004];

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].

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 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 [Gabellini et al 2006]. 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].

Additional genes located at 4q35 were found to be transcriptionally upregulated in FSHD muscle. FAT1 gene is located 3.6 Mb from the D4Z4 repeat array on 4q35. FAT1, protocadherin gene in mouse is required in migrating muscle precursors and altered muscle shapes caused by Fat1 mutations are predictive of early onset defects in muscle integrity in adult mutants. The topography of muscle abnormalities caused by Fat1 loss-of-function resembles that of human patients with FSHD. Muscle-specific reduction of FAT1 expression and promoter silencing was observed in rare cases of biopsies from foetuses with a prenatal diagnosis of FSHD1 [Caruso et al, 2013].

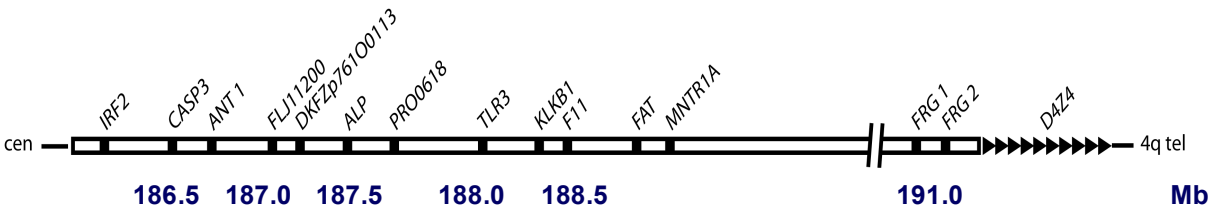


Figure 5: Schematic representation of 4q35 showing physical distances between the genes (Gabellini et al., 2002).

4 CLINICAL COMPLEXITY

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].

4.1 Atypical phenotypes associated with D4Z4 reduced alleles

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 the patients 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 [van der Kooi et al. 2000].

In the same year, Felice and coworkers 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 et al., 2000].

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 underwent 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 [Felice and Moore in 2001].

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 single 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 [Krasnianski et al. 2003]. In the same paper, 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) [Krasnianski et al., 2003].

Cardiac involvement, including hypertrophic cardiomyopathy, conduction defects and arrhythmia, has been reported in subjects carrying a DRA by several reports [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. 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 lower limb muscle involvement typical of FSHD [Reilich et al.; 2010].

Another several atypical phenotype associated with DRA is 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., 2002] was 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. described the case of a 65-years-old man complaining of low 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 of them slight weakness of foot extensors was observed. Nonetheless, none in the family presented a typical FHSD phenotype [Kottlors et al., 2010]. 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 paraspinal of thoracic and lumbar tract together with hamstrings [Jordan et al.; 2011].

Some researchers think 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. 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.

4.2 *Several reports of "double trouble" conditions in FSHD families*

In FSHD, more than in other neuromuscular disorders, several patients affected by "double trouble" conditions 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.

4.3 *Penetrance of the 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 [De Greef et al, 2010], challenging the notion that DRA alone can cause nearly full disease penetrance.

In a 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, 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 [Tonini et al. 2004].

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%.

4.4 FSHD without D4Z4 repeats contraction on chromosome 4q35 (FSHD2)

The cohort of patients, termed "FSHD2" display D4Z4 alleles of normal size on both 4q alleles and they fulfill clinical diagnostic criteria for FSHD [Tawil et al, 2010], resulting clinically indistinguishable from FSHD patients carrying D4Z4 reduced allele (also defined FSHD1).

In 2010 de Greef and coworkers have performed a cross-sectional study on 33 patients with FSHD2 from 27 families, the largest cohort described to date. The clinical presentation of FSHD2 patients appeared identical to the FSHD1. Out of 33 FSHD2 patients 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 reported 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. The most interesting difference between FSHD1 and FSHD2 is the inheritance mode. In fact, the analysis showed that the majority (20/33, 67%) of cases was sporadic, 11 were familial, and 2 had an uncertain inheritance pattern, 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 epigenetic and molecular mechanisms, also supposed to be pathogenic in FSHD associated with D4Z4 repeat contraction, are involved.

5 GENETIC COMPLEXITY

5.1 *Specific haplotypes associated with D4Z4 reduced alleles*

Since there are individuals with reduced D4Z4 alleles 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 segment of 10 kb directly distal to D4Z4 and presenting in two allelic forms, 4qA and 4qB, was identified [van Geel et al, 2002](Figure 6). Although, both alleles are equally common in the general population, it was reported that FSHD is solely associated with the 4qA allele. Lemmers and coworkers, in 2002, analyzed 80 healthy controls and 80 unrelated individuals with FSHD for the presence of 4qA and 4qB alleles. In the controls they observed almost equal frequencies of 4qA and 4qB alleles (42% and 58% respectively) on chromosome 4, but only alleles of the 4qA type on chromosome 10. By contrast, in the 80 unrelated individuals with FSHD (44 *de novo* cases and 36 unrelated familial cases) they detected D4Z4 reduced alleles exclusively in chromosomes 4 bearing the 4qA allele, and never in those with the 4qB allele [Lemmers et al, 2002]. Subsequently, [Lemmers et al, 2004a] described three families with FSHD in which each proband of the family carried two FSHD-sized alleles and was heterozygous for the 4qA/4qB polymorphism. Segregation analysis demonstrated that FSHD-sized 4qB alleles were not associated with disease, since these were present in unaffected family members. Thus, the authors supposed that, in addition to a contraction of D4Z4, other cis-acting elements on 4qA might be necessary for the development of FSHD. Alternatively, they proposed that elements present at 4qB sub-telomeres might prevent pathogenesis of FSHD. In 2007, the identification of additional sequence variations in a relatively stable Simple Sequence Length Polymorphisms (SSLP) proximal to the D4Z4 repeat were identified in the FSHD locus [Lemmers et al, 2007]. On the basis of the proximal SSLP, it was possible to distingush at least 17 genetically distinct sub-telomeric variants of chromosome 4 and 8 sub-telomeric variants of chromosome 10. It was supposed that only D4Z4 contractions in three specific sub-telomeric variants on chromosome 4, the common 4A161 and the rarer 4A159 and 4A168, are responsible for developing FSHD. Contractions in other 4q sub-telomeres were not associated with the disease. The authors presented the pedigrees of two FSHD families in which two different D4Z4 reduced allele segregate, reporting that subjects carrying the D4Z4 reduced allele with non-permissive 4qA166 haplotype did not manifest signs of disease.

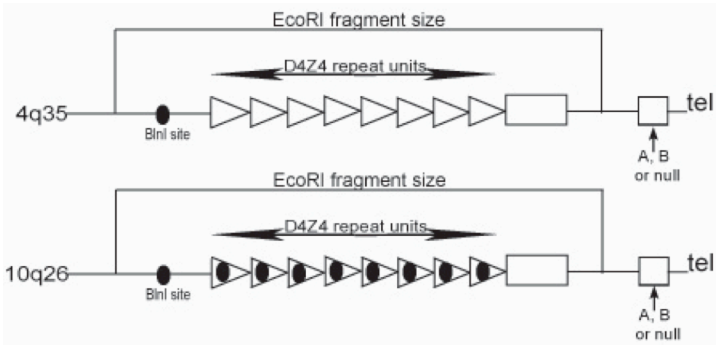


Figure 6: Schematic representation of 4qA/4qB polymorphism.

Finally, it has been suggested that FSHD patients carry specific single nucleotide polymorphism (SNP) ATTAAA in the chromosomal region distal to the last D4Z4 repeat in the pLAM sequence of the 4qA alleles, and this SNP provides a PolyAdenylation Signal (PAS) [Lemmers et al, 2010]. Thus, the specific molecular signature, named 4A(159,161,168)PAS, has been proposed to define genetic background responsible for FSHD pathogenesis (Figure 7). This specific background is characterized by (1) reduction of D4Z4 elements associated with (2) the 4qA (161/159/168) haplotype (3) and a single nucleotide polymorphism, ATTAAA, in the pLAM sequence (figure 7).

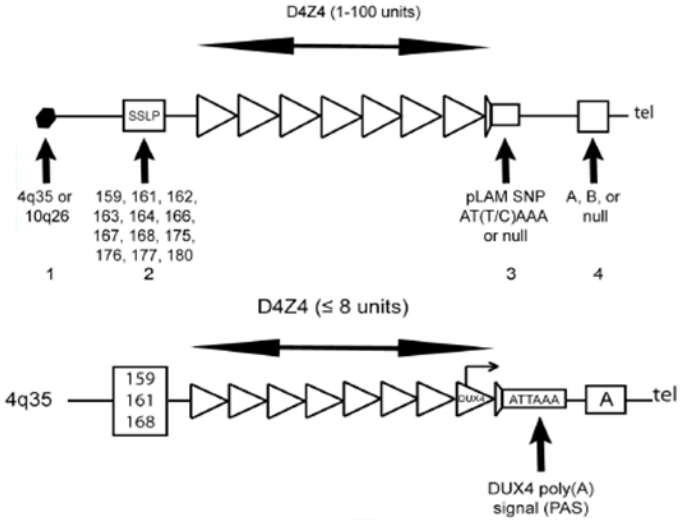


Figure 7: Schematic representation of polymorphisms at the 4q and 10q subtelomeres. a) The D4Z4 repeat array within the subtelomere of chromosomes 4q and 10q varies in size between 1 and 100 D4Z4 units (3.3–330 kb) and is indicated with triangles. Elements that distinguish subjects include: 1) The chromosomal localization of the D4Z4 repeat, chromosome 4q35 or 10q26. 2) The Simple Sequence Length Polymorphism (SSLP), a combination of five Variable Number Tandem Repeats, an 8 bp insertion/deletion, and two SNPs localized 3.5 kb proximal to D4Z4 and varies in length between 159 and 180 bp. 3) Single nucleotide polymorphism AT(T/C)AAA (SNP) in the pLAM region. 4) A large sequence variation (termed 4qA or B) that is distal to D4Z4. 4q chromosomes which do not hybridize to probes for A and B are termed "null" and their sequences vary from case to case. **b)** Schematic representation of the permissive chromosomal background. The ATTAAA variant creates a polyadenylation signal that stabilizes the DUX4 transcript and has been postulated to be the critical factor causing FSHD.

5.2 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 patient 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 [De Greef et al., 2009].. 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. However, 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. The limitation of this test is due to the 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 the 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 showed 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.
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 was 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 non manifesting 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.

5.3 *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 (figure 8). They then confirmed 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 [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% of 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%. The authors 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]. In addition, Sacconi and coworkers hypothesized that SMCHD1 may act as a genetic modifier in FSHD Sacconi et al., 2013]. The authors describe three unrelated individuals with FSHD1 presenting an unusual high clinical severity based on their upper-sized FSHD1 repeat array of nine units. Each of these individuals also carries a mutation in

the SMCHD1 gene. Familial carriers of the FSHD1 allele without the SMCHD1 mutation were only mildly affected, suggesting a modifier effect of the SMCHD1 mutation [Sacconi et al., 2013].

5.4 *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; Daxinger et al., 2015]. The D4Z4 methylation is used as a measure of D4Z4 chromatin relaxation. In particular, it has been considered an 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. 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 equally on the 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.

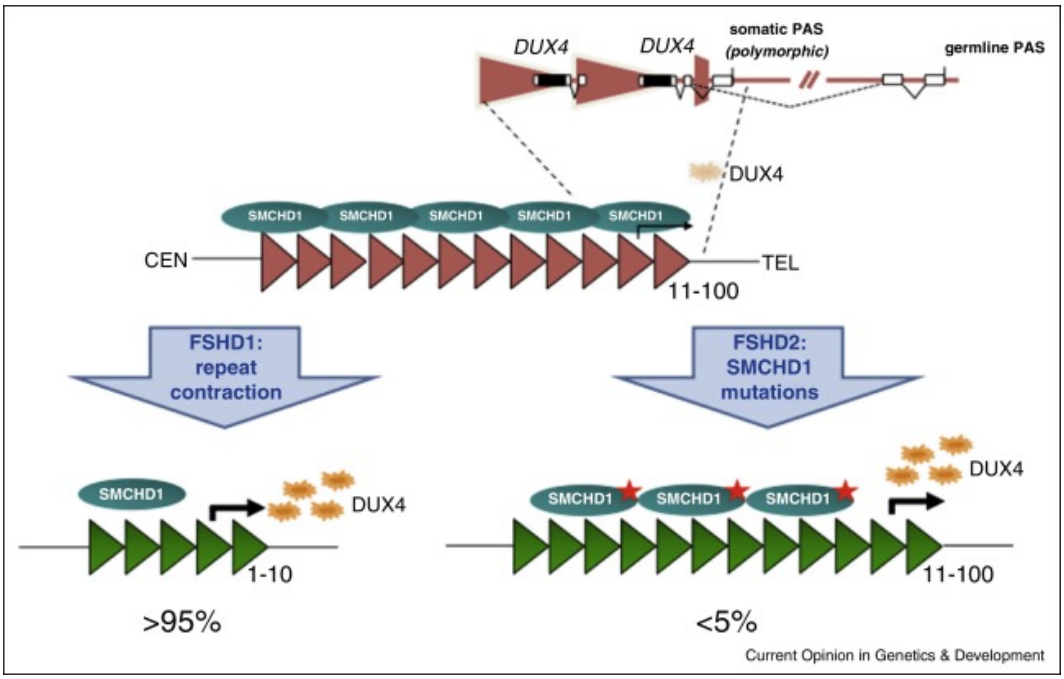


Figure 8: Genetic basis of FSHD1 and FSHD2. CEN indicates the centromeric end and TEL indicates the telomeric end. Upon contraction the D4Z4 chromatin structure becomes derepressed (green) facilitating the expression of the *DUX4* retrogene of which a copy is embedded within each unit. *DUX4* (white boxes with open reading frame in black) within the D4Z4 unit does not have a polyadenylation signal (PAS), but, in somatic cells can make use of a polymorphic PAS immediately distal to the D4Z4 repeat present on 4qA but not on 4qB chromosomes. Thus, only on 4qA chromosomes, D4Z4 chromatin derepression leads to the production of DUX4 protein. SMCHD1 is a chromatin modifier that binds to the D4Z4 repeat to keep the D4Z4 chromatin structure in a repressed state in somatic cells. Individuals with a mutation in SMCHD1 (asterisk), in combination with a *DUX4* polyadenylation signal, can express DUX4 in their muscles. This is called FSHD2. (Daxinger et al., 2015)

THE ITALIAN NATIONAL REGISTRY FOR FSHD

The Italian National Registry has been developed since 2008 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, distributed across all of Italy, from northern to southern regions. The neuromuscular Clinical Centers are the following: Department of Neurosciences, Reproductive Sciences and Odontostomatology "Federico II" University of Naples (Prof. Lucio Santoro), 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 Neurosciences, Psychiatry and Anaesthesiology University of Messina (Prof. Carmelo Rodolico), ASL8 University of Cagliari (Prof. Giovanni Marrosu).

Starting from data collected since 2008 in Italian National Registry for FSHD the following studies are ongoing:

i. classification FSHD patients and families in homogeneous sub-groups on the basis of phenotypic features;
ii. research for modifier loci/new genes through whole exome sequencing and candidate gene approach.
iii. investigation of the natural history of the disease through the prospective clinical evalution of DRA carriers;

Since 2008, 2460 carriers of a DRA within 1-10 repeats from 1273 unrelated families have been recruited and clinically evaluated. In the Clinical Centers, 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.

From the article: "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 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. 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 studies 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). 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, 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

SECTION 2: challenges in diagnosis of FSHD

From the article: "Large scale population analysis challenges the current criteria for the molecular diagnosis of fascioscapulohumeral muscular dystrophy"

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. [Am J Hum Genet. 2012]*

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., 2012]. 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]. 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.

From the article: "Large scale genotype-phenotype analyses indicate that novel prognostic tools are required for families with facioscapulohumeral muscular dystrophy."

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. [Brain. 2013]*

Several studies conducted on FSHD families have described a high variability in clinical expression among and within FSHD families, as well as asymptomatic subjects carrying DRA, doubting the notion of an almost full penetrance of disease. Thus we considered it is important to establish the penetrance of disease in FSHD families carrying DRA and to ascertain previously reported rough and inverse correlation between the size of DRA and the age at onset and severity of disease expression.

The selection process was conducted on 418 FSHD index cases carrying DRA with 1-8 repeats (Figure 9).

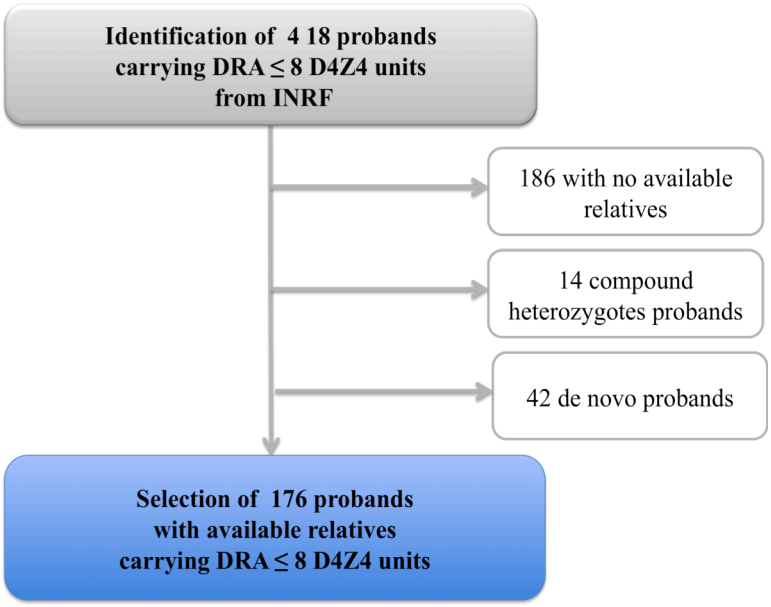


Figure 9: Preliminary selection of probands/families from the Italian National Registry for FSHD (INRF).

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 (Figure 10).

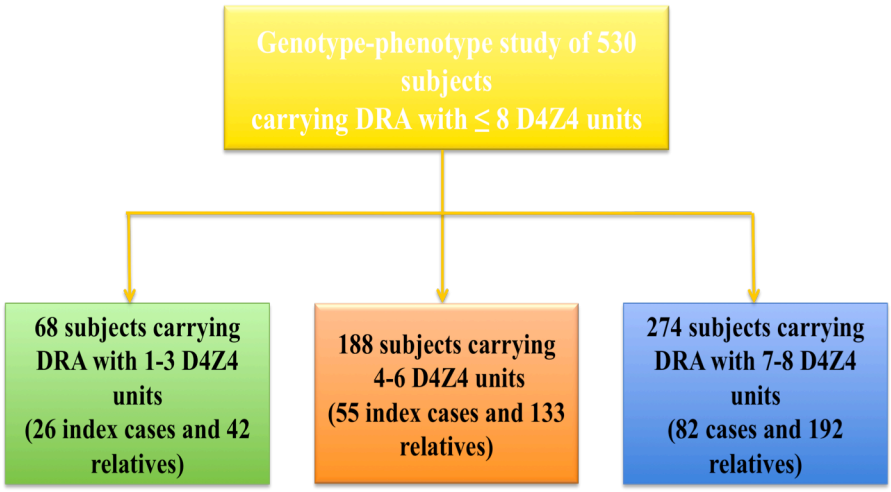
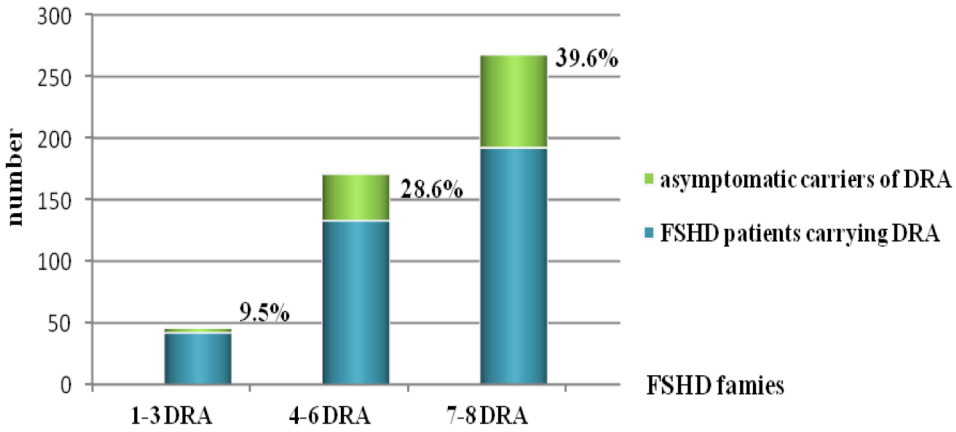


Figure 10: Selection of the cohort of probands and their relatives for genotype–phenotype correlation analysis.

The distribution of asymptomatic relatives was analyzed based on the size of DRA. Graphic 1 shows that 9.5% (4 out of 42) of all carriers of DRA with 1-3 repeats did not display motor impairment. This percentage increases among carriers of DRA with 4-6 and 7-8 repeats (28.6% and 39.6% respectively).



Graphic 1: **The distribution of asymptomatic DRA carriers in three groups.**

In addition we calculated the distribution of asymptomatic carriers based on the age at examination: subjects aged between 18-30, 31-55, 56-70 years, and subjects over 70 years of age. As shown in Table 1 asymptomatic DRA 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.

	Age (years)					
	18-30		31-55		56-70	
D4Z4 units	N of subjects	% score=0 (N)	N of subjects	% score=0 (N)	N of subjects	% score=0 (N)
1-3	8	12.5 (1)	23	8.7 (2)	9	11.1 (1)
4-6	31	25.8 (8)	65	33.8 (22)	29	27.6 (8)
7-8	42	54.8 (23)	85	40.0 (34)	39	35.9 (14)

Table 1: The percentage of asymptomatic DRA carriers in the group of 1-3, 4-6 and 6-8 DRA based at the age at onset.

We tested whether the size of DRA correlates with age at onset and disease severity. Table 2 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 (respectively 29.2 and 34.6 years) (p = 0.0002).

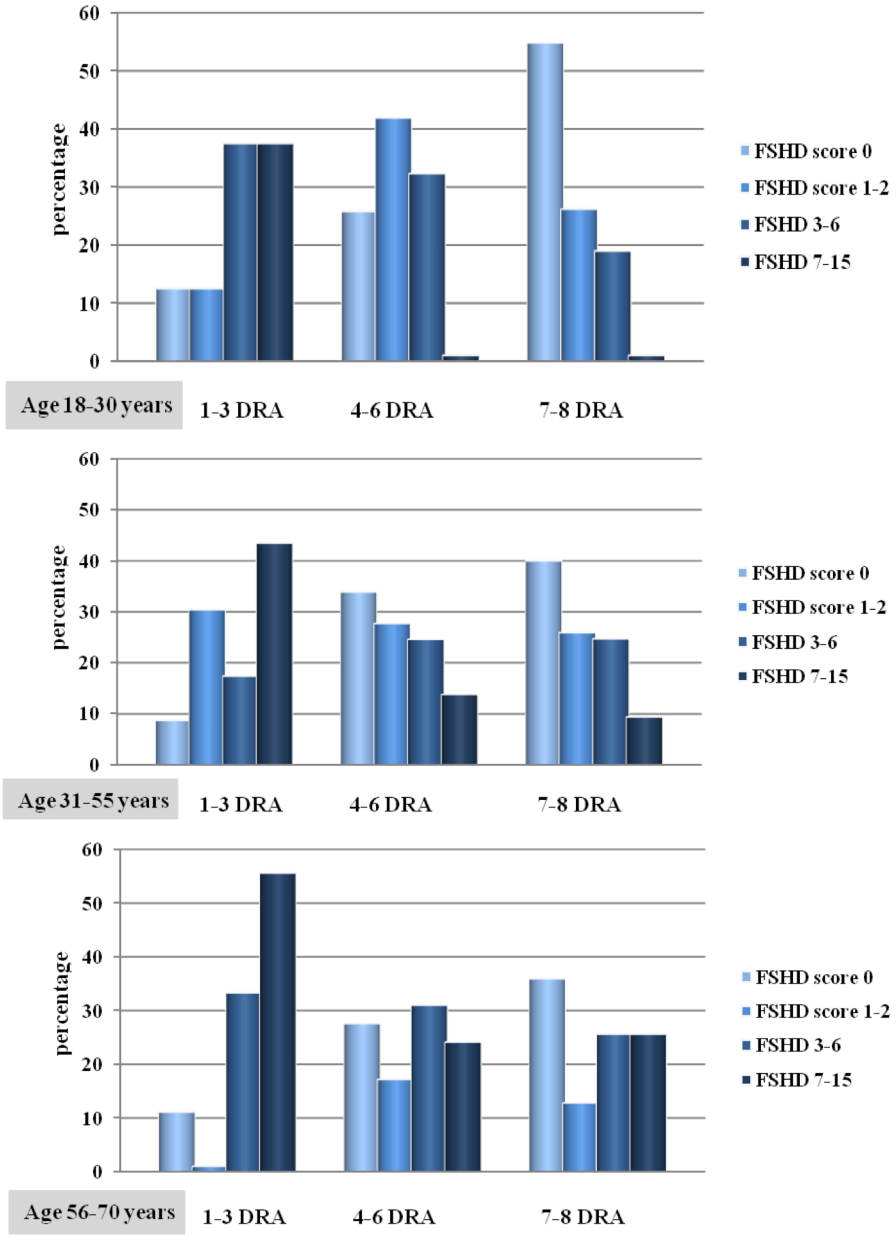
	Relatives			
D4Z4 units	N of subjects	Mean age at onset (yrs)	95% CI	p-value*
1-3	77	20.3	(15.5;25.2)	
4-6	41	29.2	(25.6:32.7)	
7-8	114	34.6	(30.1;39.1)	0.0002

Table 2: The age at onset in the group of 1-3, 4-6 and 7-8 DRA carriers.

Severity is also increased among carriers of DRA with 1-3 repeats. Indeed, as shown in Table 3, affected relatives carrying DRA with 1-3 repeats had a mean FSHD score of 7.2.

	Relatives			
D4Z4 units	N of subjects	Mean FSHD score	95% CI	p-value*
1-3	38	7.2	(5.8; 8.6)	
4-6	96	4.4	(3.8; 5.1)	
7-8	116	4.1	(3.5; 4.7)	0.0006

Table 3: The mean FSHD score in the group of 1-3, 4-6 and 7-8 DRA carriers.



Graphic 2: The degree of motor impairment among relatives was also evaluated in association with D4Z4 allele size and age at examination.

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 ($p = 0.0006$) and was obtained by using linear regression model adjusted for age at examination.

The degree of motor impairment among relatives was also evaluated in association with D4Z4 allele size and age at examination. Graphic 2 shows that approximately 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 10 shows that between age 31-55 and 56-70 a high percentage (ranging between 32% and 37%) of relatives carrying DRA with 4-8 units were asymptomatic (FSHD score equal to zero) or displayed minimal signs of functional motor impairment (FSHD score 1-2, ranging between 15% and 27%).

Our large scale genotype-phenotype study [Ricci et al, 2013] revealed 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. It was shown that DRAs with 4–8 repeats have no definitive prognostic value, and that other prognostic parameters, beside DRAs, should be considered. Instead, 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. 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. 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).

Collectively, the wide clinical variability among subjects carrying 4-8 D4Z4 repeats together with the high number of asymptomatic or minimally affected carriers suggests that additional factors, such as genetic, epigenetic and environmental factors, are involved in reaching the threshold of disease appearance and/or modifying the clinical outcome [Scionti et al, 2012]. Remarkably, the study shows a higher percentage of asymptomatic subjects between relatives with lower degree of relationship with proband, regardless age and D4Z4 size. The degree of kinship may influence the disease outcome, as result of genetic background “dispersion”, suggesting a more complex mode of inheritance of FSHD.

From the article: "Clinical expression of facioscapulohumeral muscular dystrophy in carriers of 1-3 D4Z4 reduced alleles: experience of the FSHD Italian National Registry."

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. [BMJ Open. 2016]*

The alleles of extremely short sizes (1-3 D4Z4 repeats) were described to be associated with the most severe form of disease characterized by an early onset and rapid progression of muscle weakness [Jardine et al, 1994; Lunt et al, 1995; Ricci et al, 1999; Ricci et al, 2013]. Infantile FSHD has been subsequently described as a separate entity, defined by the onset of facial weakness by the age of 5 years and shoulder girdle weakness by the age of 10. A number of reports described cases carrying very short D4Z4 alleles with 1-2 repeats characterized by childhood onset, rapid progression of muscle weakness and extramuscular clinical features [Okinaga et al, 1997; Wang et al, 2012]. However several studies reported differences in clinical expression between subjects carrying shorter alleles, varying from very severe forms of disease and complex phenotypes starting in infancy [Okinaga et al, 1997; Dorobek et al, 2004] to milder form or asymptomatic carriers [Tupler et al., 1998; Sakellariou et al, 2012]. By revising the literature, we found that the severe cases not all had a childhood onset, or carried a D4Z4 allele of very reduced size. Due to the different design of these studies it is not possible to pool various observations to obtain a complete or more defined picture of clinical features of subjects carrying "very short" D4Z4 allele. Therefore, the goal of our research is was to conduct a detailed clinical and molecular characterization of index cases carrying 1-3 DRA alleles from Italian National Registry for FSHD.

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. 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 (Figure 11). We defined *de novo* cases 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 inter-rater 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.

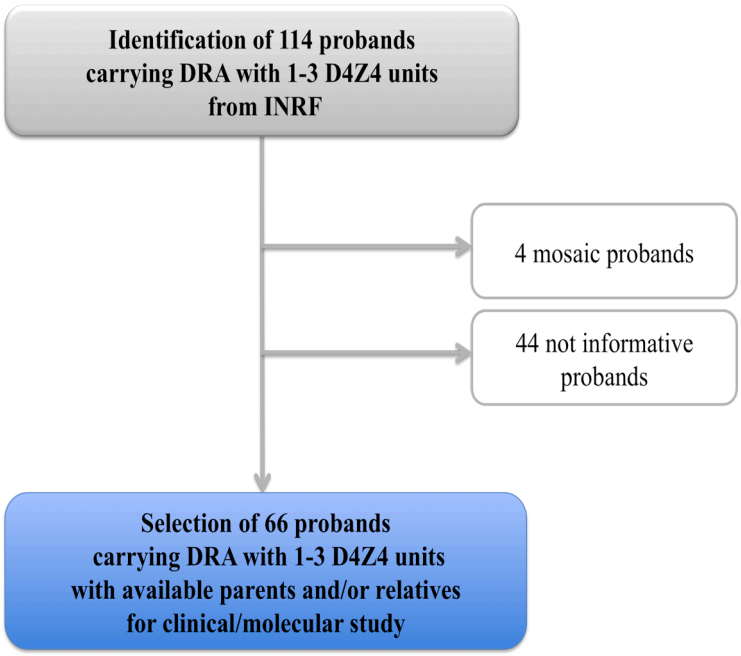
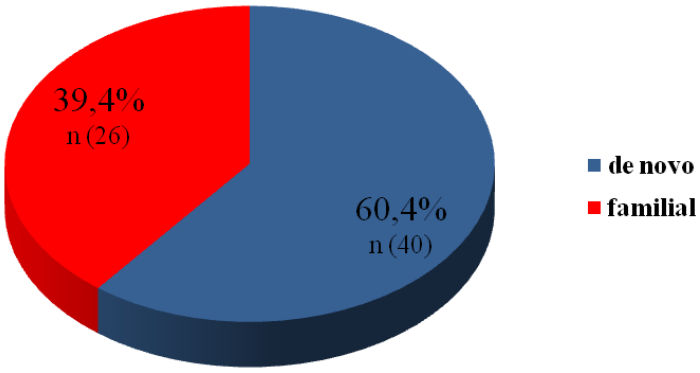


Figure 11: Preliminary selection of probands/families from the Italian National Registry for FSHD (INRF).

In 66 unrelated index cases carrying 1-3 DRA we extended molecular characterization to parents and/or other relatives. To this purpose we analyzed 226 subjects clinically and molecularly. We found that 26 probands were familial (39.4%) index cases and 40 probands were *de novo* (60.6%)(Graphic 3).



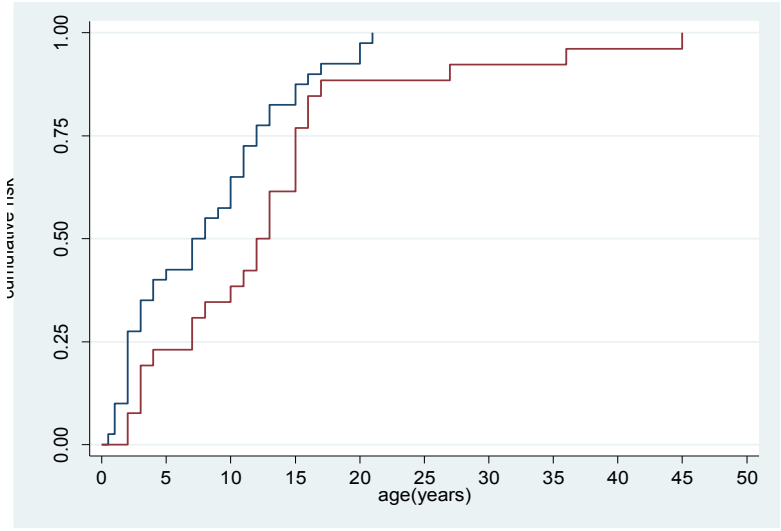
Graphic 3: Distribution of *de novo* and familial index cases, carriers of 1-3 DRA.

Interestingly, the mean age at onset observed among the *de novo* probands was 8.1 yrs; whereas it was 13.1 yrs among familial index cases (table 4). Thus muscle weakness appears significantly earlier in *de novo* cases than in familial (Long-rank p test value=0.020). The difference in the onset of disease between two groups was calculated with non-parametric Kruskal-Wallis test.

		Age at onset			
		0-10 yrs		> 10 yrs	
1-3 DRA index cases	N subjects	N subjects	%	N subjects	%
de novo	40	26	65	14	35
familial	26	10	38.5	16	61.5
tot	66	36	54.5	30	45.5

Table 4: Age at onset among *de novo* and familial index cases, carriers of 1-3 DRA.

Graphic 4 compares FSHD penetrance between carriers of *de novo* or familial 1-3 DRA calculated with the Kaplan-Meier method. Among subjects carrying a *de novo* 1-3 DRA the risk of developing motor impairment is 65% by age 10, 88% by age 15 and 98% by age 20. Among subjects carrying a familial 1-3 DRA the risk is 38% by age 10, 77% by age 15 and 88% by age 20. Therefore the risk of developing FSHD in childhood, before age 10, is significantly higher in subjects carrying a *de novo* 1-3 DRA.

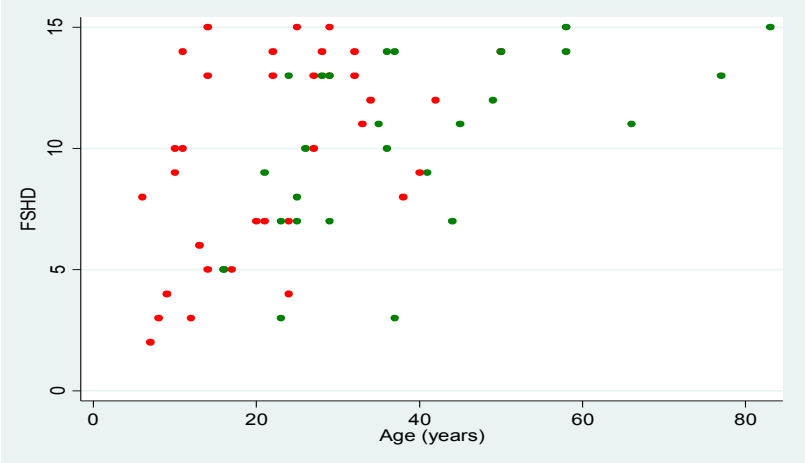


Graphic 4: Age-specific cumulative risk of reported age at onset between *de novo* and familial index cases, carriers of 1-3 DRA. Blue line refers to *de novo* index cases carriers of 1-3 DRA, red line refers to familial index cases carriers of 1-3 DRA. Long-rank test p value=0.026.

It has been reported that alleles of extremely short dimension (1-3 D4Z4 repeats) are associated with most severe form of disease [Jardine et al, 1994; Lunt et al, 1995; Ricci et al, 1999; Ricci et al, 2013].

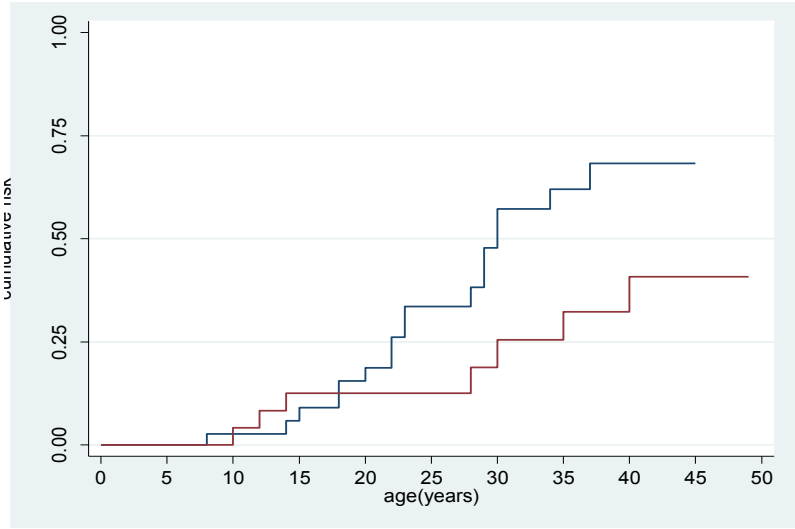
Considering the observed differences in age at onset between *de novo* and familial 1-3 DRA carriers, we tested whether the disease expression is more severe in *de novo* index cases. To this aim, we measured the motor disability by the FSHD score [Lamperti et al, 2010] in *de novo* and familial index cases. Statistical evaluation failed to detect any significant difference in mean FSHD score between the two groups (*de novo* vs familial index cases, 9.7 vs 11.1; Long-rank p test value=0.145).

Statistical evaluation failed also to detect any significant difference in mean FSHD score adjusted by sex and age, between the two groups (*de novo* vs familial index cases, 9.7 vs 11.1; Long-rank p test value=0.145) (Graphic 5).



Graphic 5: **FSHD score in correlation to the age at examination**. Red spots refer to *de novo* index cases carriers of 1-3 DRA, green spots refer to familial index cases carriers of 1-3 DRA.

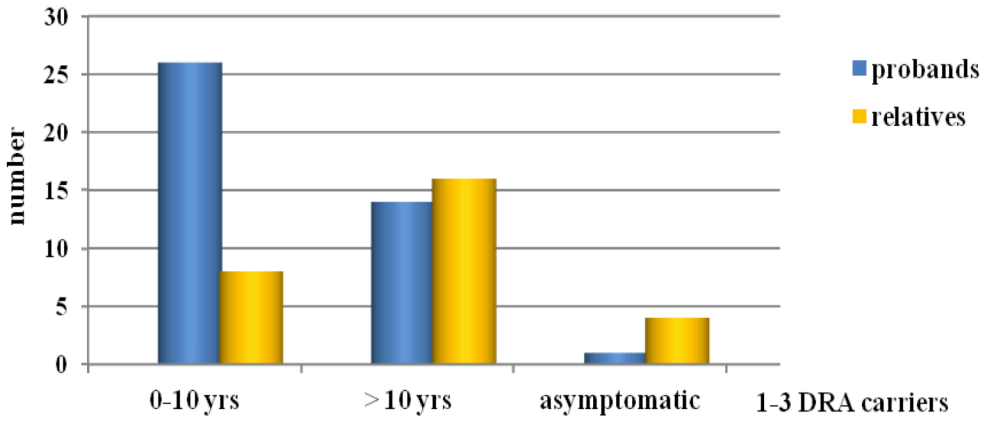
By using the Kaplan-Meier method (Graphic 6) we further evaluated the relative risk of loss of independent walking, considered as an important feature of motor disability, in carriers of *de novo* rearrangement and familial index cases. Our analysis showed that the cohort of *de novo* carriers has a higher risk of loss of independent walking versus the familial index cases, even though this difference did not reach a statistically significant value.



Graphic 6: **Age-specific cumulative risk of reported loss of independent walking between *novo* and familial index cases, carriers of 1-3 DRA.**Blue line refers to *de novo* index cases carriers of 1-3 DRA, red line refers to familial index cases carriers of 1-3 DRA. Long-rank test p value=0.062.

In familial cases analysis was also extended to 42 relatives carrying a DRA. We compared the age of the disease onset detected in the group of probands with that recorded in the group of relatives. This comparison displayed that affected relatives present a later onset of FSHD than the probands (relatives versus probands, 17.6 yrs vs 13.1 yrs (Graphic 7).

Long-rank p test value=0.019). We also compared the degree of motor impairment, recorded as FSHD score, detected in the two groups. The mean FSHD score received by relatives was significantly lower than that recorded in probands (6.1 vs 10.5, Long rank test p value<0.0001). Four relatives (6.1%), respectively aged 33, 42, 47, 50, did not present any muscle weakness.



Graphic 7: The distribution of probands and their relatives by the reported age at onset.

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 (Table 5).

Table 5

Infantile anamnestic records of 70 carriers of 1-3 DRA

			N of subjects		N of subjects		N of subjects
prenatal period	active fetal movements	normal	**69**	reduced	**2**	NA	**5**
delivery	partum	eutocic	**60**	dystocic	**14**	NA	**2**
	fetal position	cephalic	**58**	podalic	**6**	NA	**12**
birth	weight	normal	**69**	low	**0**	NA	**7**
	revived	no	**70**	yes	**2**	NA	**4**
	clubfoot	‘’	**70**	‘’	**1**	NA	**5**
perinatal period	reduced suction	no	**68**	yes	**7**	NA	**1**
	facial nerve palsy’s diagnosis	‘’	**72**	‘’	**2**	NA	**2**
	Moebius syndrome’s diagnosis	‘’	**72**	‘’	**1**	NA	**3**
	hip dysplasia	‘’	**70**	‘’	**2**	NA	**4**
	facial hypomimia	‘’	**72**	‘’	**3**	NA	**1**
	floppy	‘’	**72**	‘’	**0**	NA	**4**
psychomotor development	social smile	normal	**68**	altered	**5**	NA	**3**
	walk independently	<15 months	**65**				
		15-18 months	**7**				
		>18 months	**3**				
		NA	**1**				

In conclusion our analysis, first showed that the majority of carriers of 1-3 DRA (60.6%) are *de novo*. Notably, this percentage is higher than that previously described in the whole FSHD population [Padberg 1982; Padberg et al, 1995; Zatz et al, 1995 ; Tawil et al, 1996; Zatz et al 1998; van der Maarel et al, 2000]. We verified the percentage of *de novo* cases among 4-8 DRA probands from the INRF and surprisingly identified only 14 (5.7%) out 246 index cases, carrying 4-8 DRA. Thus we concluded that among probands from the INRF, carriers of *de novo* rearrangements are significantly more frequent in the cohort of subjects carrying 1-3 DRA, than in the cohort of 4-8 DRA carriers. This data, associated with the observation that previously 1-3 DRAs have never been detected in general population [Scionti et al, 2012], support the idea that the D4Z4 repeat array is highly recombinogenic and therefore prone to high mutation rate. Second, we found that the majority of cases presenting disease onset before age 10, are isolated and carry a *de novo* rearranged DRA (Table 4); while the majority of familial cases develops FSHD around the second decade of life. Interestingly, among the 66 carriers of 1-3 D4Z4 alleles 6.1% were asymptomatic (Graphic 7). However, even though there is a trend towards a more severe progression among *de novo* cases in comparison with familial probands (Graphic 4 and Graphic 6) and infantile disease onset is more common among *de novo* carriers of 1-3 DRA (Table 4), we failed to observe a

significant difference in disease outcome between the two groups (mean FSHD score in *de novo* versus familial index cases, 9.7 vs 11.1). In addition it is important to emphasize that in this cohort of subjects carrying the shortest D4Z4 allele, not all presented an infantile onset. Indeed 45.5% of index cases reported age at onset after 10 years of age, moreover our Anamnestic Infantile Form revealed no presence of the congenital form of the FSHD.

Collectively, our genotype-phenotype study on the large cohort of subjects carrying the shortest allele, revealed that:

1) first signs and/or symptoms were not detected in pre- and peri-natal period;

2) only half of the subjects carrying 1-3 DRA develop FSHD in infantile period (1-10 years);

3) not all subjects carrying 1-3 DRA display more severe form of disease, regardless infantile onset;

4) asymptomatic individuals were found to carry 1-3 DRA.

These observations suggest that additional factors might contribute to complex FSHD pathogenesis.

SECTION 3: a novel clinical tool

From the article: "A novel clinical tool to classify facioscapulohumeral muscular dystrophy phenotypes."

*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. [J Neurol. 2016]*

Through the systematic use of the FSHD Clinical Form [Lamperti et al., 2010; Scionti et al., 2012; 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 [Ricci et al., 2014]. Typical and atypical clinical features were combined in the Comprehensive Clinical Evaluation Form (CCEF). 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 invited 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 6) 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].

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 ≥ 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].

Table 6. *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

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 12) and the Clinical Categories (Section 4, Figure 13).

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. 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 [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 12), it is possible to assign patients to different phenotypic categories (CCEF Section 4, Figure 13). 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 12: *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 13: *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 13, Figure 14). 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 14A-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 14D, 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 14H,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 12) are described as category C1, whereas category C2 includes subjects with a neurological examination completely normal (Figure 14F,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.

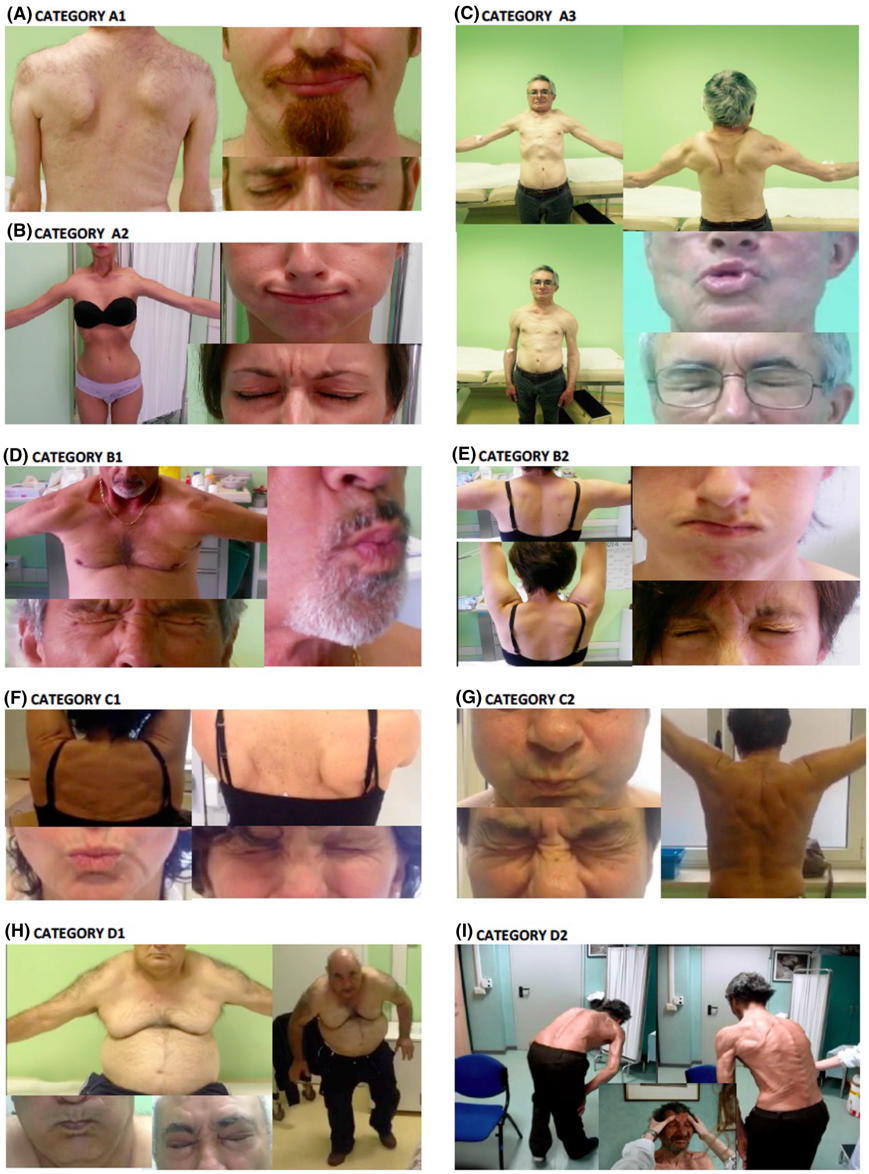


Figure 14: *Examples of clinical categories: case reports. a Category A1: male, 38-year old, showing severe upper and lower facial weakness (unable to close both eyelids completely, puff cheeks and protrude lips), and impairment of upper limb abduction with winged scapula. b Category A2: female, 31-year 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 concordance between the clinical assessments performed by the two neurologists was evaluated for the nine CCEF categories described in Figure 13. As shown in Table 7, 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 8. 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 7: *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 8: *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

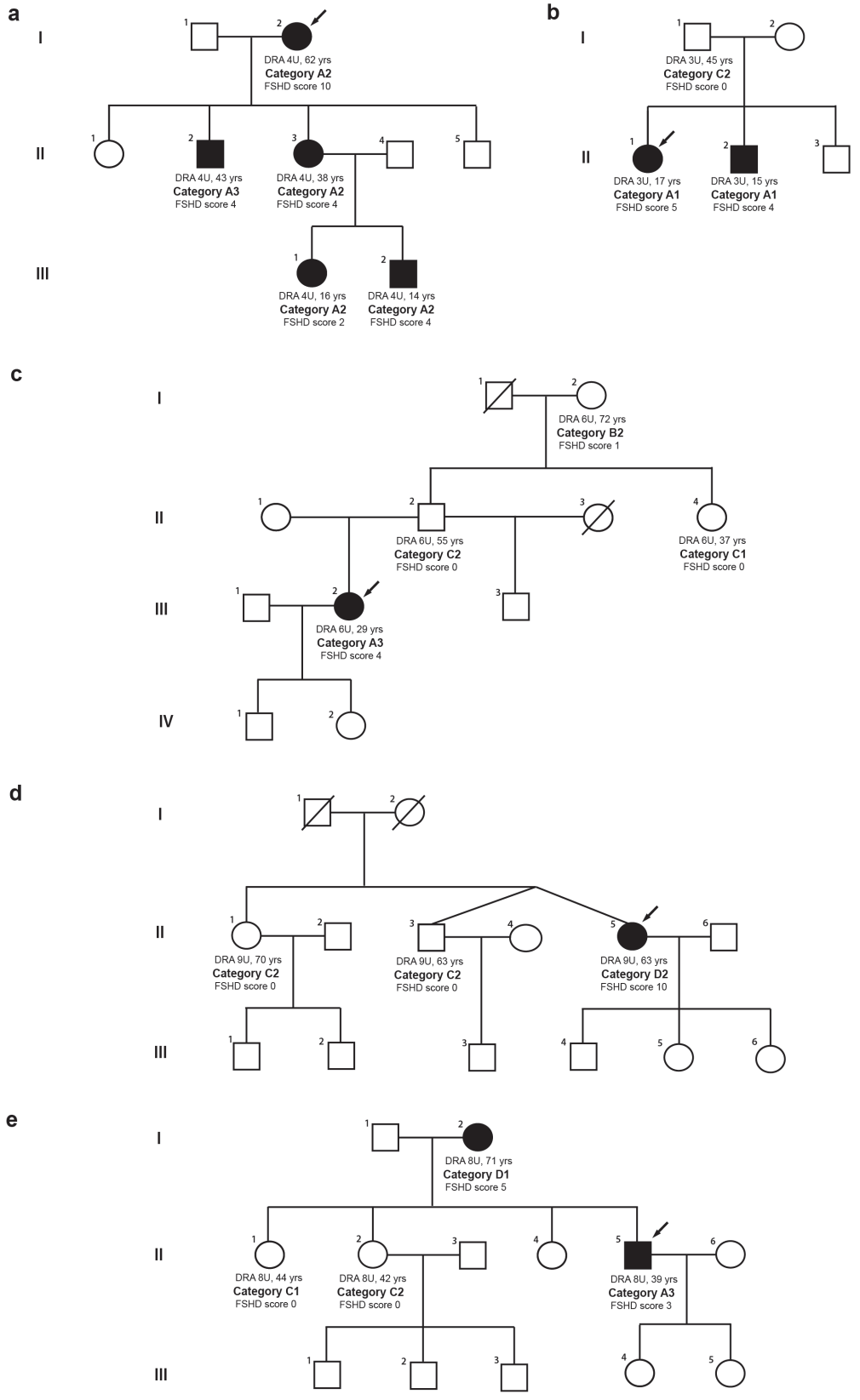
Kappa=0.90; 95%CI (0.71; 0.97)

The genetic heterogeneity in FSHD 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. 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 15 shows some examples. Figure 15A 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 15B 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 15C 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 15D 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 15E 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. 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 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].

Figure 15: *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.*



SECTION 4: the next step to dissect the genetic and clinical complexity of FSHD

From the article: Clinical expression of facioscapulohumeral muscular dystrophy in carriers of 33-35 kb D4Z4 reduced alleles: experience of the Italian National Registry for FSHD.

***Ruggiero L**, Mele F , Ricci G, Vercelli L, Govi M; Nikolic A, Louise M, Sera F, Bruzzese D, Berardinelli A, Angelini C; Antonini G, Bucci E, Filosto M, Cao M, Giardina E, Pegoraro E, Di Muzio A, Telese R, Maggi L, Portaro S, Rodolico C, Villa L, Mongini T, Siciliano G, Tomelleri G, D'Angelo G, Maioli MA, Moggio M, Santoro L, Rossella Tupler. [To be submitted]*

Methods

Study design and subject selection

We performed an observational study of 152 probands (P) and 223 relatives (R) from a consecutive group of 252 probands and 306 relatives, carriers of 8-10 DRA, from the Italian National Registry for FSHD collected between 2008 and 2016. Informed consent, according to the Declaration of Helsinki, was obtained from each participant enrolled in the study.

Clinical Examination

In this large cohort we applied the Comprehensive Clinical Evaluation Form (CCEF). Based on the distribution of muscle weakness and the combination of the clinical features suggestive or not of FSHD, it is possible to assign patients to different Phenotypic Categories. In particular, we assigned subjects with typical FSHD presenting facial and scapular girdle muscle weakness without atypical features in category A; subjects with muscle weakness limited to facial or scapular girdle muscles in category B; asymptomatic subjects without motor impairment in category C; subjects with myopathic phenotype presenting other anomalous clinical features not consistent with FSHD in category D.

Molecular characterisation

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]. 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]. Restriction fragments were detected by autoradiography or using a

Typhoon Trio system (GE Healthcare). 4qA/4qB allelic variants were defined 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].
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].

Statistical Analysis

The genotype-phenoptype correlations analysis was performed using the following statistical tests:

- p value: for evaluation of difference distribution between males and females in the clinical categories, to compare the age at onset between index cases and relatives and between the age at last neurological examination of asymptomatic relatives and age at onset of symptomatic relatives. Finally, to compare the FSHD score between index case and relatives.
- t test: to compare the age at onset and the FSHD score between males and females in the group of index cases and relatives.
- Anova test: to compare the age at onset between classical FSHD and atypical FSHD in the group of index cases and relatives.

Results

Phenotypic characterization of index cases and relatives carrying 7-8 DRA

We re-evaluated 152 unrelated index cases [84 males, 70 females, mean age at last neurological examination 53,8 ± 15,4 (range 8-87)], in males mean age was 49.8, in females was 57,9. Starting from index cases, we identified 223 relatives carriers 7-8 DRA (99 males and 124 females, mean age at last neurological examination 45,5 ± 17,3). The percentage of carriers of 33 kb DRA was 46% while of carriers of 35 kb DRA 53%, with a similar distribution of subject in two genetically subgroup **(table 9)**.

Table 9*: Characteristics of the 251 index cases and 310 relatives carriers 7-8 DRA.*

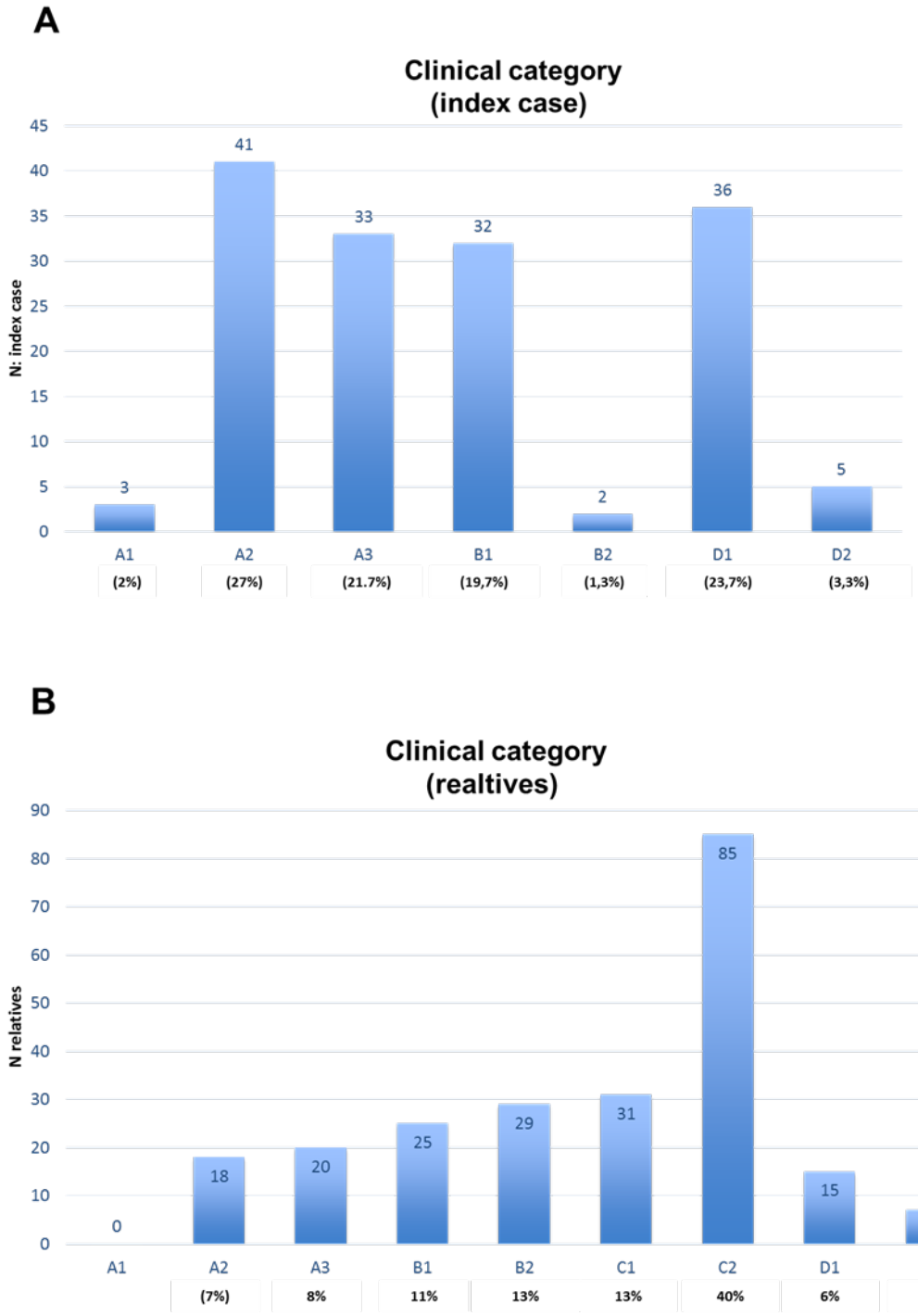
	Index Cases	**Relatives**
N subject entrolled	251	310
N subject revalued	154	221
M	84	95
F	70	126
Mean age at examination	53,8	45,5
Carriers 33 kb DRA	45%	49%
Carriers 35 kb DRA	55%	51%

Distribution of clinical categories of index cases

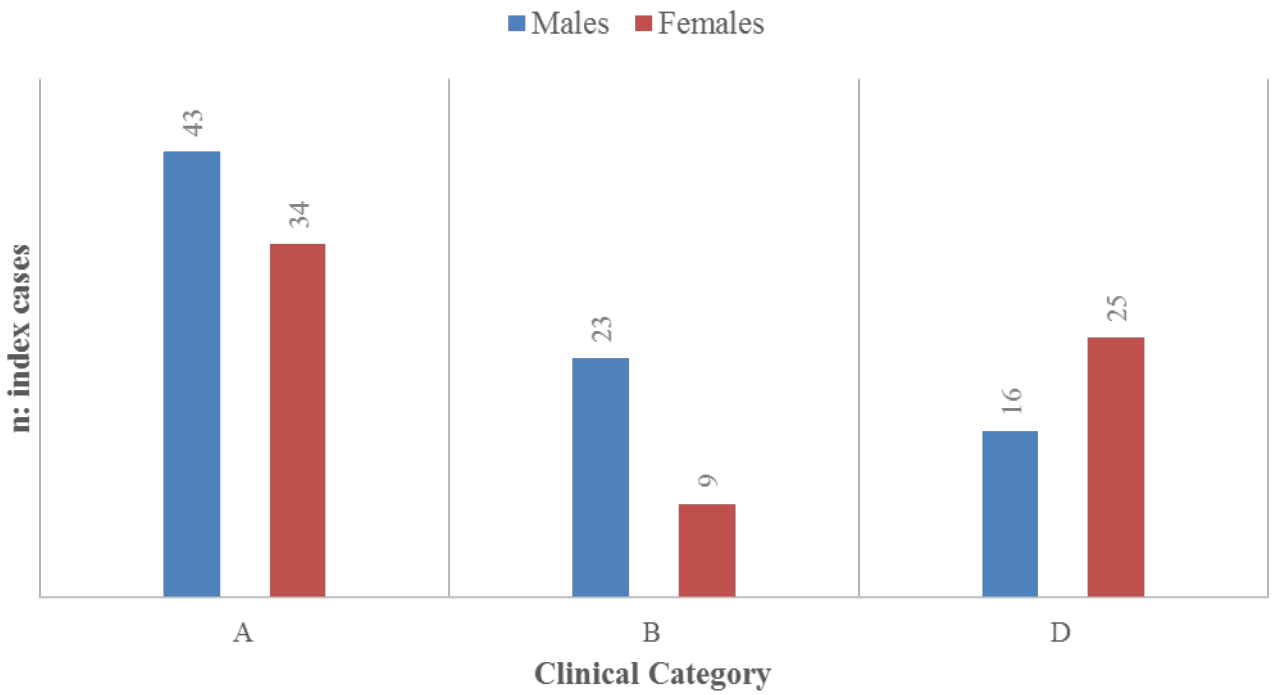
The distribution of probands in the clinical categories shows that 50,7% (n=77) of subjects display a classic FSHD phenotype, classified as category A. Interestingly, among these 77 cases, only 3 presented severe facial weakness, classified as category A1, 41 showed a moderate facial weakness (category A2) and 27 were characterized by a partial facial involvement (category A3). An incomplete FSHD phenotype (category B) has been observed in 32 index cases (20,7%). The majority of this subgroup (n: 30) is composed by patients with shoulder involvement without facial weakness (category B1). Whereas the isolated facial weakness was detected only in 2 subjects (category B2). Finnaly, 37 index cases (28.6%) presented phenotypes with clinical features not consistent with FSHD and were listed as category D. In particular 5 subjects showed a phenotype not consistent with FSHD, but suggestive of an alternative diagnosis (category D2) and 36 presented additional uncommon clinical signs suggestive of a possible comorbidity (category D1) (Graphic 8 A)**.** Interestingly, we did detect a significant difference of distribution between males and females in the clinical categories (**p-value 0,019**). The atypical phenotypes are more frequently in women group (Graphic 9).

Distribution of clinical categories of relatives

The distribution of relatives in the clinical categories shows that 52.7% of subjects were asymptomatic, classified as category C. In particular 85 showed a neurological examination completely normal (category C2), while 31 present minor signs (category C1) without motor impairment. On the other hand only 38 (15.9%) of realtives display a classic FSHD phenotype (category A). Interestingly, anyone was in category A1, 18 were in category A2 and 20 in category A3. We observed the shoulder involvement without facial weakness in 25 subjects, whereas the isolated facial weakness was detected in 29 subjects. Overall 24% of relatives displayed an incomplete phenotype. Finally, 15 relatives presented additional uncommon characteristics suggestive of a possible comorbidity (category D1) and 7 with clinical features not consistent with FSHD (category D2). The distribution of index case and relatives in different clinical category is synthesized in (Graphic 8)



Graphic 8: the distribution of index cases (part A) and relatives (part B) in the clinical category



Graphic 9: the comparison of distribution of clinical category between male and female in the group of index cases

Analysis of clinical categories in families

Based on the clinical category of index case, we assessed the clinical patterns of relatives (**table 10**) and we observed that only in 1/99 of our families, all analyzed subjects displayed classical FSHD phenotype. In contrast, in about a third of our families (36/99) the index case only presents myopatic phenotype, moreover in these families all relative carriers 7-8 DRA are non-penetrant. Finally, it is important to emphasize that if the proband is classified as "B" or "D" we do not find relatives with classic FSHD phenotype.

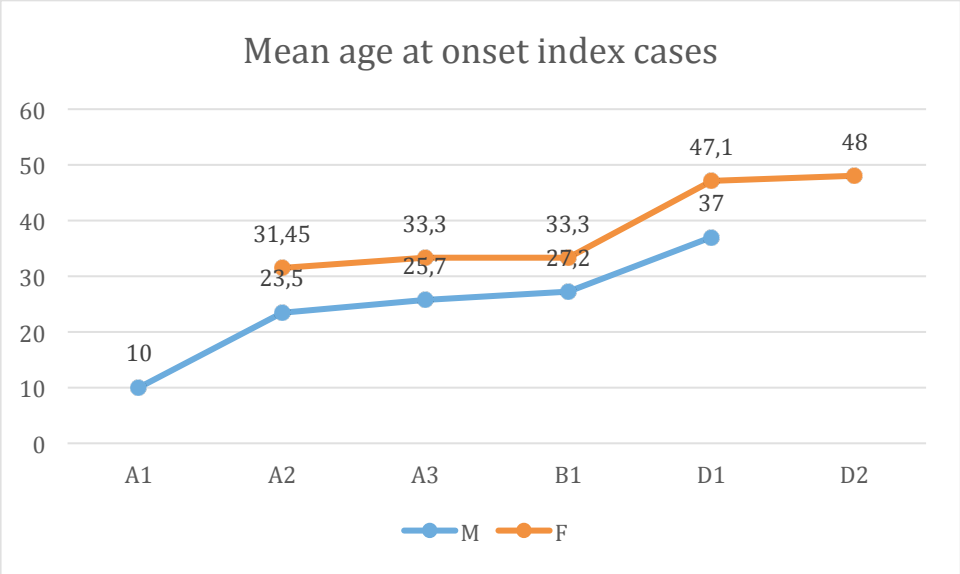
(**Table 10**): *Evaluation of clinical patterns of relatives on the basis of clinical category of proband*

	Clinical Category Probands			
Clinical patterns Familiars	A	B	D	**Total**
A	1	0	0	1
AB	1	0	0	1
ABC	5	0	0	5
ABCD	2	0	0	2
AC	7	1	2	9
ACD	1	0	0	1
B	5	2	2	9
BC	8	6	3	17
BCD	3	1	1	5
C	16	6	14	36
CD	2	2	3	7
D	4	1	0	5
Total	55	19	25	99

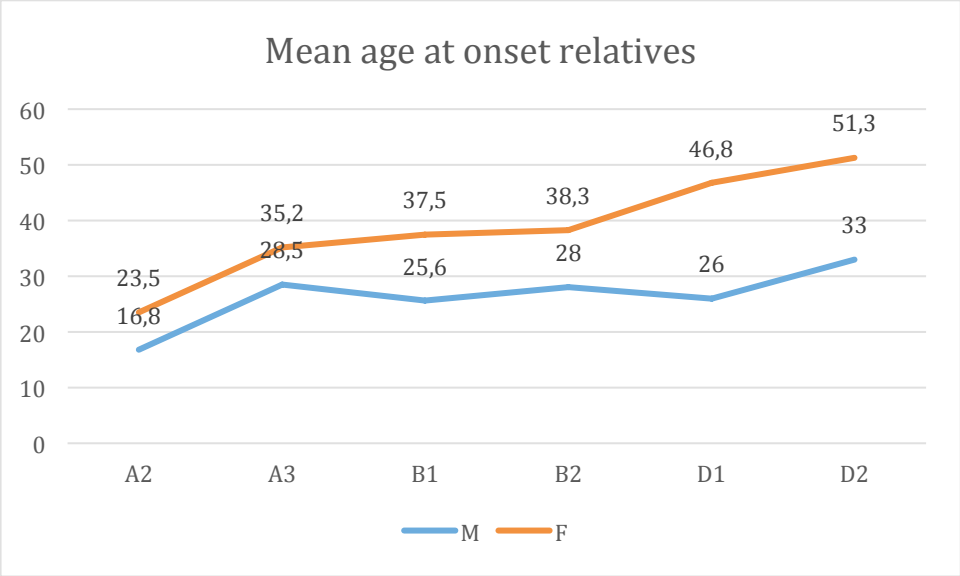
Age at onset

We observed that 62% of index cases present the first symptoms after 20 years old (mean 33,2 ±18.5). We did detect a significant difference between males and females (in males age at onset was 29,4 ±17,2 while in females 38,1 ±18.9; T test p-value: 0,002). Moreover, we evaluated the mean age at onset of index cases for each category and we observed that subject with classical FSHD phenotype had a significantly earlier onset than subjects with atypical FSHD phenotype (classical FSHD 28.4 ±17.3, in contrast atypical FSHD 42.6 ±17.8; Anova p-value >0.001) (Graphic 10). Interestingly, we found the same statistical significant data in the group of relatives (males age at onset 26.4 ±12.8 while females 38.7 ±18.1; t-test p-value 0,003; classical FSHD 29.7±18.1 while atypical FSHD 45.2 ±16.3, Anova p-value: 0.017) (Graphic 11). In line to these data when we compared the age at onset between index cases and relatives we did not detect a significant difference (index cases 33,2 ±18.5, and relatives 34.6 ±17.4; p-value 0.6). Instead the age at last neurological examination of asymptomatic relatives (39,2) was result significantly older respect the mean of age at onset of symptomatic relatives (p-value >0.01).

Finally, we observed that the 70% of patients present with scapular girdle weakness at onset, in contrast patients with facial weakness at onset are only 12 (5%). A considerable percentage of patients present pelvic girdle weakness at onset (13,7%) and in this subgroup the large majority 25/31 are women.



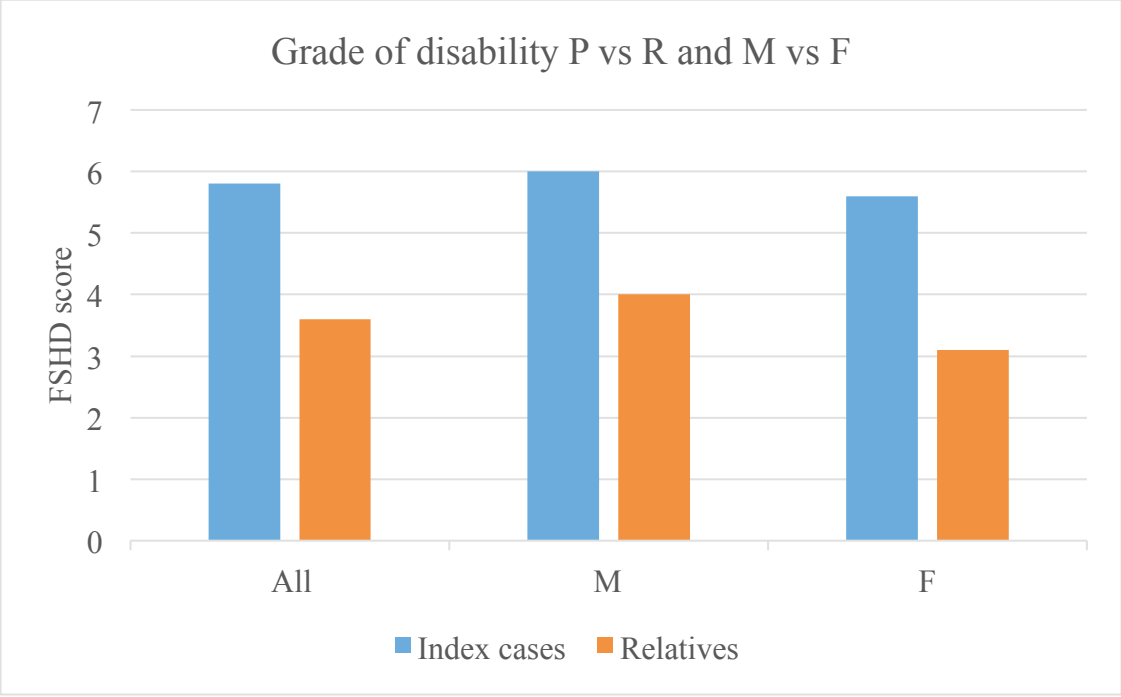
Graphic 10: the evaluation of age at onset in the group of index cases on the basis clinical category and sex



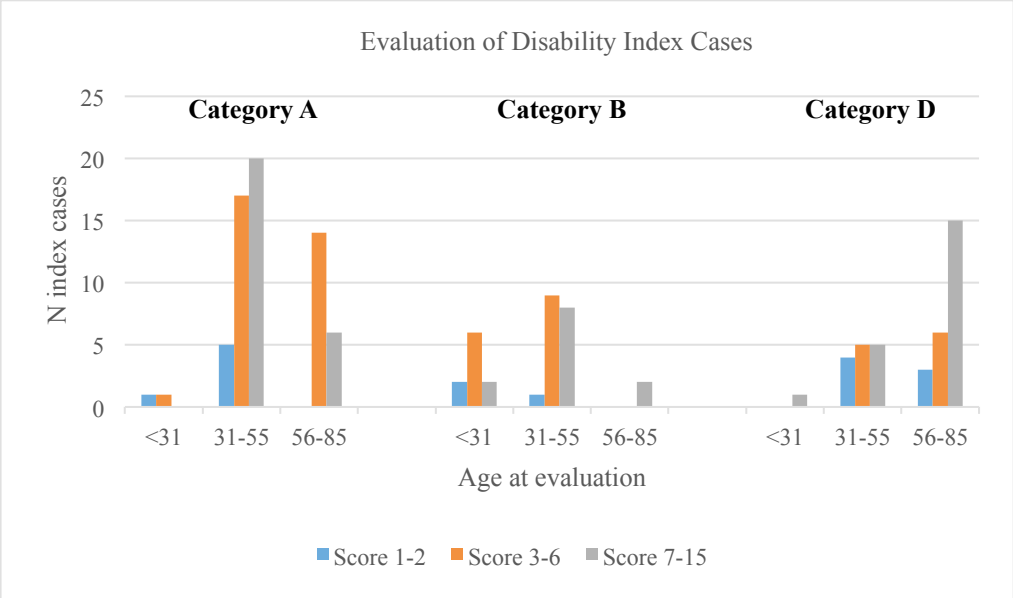
Graphic 11: the evaluation of age at onset in the group of relatives on the basis clinical category and sex

Severity of motor impairment

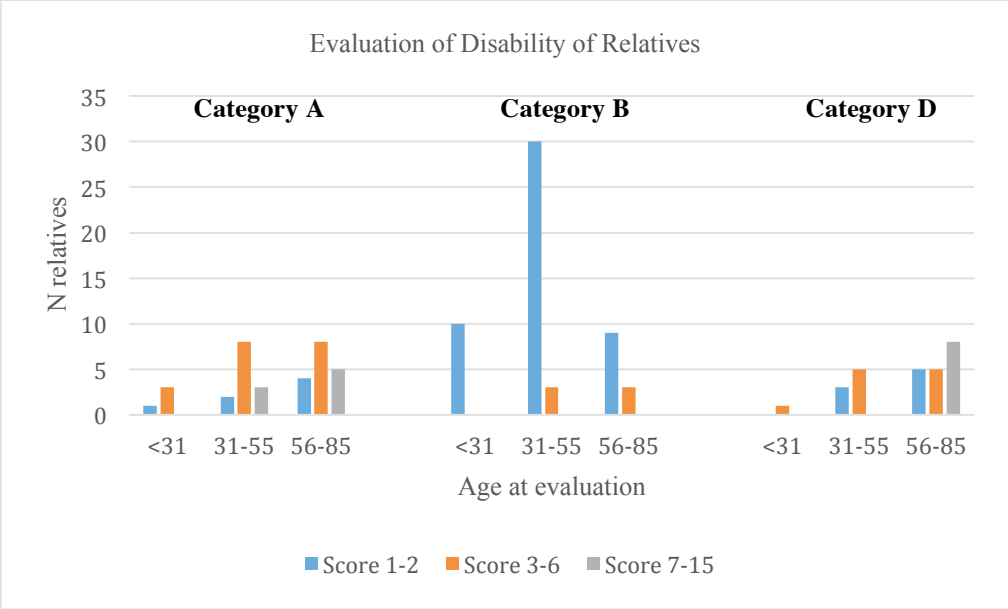
The degree of motor impairment among index cases was also evaluated, using FSHD clinical score. The mean FSHD score was 5.8 ±3.3. We did not detect evidence of a difference in term of FSHD score among males and females (males mean FSHD score 6 ±3.2, females FSHD score 5.6 ±3.3). Finally, we did detect a difference of FSHD score between index cases and relatives (p-value <0.001), in fact the last ones present less severe clinical impairment (mean FSHD score 3.6 ±3.1) (Graphic 12).



Graphic 12: the degree of motor impairment of index cases and relatives and the comparison between sexs.



Graphic 13: the degree of motor impairment among index cases evaluated in association with clinical category and age at examination.



Graphic 13: the degree of motor impairment among relatives evaluated in association with clinical category and age at examination.

Discussion:

FSHD 1 is a complex disease with peculiar clinical and genetical aspects. It is reported a wide variability of clinical expression, both in term of age at onset, severity of motor impairment and progression [Mul et al., 2017]. In addition, several studies have reported high percentage, ranging between 25-30%, of non-penetrant carriers, making uncertain the prognosis for subjects carrying or at risk of carrying D4Z4 reduced alleles [Tonini et al., 2004].

This complexity was generated by a difficult application and interpretation of molecular test. First of all, a positive result of the genetic test has been considered sufficient for definite diagnosis of FSHD regardless of the clinical characterization of subjects. Unexpectedly, by revising the literature the molecular signature of FSHD turned out poorly specific and sensitive [Nguyen K et al 2017; Scionti et al. 2012, Tawil et al., 2015]. Instead, before the discovery of genetic signature, the diagnosis was entirely based on clinical evidence.

However, the cited 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, 4-8 D4Z4 repeats are associated with the classical form of FSHD, and 9-10 D4Z4 repeats with a milder disease [Lunt et al., 1995a; Tawil et al., 1996; Ricci et al., 1999]. DRA between 38 and 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]. This last group of patients with milder phenotype seems to be in expansion including also 7-8 DRA carriers [Statland et al., 2015].

The present study is the largest standardized genotype-phenotype correlation analysis of index cases and relatives carriers of 7-8 DRA.

The first objective of our study was to evaluate the distribution of index cases in the different phenotypic categories. Very interestingly, we observed that only 50.7% of probands have the classic FSHD phenotype (category A). In addition, only 3 of 154 index cases have a severe facial involvement (category A1) and the facial weakness at onset is a very rare report. These data confirm that in this subgroup of patients the facial involvement is less frequent and less severe than in subjects carrying a smaller fragment [Felice et al., 2000]. Moreover, it is even more important to emphasize that frequently these patients present incomplete phenotypes without facial involvement (B1) or with atypical signs (category D). The clinical pattern of the relatives of index cases belonging to the B1 and D group did not show any subject with classic FSHD phenotype. For these patients it is mandatory to consider an alternative diagnosis.

The second objective was to evaluate the distribution of relatives in the phenotypic categories. The most important result was that the majority of these subjects (53%) were asymptomatic. This

percentage of asymptomatic carriers is much higher than those reported earlier [Tonini et al. 2004]. Interestingly, the age at last neurological examination of asymptomatic relatives resulted significantly older with respect to symptomatic relatives, so we may suppose that these subjects will never develop the disease. A first interesting conclusion is that for appropriate genetic counselling it is mandatory to perform systematically clinical and molecular test in the relatives. On the other hand, the NGS technology frequently discovers “new” or “note” mutation reported as pathogenic witch reveal to be non causative, thus requiring the extension of the molecular test to parents or other relatives [Savarese et al. 2016].

The majority of patients show the first symptoms after 20 years without statistical difference between the mean of age at onset for index cases and relatives so we can consider carriers 7-8 DRA as “late onset” patients [Zatz et al., 1995, Tawil et.al 1996]. In addition, atypical cases have the onset after 40 years and so we could consider them as “very late onset patients”. Moreover, none of probands classified as atypical phenotype (category D) had a relative with a classic phenotype (category A) and in none of these pedigree we can detect the classic autosomal dominant inheritance. Indeed, this subgroup has peculiar features and it is different from classical form of FSHD described by Padberg.

Moreover, it is commonly reported that women have a phenotype milder than men, but the reasons for this are still unknown [Tonini et al. 2004; Zatz et.al., 1998]. The evaluation of age at onset between males and females in combination with FSHD score and clinical category allows us to make another interesting consideration. Women have a later onset and frequently atypical phenotype but the degree of disability impairment is comparable between sexes. Therefore, our data suggest that some factor exist in woman that delay the symptom onset and influence the phenotype but these “protective factor” have a role time-related. Considering this specific trend and the mean of age at onset in woman we can hypothesize a crucial role of hormonal factors related to fertile age but the data should be confirmed with dedicated studies.

Globally the evaluation of FSHD clinical score, as before reported, confirms that the carriers of 33-35 kb DRA have a slight clinical impairment [Statland et al. 2015; Ricci et al., 2013] ,but clinical expression of index cases, showed a large variability ranging from subjects minimally affected to wheelchair patients. This variability is not completely related to the disease duration, therefore there are some other factors that influence the severity of disease at the beginning. Moreover, relatives displayed a milder phenotype than proband, supporting the notion that in this subgroup of patients, the genetic background plays a role in modulating the disease expression. The degree of disability of relatives is related to the age at last neurological examination but it does not depend on the disease duration, therefore the presence of co-morbidity may play a decisive role.

Conclusion

1) About 50% of index cases carriers of 7-8 DRA do not present a classic FSHD phenotype. Therefore for a good clinical practice, it is necessary to consider alternative myopathy, in particular because subjects with atypical phenotype are often sporadic cases. In these way, the association of a myopathic phenotype with the D4Z4 contraction may be random.

2) 53% of relatives carriers 7-8 DRA are non-penetrant and therefore a big careful attention is needed in genetic counselling.

3) To dissect FSHD clinical complexity it is mandatory to associate scales that evaluate the degree of disability with tool that evaluate the phenotype

4) Probably protective factors exist in women but their benefits play a role only during fertile age.

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